STRUCTURAL AND FUNCTIONAL ASPECTS OF DOMAIN MOTIONS IN PROTEINS

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I. INTRODUCTION

A. Flexibility in Globular Protein Molecules

The past decade has seen considerable progress in the study of the dynamic aspects of protein structure. Proteins are known to exhibit internal motions over a broad time scale, ranging from about 10^{-12} sec to more than 1 sec[1,2] in addition to rotational and translational motions of the entire molecule. Events at the lower end of this scale have time constants comparable to unhindered rotations about single bonds and probably involve motion in amino-acid sidechains or polypeptide chain termini on the surface of a molecule. Dynamic processes of this type presumably have little influence on the structure of neighboring regions of the protein. Slower processes are known to include the spontaneous "flipping" of internal sidechains,[3-5] the penetration of a protein molecule by external agents such as O_2 or I^- to quench the fluorescence of internal tryptophan residues,[6] and the well-studied exchange of hydrogen atoms between protein molecules and the solvent,[7,8] the most slowly exchanging of which are now known to be interior amide protons.[8-10] These slower processes clearly involve some concerted movement of groups of atoms within the protein, but the nature and size of the motions required remain unresolved problems.[2,8] A number of observations suggest, however, that even these motions are localized:

1. Simulations of ring flipping by internal aromatic residues of a protein indicate that motions by neighboring residues required to accommodate such a process are small; movements of more than a few tenths of an angstrom are limited to residues immediately adjacent to the ring.[11] Moreover, the energy barriers to ring flipping estimated from such simulations are found to be in reasonable agreement with experimentally observed flipping rates.[12]
2. Calculations based on a model of randomly migrating defects in the atomic packing of a protein molecule have been shown to account quite well for its bulk hydrogen-exchange kinetics.[13] As the volume associated with packing defects in proteins is small,[14] the atomic motions involved in "moving" a defect are presumably also small.
3. Single-crystal neutron diffraction studies have demonstrated that most of the internal amide protons of a crystalline protein can be exchanged without loss of order in the crystal.[15]

4. Hydrogen-exchange studies have shown that the overall exchange kinetics of a number of proteins is the same for crystalline samples as for the protein in solution.[16,17]

A number of reviews have appeared on the general topic of protein flexibility. Gurd and Rothgeb[2] provide an overview of the principle techniques used to study internal motions in proteins and review the evidence that such motions occur; Karplus and McCammon[18] give a similar overview with special emphasis on computational simulations of protein dynamics. A brief discussion of protein flexibility has also been given recently by Cooper,[19] although relatively limited references to the original literature are provided with this article. Careri et al.[1] focus on evidence for periodic or stochastic events in proteins and review model systems that allow these events to be associated with plausible molecular processes. More recently, the same authors have discussed one mechanism by which such stochastic events might contribute to enzyme catalysis;[20] a thorough review of proposals for the role that fluctuations in the structure of an enzyme might play in catalysis has been given quite recently by Welch et al.[21] An earlier review by Citri[22] on ligand-induced conformational changes in enzymes covers a number of techniques, including enzyme kinetics and differential modification studies, that can provide indirect evidence of protein flexibility but are not treated in depth in the more recent reviews cited.

Additional insight into the nature of our current understanding of protein flexibility can be obtained from a number of specialized reviews of techniques used to study molecular motions. The most versatile technique for the study of proteins in solution is NMR spectroscopy. Through the use of different experimental procedures, NMR observations can provide information about events with time constants over a considerable portion of the time scale in which motions have been found in proteins. Under favorable circumstances, a single NMR experiment can resolve and monitor motions in different regions of a protein molecule. The application of NMR techniques to biological samples has been the topic of a number of monographs and reviews in recent years; contributions that give particular emphasis to the dynamic aspects of protein structure include the monograph of Jardetzky and Roberts[23] and reviews by Wüthrich and Wagner[24] and by Jardetzky.[25]

The use of NMR to monitor the exchange of specific amide protons in a protein is emerging as an important extension to the established chemical sampling and spectroscopic methods of studying bulk hydrogen exchange, which have at best relatively coarse spatial resolution;[26] this technique is discussed in the context of other hydrogen-exchange studies in recent reviews.[8,27] Barksdale and Rosenberg[8] also provide a useful summary of molecular mechanisms proposed to account for hydrogen-exchange data. The different mechanisms have been emphasized in earlier reviews and articles: "exposed" intermediate states,[7] "breathing",[28] "local" denaturation,[29] "global" fluctuation,[24,30] solvent "penetration",[27] and "mobile defects".[31] An adequate experimental description of the molecular motions involved in hydrogen-exchange processes is not yet available.[8] Similar difficulties are faced in specifying the molecular mechanisms by which quenching agents gain access to a fluorophore buried in a protein molecule; fluorescence quenching techniques have been reviewed recently by Eftink and Ghiron.[32]

Although the structural details of the motions associated with phenomena such as hydrogen exchange and ring flipping are unclear, there is evidence (cited previously)[11-17] that these motions may be small. The statistical calculations used by Richards[13] to test the mobile defect model of Lumry and Rosenberg[31] are particularly noteworthy, as they indicate not only that one can account for hydrogen-exchange data with small motions, but also that the method can be used to describe the entire spectrum of exchange rates observed in a typical hydrogen-exchange experiment. Other computational approaches to the study of protein flexibility have been restricted by the complexity of the necessary calculations. The application of energy minimization techniques is generally limited to the study of specific dynamic processes, such as aromatic ring flipping,[11,12] while molecular dynamics simulations of

protein motions are limited to relatively short time periods.[33,34] Within these limitations, the results obtained with computational methods are consistent with experimental observations, however. The application of molecular dynamics calculations to proteins has been reviewed by McCammon and Karplus,[35] and Levitt has recently reviewed both this technique and the use of energy minimization procedures.[36] The advantage of the more complicated calculations is that they incorporate the specific intramolecular interactions of the protein molecule known from X-ray diffraction analysis, while Richards treats the protein as an isotropic matrix of the appropriate size and shape. It remains to be seen if the latter approach can be adapted to include more detailed structural information without introducing similar computational restrictions.

The techniques mentioned so far frequently dominate discussions of protein flexibility, as they provide a consistent picture of concerted motions within native protein molecules in solution. Moreover, the information obtainable with these methods covers the entire time range of interest to the study of protein flexibility with varying degrees of spatial resolution. A number of other techniques that provide somewhat less detailed structural information have also been used to study dynamic processes in proteins. In the following paragraphs, we briefly survey these techniques, with emphasis on their applicability to the relatively large-scale motions that will be of interest later.

It is well known, for example, that the optical properties of a protein are sensitive to its conformation, although optical spectra of proteins usually cannot be interpreted in much detail because the contributions from different chromophoric groups cannot be resolved. However, when the protein contains a small number of chromophores with distinctive spectral characteristics, one can often obtain information about the conformation and local environment of these chromophores. An obvious example of this approach is the fluorescence quenching technique mentioned earlier, which takes advantage of the small number of tryptophan residues in many proteins and of their unique fluorescence properties. Optical spectroscopy of proteins has been reviewed recently by Cantor and Timasheff.[37] Optical methods which are of particular interest to the study of protein dynamics are vibrational spectroscopy[38-40] and time-resolved fluorescence spectroscopy.[41,42] The application of fluorescence depolarization techniques to the study of immunoglobulins is a good example of the use of a suitably anchored spectroscopic probe to provide information about large-scale motion of the protein in addition to information about the local environment of the probe; these experiments will be discussed in more detail later. Similar information can be obtained from fluorescence energy-transfer experiments in principle, although this technique has been used primarily to measure intramolecular distances that are constant on the time scale of the fluorescence decay process.[43,44]

The local environment and motion of a paramagnetic probe can be studied by electron spin resonance spectroscopy.[45] Although the time range normally observed in spin resonance experiments (10^{-7} to 10^{-11} sec) is at the lower end of the range of interest in proteins, experimental techniques exist that allow much slower motions to be studied.[46] Changes in the chemical modification pattern of a protein in response to perturbations such as ligation can indicate movement of reactive groups on the protein;[47] such studies are particularly useful when the three-dimensional structure of the protein is known. As with optical chromophores, both spin resonance and chemical probes can provide information about large-scale motions of the protein in favorable circumstances. With Mössbauer spectroscopy, one can measure the motion of specific atomic nuclei directly. The number of nuclear species that can be studied by this technique is limited by the requirement for a source of gamma rays with a suitable wavelength and narrow bandwidth, however.[48] Moreover, the motions that can be detected are limited to those with time constants on the order of or faster than the characteristic time, τ_c, of the excited state of the nucleus responsible for the gamma emission. Mössbauer spectroscopy of proteins to date has been confined to the study of iron (^{57}Fe; τ_c

$= 10^{-7}$ sec) in heme proteins.[49,50] These studies have been interpreted in terms of motions of the protein molecule, on the assumption that the motion of the iron atom is linked to that of the protein. A novel application of time-resolved nuclear γ-ray spectroscopy[417] to the study of domain flexibility in proteins has also been reported recently.[200] In this technique, the angular correlation of γ-ray emissions from an excited nucleus is used to provide information about motions of the nucleus in the nanosecond time range. The use of perturbed angular-correlation spectroscopy in the study of trypsinogen will be discussed in more detail later. Perturbed angular correlations of gamma radiation were used previously to determine the tumbling of ribonuclease in solution on the basis of an indium derivative of the reduced protein.[417]

B. Domain Flexibility

In this article we will focus on an aspect of protein flexibility that has received relatively little attention in earlier reviews, the phenomenon for large-scale, concerted or cooperative motion of large segments of a protein molecule. We mean here "large" in relation to the concerted motions that appear sufficient to account for the fluctuational processes described earlier, such as aromatic ring flipping or hydrogen exchange. The examples that we will discuss in detail all involve movements of at least 15% of the mass of the protein over distances sufficiently large that there is little doubt about their experimental significance. As these large-scale motions can be described either as the destabilization of a contiguous region of the protein molecule or as the rigid-body movement of parts of the molecule relative to one another, we use the phrase "domain flexibility" to refer to such processes collectively and to distinguish them from other concerted fluctuations in the structure of the molecule. The term "domain" implies here only a region of the protein molecule that can be distinguished from the rest on the basis of such concerted or cooperative motions; a "domain" by this criterion clearly need not be related to the domains of interest to students of protein folding.[51]

We shall see later that some types of domain flexibility involve the relative motion of domains that are found by crystallographic analysis to have stable tertiary structures. From the brief summary of protein flexibility given above, it is clear that a protein molecule is a dynamic system subject to a variety of fluctuational processes, even in the crystalline state. The same behavior is to be expected in the large, stable domains found in a number of the examples of domain flexibility discussed later. To emphasize this point, we will use the phrase "essentially rigid" to describe domains or proteins with stable conformations, with the understanding that they are both governed by thermodynamic principles that make thermal fluctuations in their structures inevitable.[158]

A variety of experimental techniques can be used to study domain flexibility. As we emphasized earlier, even techniques which in principle provide only localized information about protein structure in the vicinity of a spectroscopic probe are applicable to domain flexibility under some circumstances. The interpretation of such information in terms of domain motions often requires some knowledge of the three-dimensional structure of the protein from other sources, however.

In contrast, the large conformational changes found in some examples of domain flexibility can often be observed directly with electron microscopy or small-angle X-ray scattering. Changes in the overall shape of a molecule due to domain motions can be detected more easily with electron microscopy, but the effects of sample preparation for this technique are difficult to assess when domain flexibility is involved. Electron microscopy of proteins has been reviewed by Finch[52] and Ottensmeyer,[53] although neither author discusses the study of sample flexibility explicitly. Small-angle X-ray scattering provides information about the structure of the protein in solution, usually in terms of the radius of gyration or volume of the molecule; information about the shape of the particle can also be obtained with this

technique, but considerable additional effort is required. Kratky and Pilz[54] and Pilz et al.[55] have reviewed small-angle scattering studies of proteins and discuss the detection of conformational changes. More recent developments in this field are reviewed by Luzzati and Tardieu,[56] although the examples discussed by these authors do not include applications of small-angle scattering to the study of domain flexibility per se. We will discuss a number of examples of the use of both electron microscopy and small-angle scattering later in this review.

Neutron scattering can, in principle, provide the same information as small-angle X-ray scattering but has been used relatively little in the study of domain flexibility. Neutron scattering studies of proteins are more often designed to take advantage of the facility with which one can employ contrast-matching methods using this technique;[57] under favorable circumstances, contrast matching permits the study of individual components in complex systems.

Light scattering[58,59] and hydrodynamic studies[60] can also be used to measure parameters that depend on the shape of a protein molecule, such as diffusional or frictional coefficients. A disadvantage to these techniques is the difficulty of calculating the expected value of such hydrodynamic parameters for a protein of known structure; calculation of the radius of gyration observed in small-angle X-ray or neutron scattering experiments is relatively simple in comparison. As light scattering and hydrodynamic techniques can be used to advantage in the study of very long, rod-like particles, such as DNA molecules or rod-shaped viruses, they have seen less application to the study of globular proteins in general and of domain flexibility in particular.

In addition to the structural studies mentioned so far, the conformation of a protein and transitions between different conformations can be characterized thermodynamically with calorimetric techniques.[161,162] The primary application of calorimetry to the study of domain flexibility has been to establish the independent folding of domains in a protein, although the conformational changes involved in domain flexibility have also been studied directly and will be discussed later.

C. Crystallographic Study of Protein Flexibility

The high-resolution structure of a protein can presently be obtained only with single-crystal X-ray or neutron diffraction. A number of crystallographic studies in recent years have demonstrated the existence of domain flexibility in several proteins and have provided detailed information about the nature of domain flexibility in others. Brief reviews of some of this work have been given by Huber,[159] Cooper,[19] Steitz et al.,[262] and Huber and Bennett.[160] Since it is largely the detailed, structural information provided by X-ray crystallography that allows one to define domain flexibility as such in the first place, we will concentrate in this review on examples of domain flexibility that have been characterized by single-crystal X-ray diffraction studies. Single-crystal neutron diffraction has had little application to the study of domain flexibility; as is the case for low-angle neutron scattering, single-crystal neutron diffraction experiments usually exploit the unique capabilities of this technique, such as the opportunity to localize hydrogen atoms.[61]

Many discussions of protein flexibility have characterized the results of X-ray crystallography as a "static" view of protein structure. Although this oversimplification may be adequate for the discussion of other aspects of protein flexibility, some understanding of the information one can obtain about dynamic processes from X-ray diffraction and the limits that must be placed on its interpretation will be useful for readers of this review. In the following paragraphs, we summarize a few of the basic principles of X-ray diffraction relevant to the study of protein flexibility. More detailed discussions of the application of X-ray diffraction analysis to proteins are given by Blundell and Johnson[62] and by Matthews;[63] a volume of Methods in Enzymology dealing with contemporary techniques of protein crystallography is also in preparation.[64]

The scattering of X-rays by matter per se is essentially instantaneous, occurring in a time of about 10^{-15} sec for the X-ray wavelengths commonly used for studies of proteins.[65] However, the diffraction of X-rays from a crystal involves the interference of X-rays scattered from different points in the lattice; for this reason, the diffraction pattern of a crystal is sensitive to deviation of the scattering centers (atoms) from their ideal positions in the crystal lattice. The view of the protein provided by X-ray diffraction is in effect an average over all the molecules in the crystal. Since a typical diffraction experiment with a protein crystal takes at least a few hours, any atom in the molecule that is moving is "seen" at many different points within its range of motion. The crystal structure of a protein is thus an average over both time and space; all deviations from an ideal lattice contribute to the average, including those due to motion of or within the molecule, to variations in the structure of different molecules in the crystal, and to imperfections in the packing of molecules in the crystal lattice.

The end effect of deviations from a perfect lattice is to broaden the apparent electron density distribution of the atoms involved in any electron density map calculated from the resulting diffraction data, even if the structure solution (i.e., the determination of the phase of each diffracted ray) is perfect. Regions of a structure that have low or poorly defined electron density relative to other parts of the molecule are thus said to be "disordered", and the well-defined parts are described as "ordered".

It is often useful to make the conceptual distinction between "dynamic" disorder, which includes the contribution of both molecular and intramolecular motions of the protein, and "static" disorder, which incorporates both crystal packing imperfections and stable differences in the conformation of different molecules. These two components of the total disorder *in the crystal cannot be resolved with a single* X-ray diffraction experiment. In principle, one can distinguish static and dynamic disorder through the use of techniques such as Mössbauer spectroscopy that are insensitive to one component or the other or by study of the order in the crystalline molecule at different temperatures; dynamic motion should be reduced at lower temperatures, while static disorder should not be affected. In practice, however, it is not at all straightforward to separate the static and dynamic components of disorder, as we shall see in some of the examples to be discussed later.

Techniques for the quantitative analysis of disorder are well known from small-molecule crystallography.[66] In the course of refinement of a crystallographic model, one can adjust both the average coordinates and a disorder parameter for each atom to optimally account for the diffraction data. The most common disorder parameter, B, is related to the mean-square displacement of the atom from its average position, $\langle u^2 \rangle$, by the expression $B = 8\pi^2 \langle u^2 \rangle$, although some protein refinement procedures[67] employ an equivalent parameter, the effective atomic radius $a = \sqrt{B/4\pi}$. Since the displacement of an atom from its ideal position in the lattice of a small-molecule crystal is commonly associated with thermal vibration, the parameter B is often called the "temperature factor". It is clear from the previous discussion, however, that B values include the effects of both static and dynamic disorder. Moreover, it is rarely practical to refine a separate disorder parameter for each atom of protein model, as the degree of overdetermination of a protein refinement (i.e., the ratio of data to parameters) is usually insufficient to allow independent determination of all positional and disorder parameters with reasonable accuracy. For this reason, the disorder parameters used in the refinement of a protein are generally constrained to be similar for adjacent atoms or constant over an entire amino acid residue; thus, the B values reported for a protein generally provide only a measure of the degree of order in a localized region of the molecule, rather than detailed information about atomic displacements.

With this perspective, we can identify three levels at which one can obtain information about protein flexibility from X-ray diffraction. Even before refinement, the electron density of a structure can suggest regions of flexibility at an all-or-none level: either the density is

well-ordered or it isn't. Although one generally cannot distinguish between static and dynamic disorder, it is clear in some circumstances (e.g., when parts of a molecule are notably more disordered than others) that some conformational variability must exist in the disordered region. One must interpret the electron density of an unrefined protein structure with caution, however, as poorly defined density can also arise from errors in the structure determination. For this reason, most authors place relatively little emphasis on indications of flexibility in unrefined structures, unless relatively large segments of the protein are involved.

Refinement of a protein structure generally improves the quality of the electron density substantially, so that one can identify disordered regions of the structure with reasonable confidence. In addition, another level of information is available for a refined structure in the quantitative measure of the degree of order in different regions of the molecule provided by the B values.

For the large motions that characterize domain flexibility, crystallographic studies can provide information about flexibility at quite another level. These motions are often so large that they cannot occur within the confines of a crystal lattice. In a number of cases, however, it has been possible to crystallize a protein under different conditions in two (or more) different crystal lattices, each of which accommodates a different conformation of the protein. Although it is clear in such instances that the protein is flexible, one cannot tell without additional information whether the structures seen in the crystal are stable conformations in solution or whether the process of crystallization has "trapped" a few of many possible conformations available to the molecule in solution. We shall discuss this question in more detail later in the context of specific examples.

D. Scope of the Review

We have chosen to restrict the scope of this review to domain flexibility as determined by X-ray crystallography for several reasons. Perhaps most important is that, in a number of cases, domain flexibility has a clear biological function and is thus interesting in its own right. By concentrating on crystallographic results, we restrict our attention to systems for which the nature and magnitude of flexibility is well established. Obviously no one technique can provide a full description of domain flexibility; X-ray diffraction data in particular contain no information about the kinetics of the processes responsible for the observed conformational changes or disorder. It is clear that no useful review of protein flexibility can be limited to crystallographic results alone, and we include the results of solution studies in our presentation of specific examples insofar as they can be related to the crystallographically determined flexibility. By concentrating on examples of the large-scale motions we have defined as domain flexibility, we restrict our attention to systems for which the observed flexibility is more likely to have measurable physicochemical and thermodynamic consequences. We think it likely that the study of systems such as these, for which the changes in solution properties can be correlated with well-defined structural transitions, will yield considerable progress in our understanding of protein chemistry and folding in coming years.

We wish to emphasize from the outset that our definition of domain flexibility is quite arbitrary. There is no reason to expect any sharp division between domain flexibility and flexibility in smaller segments of a protein; crystallographic studies have shown disorder not only in surface sidechains, chain termini, and domains, but also in polypeptide loops of intermediate size. However, the number of such observations increases rapidly and their functional significance becomes less certain as the size of the flexible unit decreases. Indeed, we are unaware of any protein structure that does not show some degree of localized disorder, so that a detailed review of the occurrence of crystallographic disorder per se would appear to be both uninteresting and impractical. We will draw on selected examples of small-scale flexibility in proteins to complement our discussion of domain flexibility but will make no attempt to provide a complete survey of such observations.

We also wish to stress that domain flexibility has no monopoly on functional significance. The reorientation of tyr248 upon substrate binding to carboxypeptidase[68] and the subtle changes in the disposition his57 and ser195 upon substrate binding to trypsin[69,70] are well-known examples of localized conformational changes in proteins for which specific functional roles have been proposed. In addition to the relatively obvious importance of such changes in the position of active site residues of enzymes, it is also possible that small-scale fluctuations outside the active site play a role in enzyme catalysis.[21] We suggest therefore that the examples of domain flexibility presented in the following section should be viewed as limiting cases of the types of flexibility to be expected at all levels of protein structure.

A final point to be made prior to consideration of specific examples of domain flexibility is that crystallographic studies of large-scale flexibility in proteins are by no means immune to the question of the relationship of the crystal structure of a protein to its structure in solution. On the contrary, a flexible protein presents special problems in correlating crystal structure with solution properties as compared to an essentially rigid protein. We will devote particular attention to this question in the balance of this review.

II. EXAMPLES OF DOMAIN FLEXIBILITY

In the following sections we present several examples of domain flexibility. We have found it convenient to group the examples into three broad categories which illustrate the three distinct types of domain flexibility characterized to date. In each category we will consider the best-studied system available in detail and will review others more briefly. As most forms of crystallographic evidence for protein flexibility are encountered in the study of the immunoglobulins, the first system to be reviewed, we will discuss the interpretation of crystallographic results suggestive of flexibility in more detail for immunoglobulins than for systems covered later. We hope in this way to illustrate empirically the general considerations outlined in the introduction for readers less familiar with the principles of crystallographic analysis.

A. Proteins with Flexibly Linked, Globular Domains

1. Immunoglobulins

The structure and properties of immunoglobulin molecules are the subjects of a number of reviews, and we will attempt to duplicate as little of this information as possible. In this section we deal only with the structural details of flexibility in the human IgG class of immunoglobulins and related molecules from other species, as these are the immunoglobulins for which the most detailed crystallographic information is available. Crystallographic studies of immunoglobulins have themselves been the subject of several reviews, the most recent of which are those by Amzel and Poljak,[71] Huber,[72] and Marquart and Deisenhofer.[73] Davies et al.[74] discuss earlier crystallographic results and give a useful bibliography of structural studies of immunoglobulins by a variety of techniques.

The essential features of the IgG molecule are summarized in Figure 1. The molecule consists of four polypeptide chains, two chemically identical heavy chains with molecular weights of about 50,000 and two identical light chains with molecular weights of about 25,000. Each heavy chain consists of four domains and each light chain of two domains. Three of the domains of the heavy chain (CH_1, CH_2, and CH_3 in Figure 1) and one domain of the light chain (CL) are mutually homologous, and their amino-acid sequences are essentially constant for a given class of immunoglobulin. The remaining domain of each chain (VH and VL) has a notably more variable amino acid sequence and forms part of the antigen recognition site. Within each chain, the domains are connected by short lengths of extended polypeptide and tend to have relatively little contact with one another; such contacts between domains of the same chain are often called longitudinal interactions. Individual domains

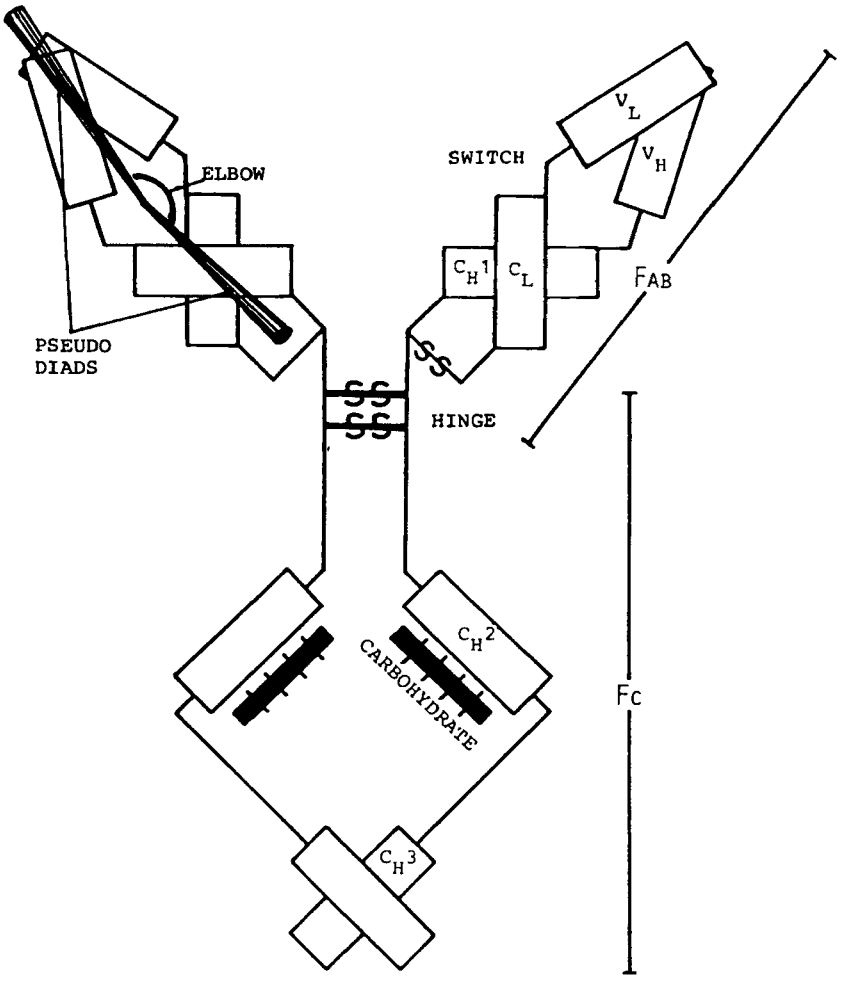


FIGURE 1. Schematic diagram of an immunoglobulin G molecule of the subclass IgG1. Each rectangle represents one domain of the molecule. Interchain disulfide bridges are indicated by the symbol "SS". (Adapted from Reference 72.)

make extensive pair-wise interactions with domains from other chains, as illustrated in the figure; these contacts are called lateral interactions. The three-dimensional structures of all the domains are quite similar (Figure 2), as was originally proposed on the basis of amino-acid sequence studies.[75] The molecule is readily cleaved into fragments consisting of either the VH-VL and CH_1-CL dimers (the Fab fragment) or the CH_2 and CH_3 dimers (the Fc fragment); a fragment containing two Fab units linked by disulfide bonds is also easily produced and is denoted $(Fab')_2$. Fragments consisting of the VH-VL dimer alone or the CH_3 dimer alone can be prepared, although not as easily as the Fab and Fc fragments are separated from one another.

a. Segmental (Hinge) Flexibility

Flexibility in IgG molecules was first suggested on the basis of a number of physico-chemical and electron microscopic studies. Due to the relatively low resolution of the techniques used, these early results dealt only with movement of the Fab arms of the molecule relative to the Fc region and to one another. For this reason, the regions of polypeptide chain connecting the Fab and Fc segments are called the "hinge" peptides. The amino-acid

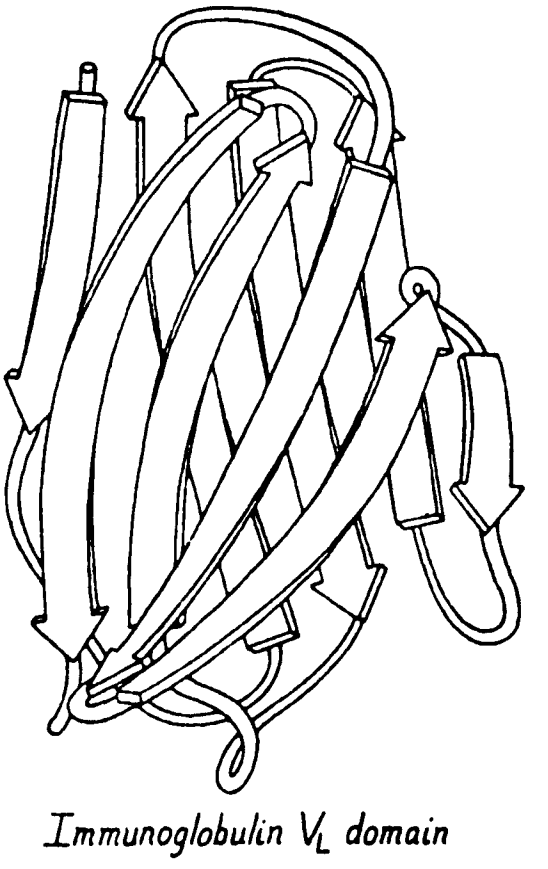


FIGURE 2. Schematic diagram of the folding of an immunoglobulin domain. Each arrow represents a strand of β-sheet. (From Reference 51.)

sequence of these peptides has a high proportion of proline residues and includes the cystine residues that form interchain disulfide bonds between the two heavy chains; the IgG1 subclass, which accounts for about 80% of the IgG molecules in normal human sera,[76] has two such hinge disulfide linkages.

Noelken et al.[77] were the first to suggest that an IgG molecule consisted of flexibly linked globular units. This proposal was based in part on hydrodynamic data presented by these authors which indicated that the Fab and Fc fragments have essentially compact, globular structures, but that the intact IgG does not; the latter conclusion had also been reached in earlier small-angle X-ray scattering studies. Although recognizing that there are several explanations for these observations, including an elongated, rigid structure or a highly hydrated, swollen molecule, Noelken et al. showed considerable insight in suggesting flexible linkage of the fragments. In support of this idea, they drew on earlier steady-state fluorescence depolarization data and evidence indicating that the hinge region is highly exposed to proteolytic enzymes and to disulfide reducing agents; a detailed review of these arguments and original references are given by Dorrington and Tanford.[78] Noelken et al. also recognized that such flexibility might play some role in immunoglobulin function. At about the same time, suggestions of hinge flexibility were also provided by electron microscopy; this early work has been reviewed by Green.[79] Particularly striking were the studies of Valentine and Green,[80] who used a divalent hapten to form closed rings of Fab arms from two, three, four, or more IgG molecules. The observation of closed polyhedral complexes of different order clearly demonstrates the existence of considerable variability in the hinge angles (i.e., the angle between Fab arms) of the molecules involved.

An important consideration in the interpretation of these early indications of flexibility is that the samples studied were heterogeneous populations of IgG molecules, which left open the possibility that many of the properties suggestive of flexibility were due instead to static variations in structure among different molecules in the population. The first direct evidence for flexibility was provided by the nanosecond fluorescence-depolarization studies of Yguerabide et al.[81] These authors made two important contributions to the study of antibody flexibility with fluorescence techniques. One was the study of antibodies raised against the fluorescent probe, dansyl-lysine, in contrast to earlier studies which used nonspecifically attached fluorophores. The latter experiments are subject to the criticism that independent rotation of the probe molecule may account for a considerable fraction of the depolarization.[78] As in earlier studies, Yguerabide et al. were able to interpret their results in terms of motion of the Fab arms relative to the molecule as a whole by comparing the behavior of intact molecules with that of isolated Fab fragments. A second improvement of technique introduced by these authors was the measurement of depolarization as an explicit function of time after a very short exitation of fluorescence, in contrast to the steady-state measurements performed in previous studies of antibodies. With this approach, two relaxation processes could be resolved, which were interpreted in terms of a global tumbling process and one internal mode of motion. Although the detailed conclusions of Yguerabide et al. have since been challenged and refined, the experiment did provide strong evidence for the existence of flexibility in all of the chromophore-binding IgG molecules of their sample and was consistent with the hinge flexibility suggested by earlier workers. We will return to more recent interpretations of fluorescence-depolarization data at the end of the section. In general, the basic conclusions about flexibility obtained from early work with heterogeneous populations of IgG molecules has since been confirmed using human myeloma proteins or monoclonal mouse antibodies, both of which are sources of homogeneous antibody.

Crystallographic studies of immunoglobulins have provided detailed structural information about Fab and Fc fragments and about most of the hinge region of the IgG molecule, but the basic picture of hinge flexibility suggested by earlier work remains unchanged. Possibly as a result of flexibility in the hinge region, it has proven quite difficult to crystallize intact immunoglobulins at all. Of the four intact IgG molecules that have been crystallized, two (Dob[82] and Mcg[83]) have the hinge deleted;[84,85] the Dob IgG is known to be defective in a number of biological functions,[86] presumably as a result of the hinge deletion. The two crystalline IgG molecules with normal hinge regions, Kol[87] and Zie,[88] both have disordered Fc regions in the crystal.[88,89] The two molecules with deleted hinges by contrast appear to have ordered Fc components; this point has been established clearly by crystallographic studies at intermediate resolution for Dob[90,91] and is probably the case for Mcg.[92] These observations are clearly consistent with the idea that the hinge provides a flexible attachment point for the Fab and Fc segments but do not give any additional insight into the nature of hinge flexibility. Although the relative orientation of the Fab arms is different for the two intact IgG molecules whose structure has been reported (Dob has the form of a "T",[90] while Kol suggests more of a "Y"),[89] the absence of a hinge in the Dob molecule makes it impossible to draw any conclusions about hinge flexibility from this comparison.

b. Kol Fc Disorder and C-Terminal Hinge Flexibility

More detailed information about hinge flexibility is provided by the structure of the intact Kol molecule, a human immunoglobulin of the IgG1 subclass which has been refined at 3.0 Å resolution.[93] In this crystal, the Fab arms and most of the two heavy-chain segments that comprise the hinge-are related by a crystallographic twofold axis; all ordered regions in these two halves of the structure are thus identical[89] and only one chain need be considered. The hinge segment is ordered from the Fab up to the second disulfide bond between the heavy chains, cys229. (For the reader's convenience, we use the amino acid numbering of

the Eu immunoglobulin, another IgG1 molecule whose sequence has been completely determined; this work is summarized by Edelman.[94] The relationship between the Eu sequence and that used in crystallographic studies of the Kol molecule is given by Marquart et al.[93]) Following cys229 in the Kol crystal, pro230 is poorly ordered. There is no interpretable electron density at all for the remaining residues of the heavy chain, including all of the Fc segment. Refinement of the Kol structure substantially reduced the electron density in the region of the map where the Fc segment must be,[93] which demonstrates that the disorder is genuine.

The observation that Kol disorder begins in the C-terminal region of the hinge is of interest in itself, as it demonstrates that the polypeptide segments which link the Fc segment to the rest of the molecule are flexible. Earlier observations of variable orientation or motion of the Fab arms indicated only that the N-terminal part of the hinge is flexible. The structures of the human Fc fragment[95] and its complex with fagment B of Protein A from *Staphylococcus aureus*[96] (FB:Fc complex) are also consistent with flexibility in the C-terminal region of the hinge, as the hinge is disordered in both crystals. The hinge disorder in the Fc crystals in particular may be quite substantial, as covalent labeling of the N-terminus of the fragment with 5-iodo-2,4-dinitrofluorobenzene did not allow the N-terminus to be located in the electron density map.[95] The electron-dense iodine atom in the label would be easy to locate by difference Fourier techniques if it were ordered. However, it is possible that the labeling group could be disordered even if the N-terminus were not, so this result is only suggestive of a large degree of disorder in the hinge.

The Fc structure has been refined at 2.9 Å resolution and the FB:Fc complex at 2.8 Å resolution;[97] in both refined models, the polypeptide chain is disordered up to residue pro238 (Eu numbering). As we will discuss in the next section, the crystal packing is quite different for these two structures and gives rise to varing degrees of disorder in other regions of the Fc fragment. The similarity in the points at which hinge disorder begins in these crystals thus suggests that the disorder is a property of the hinge itself. Although the Fc preparation used to obtain the crystals studied by Deisenhofer et al.[95] was heterogeneous with respect to IgG subclass, the predominance of IgG1 in normal human serum[76] makes it unlikely that heterogeneity contributes much to the observed hinge disorder.

Another interesting aspect of the disordered Fc segment in crystals of the intact Kol molecule is that it is currently the only example of large-scale crystallographic disorder for which the distinction between static and dynamic disorder can be made with confidence on the basis of crystallographic data alone. (A probable classification of the disorder in other examples can also be made, as will be discussed in the next section). The demonstration that the Fc disorder in Kol crystals is static in nature was made possible by crystallization of the $F(ab')_2$ fragment of the Kol molecule under the same conditions used to crystallize the intact molecule.[93] As the Kol $F(ab')_2$ crystals proved isomorphous to intact Kol crystals, Marquart et al.[93] could directly compare the diffracted intensities of the intact molecule with those from a crystal containing only the ordered segments of the same molecule. Using an analysis based on a study of isomorphous substitutions in protein crystals by Crick and Magdoff,[100] these authors found that static and dynamic Fc disorder would have quite different effects on the variation in the fractional difference in diffracted intensities from the two crystals, $\phi\Delta I$, as a function of resolution: for static disorder, $\phi\Delta I$ should be constant, while for dynamic disorder, $\phi\Delta I$ should decrease with increasing resolution. The experimental results indicate predominantly static disorder of the Fc part.

It is also possible in principle to calculate the number of different sites the F segment must occupy in the crystal lattice from $\phi\Delta I$, if the sites are assumed to be of equal occupancy. The comparison of intact Kol crystals with Kol $F(ab')_2$ crystals suggests that about five different sites are required to account for the observed overall $\phi\Delta I$. Unfortunately, the variation of $\phi\Delta I$ values in different resolution ranges indicates that there may be a rather

large error in this estimate. An alternate empirical estimate of the minimum number of different Fc positions required to account for the observed disorder can be obtained by comparing the size of the highest electron density peaks in the region of the map where Fc should be to the height of the electron density in a well-ordered region of the map; the disordered segment must be distributed among at least as many sites as the factor between these values. By this technique Marquart et al. concluded that at least four different sites are required to account for the Fc disorder. The empirical method of estimating the degree of disorder clearly underestimates the number of sites required, as it ignores contributions of noise to the electron density in disordered regions of the map. Moreover, the empirical method, unike the Crick-Magdoff approach, depends on the quality of the structure solution since the noise level of the map is increased by errors in the structure determination. In view of these difficulties, the agreement between these two estimates of the degree of disorder in the Kol Fc region is quite reasonable. We should emphasize, however, that neither the Crick-Magdoff approach nor the empirical method allows one to determine how many different conformations of the protein contribute to the disorder. Although some flexibility in the protein is required so that molecules in the crystal do not all have the same conformation, a random distribution of conformers in different lattice positions may then account for some of the disorder (Figure 3).

c. Kol Hinge Conformation and N-Terminal Hinge Flexibility

A substantial fraction of the hinge is ordered in the refined structure of the intact Kol molecule,[93] providing some information about the region of the IgG molecule that is responsible for the variable orientation of the Fab arms. From the disulfide bridge linking the heavy and light chains (cys220 in the Eu heavy chain), the heavy chain forms a single turn of "open" helix (i.e., a helix that is not stabilized by main chain hydrogen bonding) between cys220 and thr225, followed by a short segment with the conformation of a polyproline helix (Figure 4); the sequence of this region is given below in Eu[94] numbering; it is constant in several IgG1 heavy chains.[75,99,101]

220					225	226			229	
- cys	- asp	- lys	- thr	- his	- thr	- cys	- pro	- pro	- cys	-

Residues cys226 and cys229 form the interchain disulfide bonds which link the two heavy chains of the molecule.[102] Aside from the interchain disulfide linkages, this region of the hinge forms few specific intramolecular interactions that might stabilize the conformation observed in the crystal. As expected from the hydrophilic character of the hinge sequence[94] and its susceptibility to proteolytic attack,[103] the hinge region of the Kol molecule is accessible to either solvent or macromolecules.

Accessibility of the hinge region in crystals of the intact Kol molecule is demonstrated by the observation that this region of one molecule makes extensive interactions with neighboring molecules in the lattice.[89,93] It is quite likely that the conformation of the hinge found in the crystal is dictated by these intermolecular interactions. This conclusion is suggested both by the paucity of intramolecular interactions in the hinge region and by a comparison of the intact Kol molecule to its Fab fragment. The Kol Fab fragment is released by papain,[104] which makes the expected cleavage in the hinge region[103] and leaves his224 as the new C-terminus of the heavy chain;[105] its crystal structure has been solved[105] and refined at high resolution.[93] Although crystals of the intact Kol molecule and its Fab are in completely different space groups, the principal contacts between molecules in the crystal lattice are similar and involve the interaction of the antibody combining site of one molecule with segments near the hinge region of another (Figure 4). These are apparently specific contacts[93] and may be involved in cryoprecipitation of the Kol molecule;[104] as such they may be a

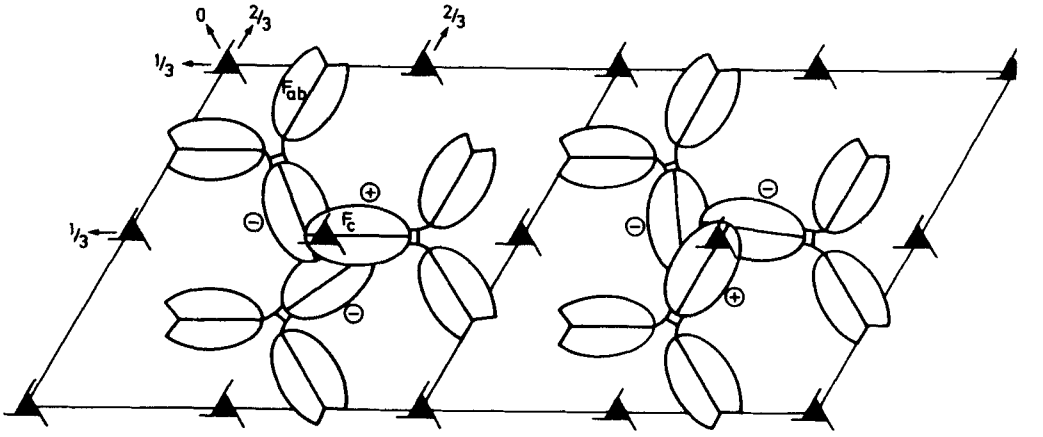


FIGURE 3. Hypothetical packing scheme showing the combined effects of conformational disorder and crystallographic disorder resulting from random packing of conformers in the crystal lattice. The packing around the central threefold axis is approximately that found for the intact Kol molecule[89] in space group $P3_221$. As indicated here, there is not room for all three Fc segments in the channel around the central threefold screw axis if all three segments obey the crystallographic twofold symmetry relating the Fab arms of the molecule. For the sake of illustration, we imagine that one Fc segment (+) of each such trimer is in the plane of the Fab arms and that the other two Fc units (−) are directed down and tilted relative to the "+" segment. Either the difference in the Fc locations in the crystal or the deviation from crystallographic symmetry alone would result in crystallographic disorder, even if this arrangement were reproduced in each such Fc cluster of the crystal. In addition, the disposition of + and − Fc conformers in different clusters would also vary in the packing we have drawn, since the Fc segments in one channel have no contact with Fc segments in adjacent channels and thus do not influence their packing. We emphasize that any number of similar schemes can be invented to account for the disorder in Kol crystals.[89]

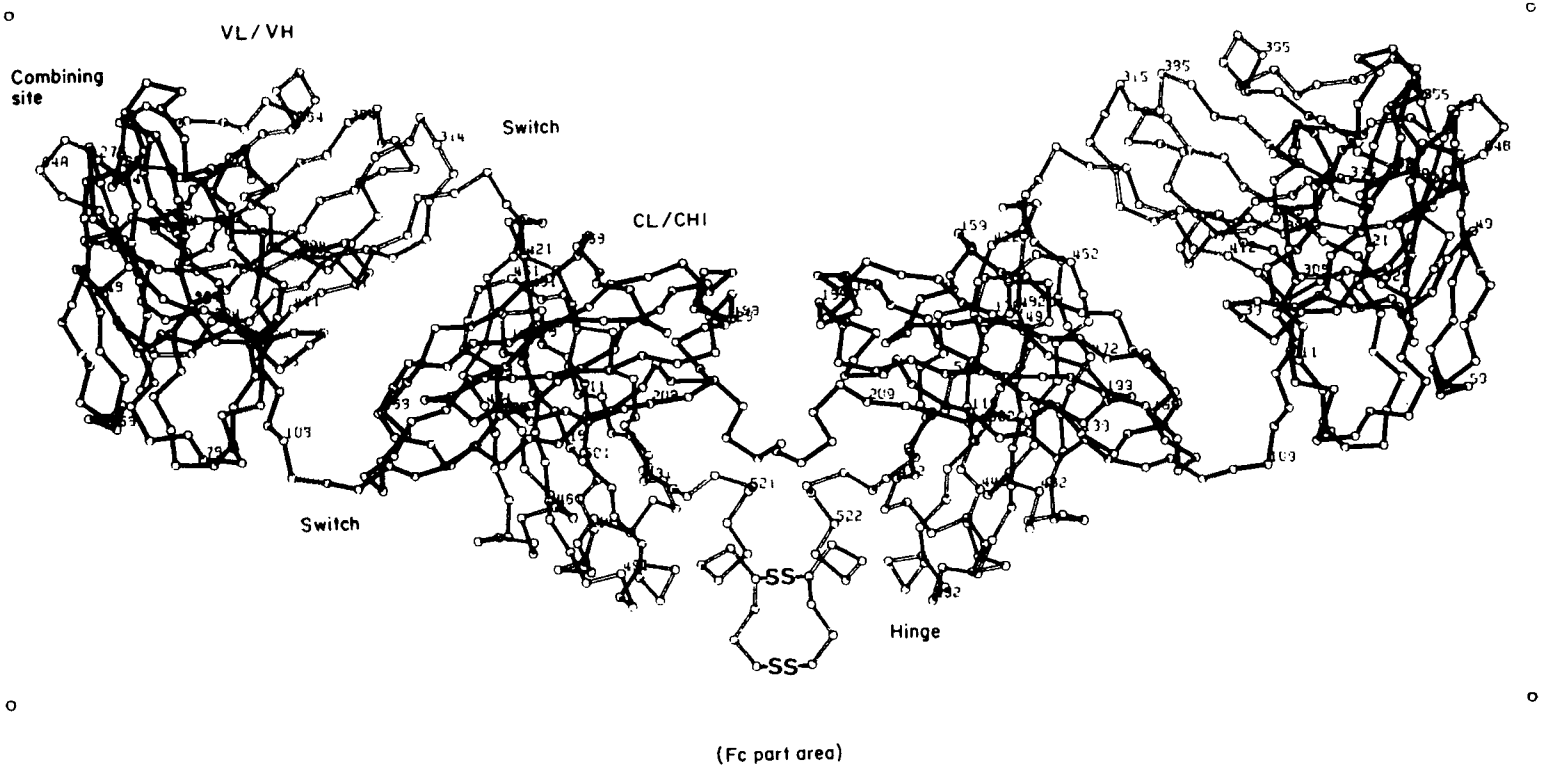


FIGURE 4. C_α-drawing showing the folding of the ordered regions of the crystalline Kol immunoglobulin. The heavy chain is drawn with open lines and the light chain with solid lines. Disulfide bonds in the hinge region are indicated by the symbol "SS"; other disulfide bonds in the molecule are not shown. The combining sites of adjacent molecules of the Kol crystal lattice approach the hinge from the front and back as seen in this drawing. The residue numbers of the heavy chain do not correspond to those used in the text; in the switch and hinge regions, residue numbers for the Eu heavy chain[94] can be obtained by subtracting 301 from the numbers shown in the figure.[93] (Adapted from Ref. 93.)

useful model for interactions of antibodies with macromolecular antigens.[93,105] Earlier evidence indicating that such interactions can affect the conformation of a protein antigen was reviewed by Celada and Strom.[106]

In the refined Kol Fab structure, the C-terminus of the light chain is disordered from arg211 to ser214 and the heavy chain from pro217 to his224. These segments include the interchain disulfide bond, cys213 (light chain) to cys220 (heavy chain). (Note that the Kol light chain sequence differs from that of Eu, as the former is a λ chain[105] and the latter a κ chain;[94] sample λ and κ sequences are given by Edelman and Gall,[75] and a recent comprehensive collection of immunoglobulin sequences can be found in Kabat et al.[165]) It is particularly interesting that this region of the Kol Fab structure is disordered in view of the contacts it makes with the antibody-combining regions of adjacent molecules in crystals of the intact molecule. The intermolecular interactions in which these segments participate include a salt bridge between ser214 (light chain) of one molecule and arg49 (light chain) of another.[93] It would appear that either the interactions provided by residues 217 to 229 are required to stabilize the C-terminal regions of the Fab fragment in the conformation found in crystals of the intact Kol molecule or that the Fc segment of the intact molecule provides some nonspecific tethering effect that serves the same purpose. Whichever is the case, it seems clear that this region of the Fab must have some flexibility to account for the crystallographic disorder seen in Kol Fab crystals. Flexibility in segments at the C-terminus of the Fab fragment is also suggested by the crystal structure of the Fab′ fragment of another human IgG1(λ), Fab′ New,[107,117] in which this region of the fragment is poorly defined. Fab′ New is not yet refined, however, so the details of the disorder are less reliable.

The general picture of hinge flexibility suggested by the detailed information obtained from crystallographic studies of immunoglobulins is clearly consistent with the segmental flexibility proposed on the basis of hydrodynamic studies, electron microscopy, and fluorescence depolarization, but the extent of the hinge region indicated by crystallographic studies is somewhat larger than expected. Consideration of the homology among immunoglobulin domains suggested that the hinge would extend from about asn221 of the heavy chain, which comes immediately after the disulfide bond between the light and heavy chains, to about glu233 of the heavy chain[94] (Eu numbering). The more recent discovery that the hinge region in a rabbit IgG is coded by a separate DNA segment that corresponds closely to the identical sequences deleted from the Dob and Mcg heavy chains[118] suggested a genetic definition of the hinge; this segment extends from val216 to pro230 of the heavy chain.[84,85]

The crystallographic results described above indicate that the functional "hinge" region encompasses at least the segments between pro217 and gly237 of the heavy chain and between arg211 and the C-terminus of the light chain. As this functional hinge region extends beyond the region deleted in the Dob and Mcg molecules, their structures may provide additional information about hinge flexibility. Work on the Mcg structure is presently in progress,[111] but has not yet been reported in detail. The most recent studies of the Dob structure indicate that some ambiguities remain in the interpretation of connections between the CH_1 and CH_2 domains of the molecule.[119]

d. Flexibility in the Elbow Region of the Fab Segment

Another region of flexibility in the immunoglobulin molecule is the "elbow" of the Fab arm, which is formed by the "switch" peptides between the variable and constant domains of each chain (Figure 1). The evidence for flexibility in the elbow region, unlike that for hinge flexibility, has appeared relatively recently. Although it was known soon after the domain structure of immunoglobulins was suggested that proteolytic cleavage of Fab and Fc fragments produced subfragments consistent with the proposal,[94] there was no direct evidence for flexibility in the segments linking V and C domains or CH_2 and CH_3 domains prior to crystallographic studies of immunoglobulin fragments.

Table 1
ELBOW ANGLES OF CRYSTALLINE IMMUNOGLOBULINS

Structure	Angle	Structure	Angle	Classification
Fab and Fab′ fragments[a]		**Intact molecules**		
—	—	Dob [91]	147°	Human IgG1(κ)[b]
New [71][c]	137°	—	—	Human IgG1(λ)
Kol [105]	166°	Kol [105]	174°	Human IgG1(λ)
McPC603 [108]	135°	—	—	Murine IgA2(κ)
J539 [109]	136°	—	—	Murine IgA2(κ)
Light-chain dimers				
Mcg (trigonal) [110]	115°			
Mcg (orthorhombic) [110]	132°	Mcg [111]	128°	Human IgG1(λ)[b]

[a] The Kol fragment is an Fab (produced by papain cleavage), while the others are Fab′ fragments (produced by pepsin cleavage).
[b] Residues 216 to 230 (Eu numbering [94] are deleted from the heavy chains of these molecules.
[c] The number in brackets is the reference.

Flexibility in the switch peptides was first suggested by comparison of the overall shape of different Fab segments. Although the Fab segment appears to be a straight rod in electron micrographs, all crystalline Fab segments whose structures are known are bent at the elbow to varying degrees.[71] The conformation of an Fab can be conveniently described by its "elbow angle", which is defined as the angle between the approximate twofold symmetry axes relating the V domains and the C domains to one another (Figure 1); even though each domain of a laterally associated pair (i.e., VL and VH or CL and CH_1) is chemically distinct, their structures are sufficiently similar to produce this three-dimensional symmetry.[71] The elbow angles of Fab segments whose structure is known are given in Table 1. Angular differences of about 5° are significant for refined structures, while larger differences can be seen clearly in low-resolution studies.

It is difficult to interpret most of the differences in elbow angle seen in Table 1 in terms of flexibility, however, as chemically distinct molecules are being compared. Thus one cannot exclude the possibility that the different molecules simply have different, fixed elbow angles. Direct crystallographic evidence for flexibility in the elbow region can be obtained only by the study of the same protein in different crystalline environments. Similar, but less definitive, information is provided by studies of immunoglobulin molecules of the same heavy-chain subclass and light-chain type, such as Kol, New, and Mcg.

The immunoglobulin chain that has been studied in the largest number of crystallographically independent environments is the λ-type light chain from the human Mcg myeloma protein. A dimer of Mcg light chains can be isolated as a Bence-Jones protein (i.e., a light-chain dimer found in the urine of a multiple myeloma patient) or can be reconstituted from light chains separated from the Mcg serum immunoglobulin under appropriate conditions.[112] Two crystal forms of this light-chain dimer have been obtained,[110] a trigonal form that crystallizes from ammonium sulfate solutions and an orthorhombic form that crystallizes from cold, distilled water. The structure of the molecule in the trigonal crystal form has been more extensively studied[113,114] and has been partially refined at 2.3 Å resolution;[115] its structure in the orthorhombic form has been reported only at low resolution so far.[110] Crystallographic studies of the Mcg proteins have been reviewed by Edmundson et al.[164]

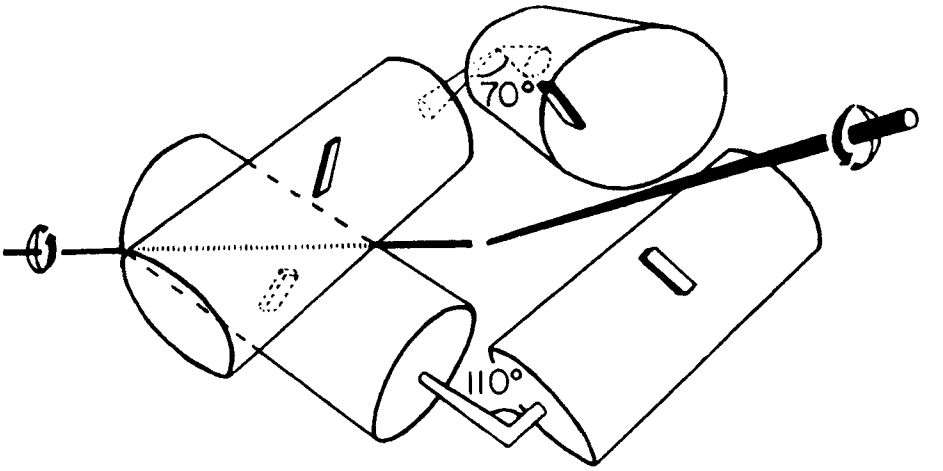


FIGURE 5. Schematic drawing of the conformation of the Mcg light-chain dimer found in the trigonal crystal form. Each large cylinder represents a domain of the molecule. The switch peptides linking the domains of one chain are drawn as open rods, and the local twofold axes relating laterally associated domains are indicated by solid bars. The small rectangles at the center of each cylinder show the location of intrachain disulfide bonds. (From Ref. 113.)

Flexibility in the switch peptide of the Mcg light chain is demonstrated by two types of observations. First, the overall structure of the light-chain dimer is similar to that of an Fab fragment;[113] the folding of the domains and their pair-wise association into V and C regions are essentially indistinguishable from those of an Fab. The striking feature of the dimer, however, is that it has a pronounced elbow bend (Table 1); thus the twofold axes relating the domains of the V and C regions (in this case proper axes, since the chains of the dimer are chemically identical) are not colinear, and the switch peptides of the two chains have different conformations (Figure 5). In effect, one chain of the dimer has the conformation of an Fab light chain, and the other has the conformation of an Fab heavy chain[114] (Figure 5). Further evidence for flexibility in this switch peptide comes from the observation that the elbow angles are different in the trigonal and orthorhombic crystal forms of the dimer (Table 1). As the conformation of each chain of a bent dimer must be different, this means that the switch region of the Mcg light chain can assume at least four different conformations, two for each elbow bend. It is likely that the light-chain switch peptide assumes a fifth, distinct conformation in crystals of the intact molecule[111] (Table 1), but this structure has not been reported in enough detail to allow one to assess whether the elbow angle of the intact molecule is significantly different from that of the orthorhombic light-chain dimer.

Similar observations of flexibility have been made for the switch region of the Kol molecule[93,105] and for the segments connecting CH_2 and CH_3 domains of the human Fc fragment.[95-97] The switch peptides of both the heavy and the light chains of Kol adopt conformations in crystals of the intact molecule different from those found in crystals of the Kol Fab;[105] these differences are reflected in the elbow angles in the Fab parts of the two structures (Table 1). Differences in the conformation of the two crystallographically independent chains in Fc crystals must be expressed in slightly different terms than we have used in our discussion of switch-peptide flexibility. The approximate twofold axis relating the CH_2 domains deviates only 2° from the axis relating the CH_3 domains; however, the CH_2 domains are related by a rotation that differs by about 5° from a strict twofold rotation coupled with a small translation, while the CH_3 domains are related by a simple twofold rotation within experimental error.[97] As with the elbow bending found in Fab segments,

these deviations from strict twofold symmetry in the Fc fragment require differences in the conformation of the chemically identical polypeptide segments connecting the CH_2 and CH_3 domains of each of the heavy chain fragments. In the FB:Fc complex, the two heavy-chain fragments of the Fc component are related by an exact crystallographic twofold axis,[96,97] indicating that the connecting peptide can assume a third, distinct conformation. Although both the Kol structures and the Fc-containing structures are refined, the differences in the conformations of the connecting peptides which must accompany the observed differences in domain orientation are too small to be detected with significance; the differences seen in the Mcg light chain structures are much larger.

The observations discussed so far show clearly that the polypeptide segments linking domains in the Fab and Fc segments of an IgG can assume different conformations under different conditions, but they do not indicate whether the structures observed in the crystal are stable in solution. The question is whether the crystal lattice "traps" one of many interconvertible conformations (perhaps a continuum) of a highly flexible structure in solution or whether the structure is essentially constant for a given set of conditions in solution and happens to be crystallizable. This question is of particular interest when one notices that the largest of the conformational differences we have described are associated with the most notable differences in solvent composition. The trigonal crystal form of the Mcg light-chain dimer is obtained from a high concentration of salt, while both the orthorhombic form of the dimer and the intact Mcg crystals are obtained from solutions of low ionic strength.[83,110] Crystals of the intact Kol molecule and its Fab are obtained under similar high-salt conditions,[87,105] and the two chains in the Fc crystals are obviously in the same solvent (although their crystalline environments differ). Only when comparing the Fc structures from different crystal forms does one find similar conformations under quite different conditions. The FB:Fc complex crystallizes from solutions of high ionic,[96] while Fc crystals are grown under low-salt conditions;[98] the pH of crystallization is also different for the FB:Fc complex and the Fc fragment alone.

Two observations suggest, however, that the connecting regions of the Fab and Fc segments are flexible in solution. First, these segments are in extended conformations that are readily accessible to solvent and make few contacts with the rest of the molecule; this is seen clearly in Figure 4 for the Fab region of the Kol molecule and is also observed for all other structures listed in Table 1.[71] Moreover, the switch peptides of the refined Kol Fab are relatively poorly ordered,[93] indicating some flexibility in this region. None of the other structures is sufficiently well refined to establish whether the same is true for all crystalline Fab segments; in the trigonal Mcg crystals the conformation of the switch peptides may be stabilized by contacts with the hapten-binding region of a neighboring molecule.[116] An extended, accessible conformation is also observed for the peptide segments linking the CH_2 and CH_3 domains of the Fc fragment.[95-97] It is thus unlikely that the connecting regions themselves can contribute much to the stability of the Fab or Fc conformation in solution.

Second, there are relatively few longitudinal interactions between the V and C regions of a Fab[71] or between the CH_2 and CH_3 domains of the Fc fragment.[97] If the connecting peptides between domains are flexible, the stability of the conformation of an Fab or Fc segment in solution will be determined by such interactions; for a crystalline Fab or Fc, the conformation would also be influenced by crystal packing interactions, if these provide a net energy of stabilization comparable to that available from longitudinal interactions. A comparison of the Fab conformations in the similar Kol structures to that of Fab' New suggests that the longitudinal interactions in an Fab are in fact relatively weak. As we have seen, the Kol and New molecules are of the same heavy-chain subclass and light-chain type and should be directly comparable. The New structure has been solved at 2.0 Å resolution[107] and a preliminary refinement has been performed at the same resolution.[117] A small number of longitudinal contacts is found in the New structure,[117] as is also the case for other Fab

structures with an elbow angle near 135°[71] (Table 1). The two Kol structures, by contrast, have substantially larger elbow angles and make essentially no longitudinal contacts,[89,93,105] suggesting that the extensive, specific intractions between neighboring molecules in the two Kol crystals is responsible for the distinctive elbow angles observed in these structures. However, this argument does not explain the preponderance of elbow angles near 135° seen in Table 1, other than to suggest that weak longitudinal interactions are sufficient to determine the conformation of a crystalline Fab in the absence of stronger, specific contacts such as those formed in the Kol crystals. A detailed analysis of packing contacts has been presented only for the trigonal Mcg crystals,[116] so it is not known if the Fab structures with elbow angles near 135° share any common features in this regard. Refinement of these structures with high-resolution data will probably be required before a satisfactory answer to this question can be obtained, as detailed information about specific intra- and intermolecular interactions is needed; given the overall similarity in the shapes of these molecules, some general similarity in their crystal packing schemes would not be surprising.

Comparison of the Kol and New Fab structures[93] reveals one other interesting aspect of elbow flexibiity. The conformational differences in the elbow regions of these molecules are confined to a short region of the peptide chain consisting of only a few residues (Figure 6). This suggests that a fairly specific hinge point exists in these sequences. Similar observations have been made for the order-disorder junctions in the Kol structures[89,93,105] and at the N-termini of the refined Fc structures.[97] These regions of the polypeptide backbone show an abrupt transition from full order to disorder within a span of one to two residues; other examples of abrupt order-disorder junctions will be encountered in later sections of this review. The suggestion made by these observations is that such discrete hinge points may be characteristic of large-scale flexibility in proteins. The IgG "hinge" region itself seems to consist of several such hinge points; three identified so far are (1) near asn210 of the light chain and glu216 of the heavy chain in the Kol Fab structure,[93] (2) near pro230 in the intact Kol structure,[93] and (3) near pro238 in the Fc structures.[97] Since these three examples involve disorder of the polypeptide chain on one side of the apparent hinge point, however, more delocalized flexibility in the hinge region cannot be ruled out. The conformational differences in the switch peptides of the trigonal Mcg structure also appear to be localized to the region around gly111,[114] but the interactions made by one of these peptides with a neighboring molecule of the crystal lattice[116] may influence its conformation.

e. IgG Flexibility in Solution

An outline of the early evidence for segmental flexibility in immunoglobulins was given at the beginning of this section. Two reviews on the topic of immunoglobulin flexibility in solution have appeared recently. Cathou[120] has reviewed segmental flexibility with particular attention to fluorescence depolarization studies, and Pecht[121] discusses a number of dynamic processes in immunoglobulins, including segmental flexibility, ligand-binding kinetics, and folding. Here we mention only a few very recent results on antibody flexibility in solution that complement our discussion of crystallographic indicators of flexibility.

Two important questions on which crystallographic studies can provide little or no information are the range of motion allowed by a flexible joint between globular domains and the kinetics of this motion. The first of these questions has been addressed recently by Schumaker et al.;[122] extending the observation of Valentine and Green[80] that a divalent hapten will form closed, polyhedral complexes of 2,3,4,..., n antibody molecules, these authors measured the distribution of complexes formed with a combination of analytical ultracentrifugation and electron microscopy. Then using an analysis based on the thermodynamic model of Archer and Krakauer,[123] Schumaker et al. estimated an effective strain energy for various Fab angles (i.e., the angle between Fab arms) by comparing the observed and predicted distributions of n-mers. The results of this interpretation, presented in Table

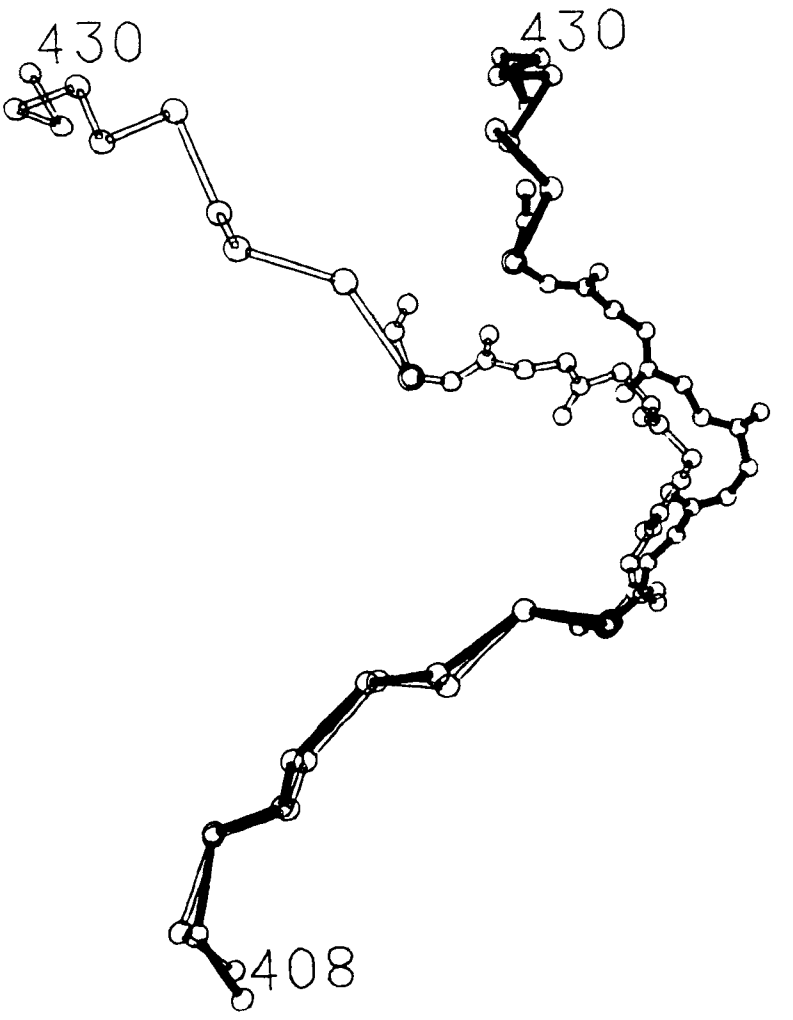


FIGURE 6. Cα-drawing comparing the switch peptides of the heavy chains of IgG Kol and Fab′ New. The backbone conformation of the New peptide is drawn with open lines and that of the Kol peptide in solid lines. Residue 408 in the figure corresponds to residue glu107 in the amino-acid sequence of the Eu heavy chain[94] and residue 430 in the figure to residue ala129 of Eu. (From Ref. 93.)

2 in terms of effective strain energy per antibody molecule, suggest that there is little strain associated with Fab angles up to about 140°; even the effective strain energies indicated for smaller angles are low. Larger Fab angles are not observed with this technique because complexes of order $n > 9$ are not formed in detectable quantities. Schumaker et al. note that their analysis ignores two factors that could affect their quantitative results. First, there must be heterogeneity in the affinities of antibodies in their unfractionated sample for hapten and, second, monomeric hapten used in the purification of hapten-specific antibodies is known to contaminate their IgG preparation; as pointed out by these authors, the use of monoclonal antibodies could circumvent both of these problems. It seems unlikely that these factors would change the basic conclusions of Schumaker et al., although the magnitudes of the apparent strain energies may be affected.

While the results of Schumaker et al. suggest that the Fab arms of an IgG molecule are free to rotate over a considerable angular range, recent nanosecond fluorescence studies of monoclonal anti-dansyl antibodies by Reidler et al.[124] indicate some restriction of the seg-

Table 2
EFFECTIVE STRAIN ENERGY AS A FUNCTION OF FAB ANGLE[122]

Order of complex (n)	Ideal Fab angle (degrees)	Effective strain energy (kcal/mol)
2	0	0.36
3	60	0.13
4	90	0.05
5	108	0.02
6—9	120—140	0

mental motion of the Fab arms. This conclusion is based on the observation that the average rotational correlation time of a dansyl probe bound to the Fab combining site was highly sensitive to the binding of "anchor" proteins to the Fc region of the molecule and to the presence of reducing agents, which presumably cleave the hinge disulfide. In addition, these authors found that the average rotational correlation time observed for the intact molecule was larger than that predicted for a molecule with a highly flexible hinge using the hydrodynamic models of Wegener et al.[125] and Wegener.[126] It is unclear, however, whether this apparent restriction of Fab motion on the nanosecond time scale is inconsistent with the equilibrium results of Schumaker et al.

The formalism of Wegener et al.[125] has also been used in a recent reinterpretation[127] of the fluorescence depolarization experiments first performed by Yguerabide et al.[81] In these initial experiments, the decay of anisotropy in the polarized fluorescence from a dansyl hapten bound to an IgG molecule was found to have two components, one with a correlation time (φ) of about 30 ns and the other with a φ of about 170 ns; the shorter correlation time, φ_s, was interpreted in terms of restricted rigid-body motion of the Fab arms of the molecule, and the longer correlation time, φ_L, was interpreted as rotation of the whole molecule.[81] The techniques introduced by Yguerabide et al. have been refined and extended in a number of laboratories.[120,121] Removal of aggregates from the anti-dansyl antibody sample reduces φ_L to about 110 ns for example,[128] and other probes have been used to look for motions with somewhat longer correlation times;[129] a two-component decay is consistently observed, however. Hanson et al.[127] repeated the basic experiments of Yguerabide et al. with careful attention to sample preparation and more sophisticated techniques of data analysis, obtaining values of about 16 ns and 90 ns for φ_s and φ_L, respectively. These authors argue that φ_L is too short to be the rotational correlation time of the whole molecule (estimated from the sedimentation coefficient of the sample to be about 170 ns); they suggest instead that φ_L corresponds to "waggling" or "wobbling" motions of an Fab arm and so "twisting" of the arm or to flexibility in its elbow region (Figure 7).

What remains unclear from these recent fluorescence depolarization studies is the precision to which one can determine φ_L and φ_s. Reidler et al.[124] used analytical methods similar to those described by Hanson et al. and obtained similar values of 23 ns and 99 ns for φ_s and φ_L. However, the former authors found that their anisotropy data could be fit by a range of φ_s and φ_L values. In the absence of numerical estimates of the precision of φ_s and φ_L, it is difficult to assess the degree of confidence with which one can reject a given model for Fab motion or confirm the available theoretical formalisms, although there can be little doubt that the Fab arms of an IgG molecule display independent motion on the nanosecond time scale. If the analysis of Hanson et al. is reasonably correct, it would appear that the motion detected by fluorescence depolarization studies can be described to a first approximation as

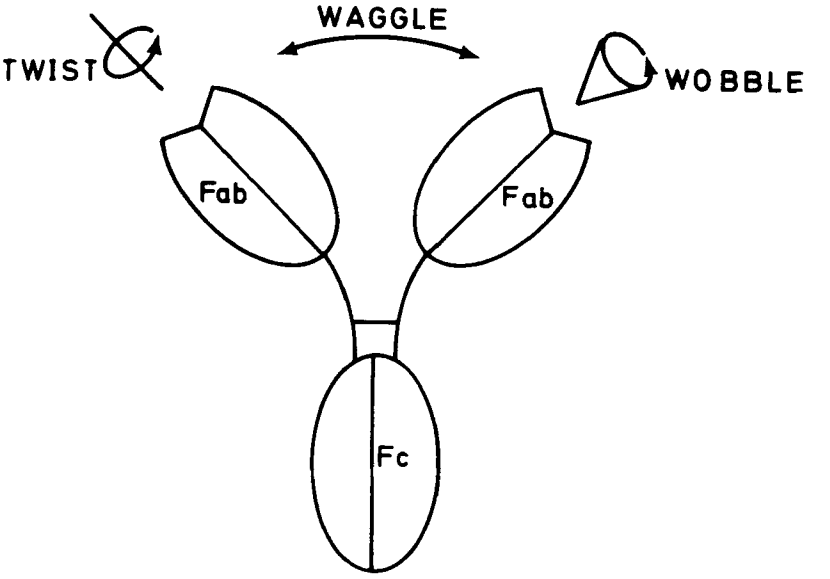


FIGURE 7. Schematic diagram of an IgG molecule illustrating the definitions of the terms "twist", "waggle", and "wobble". (Redrawn from Ref. 127.)

simple rotational diffusion about joints in the hinge and possibly the elbow regions of an IgG molecule; as emphasized by these authors, the calculation of rotational correlation times involves too many approximations to accurately characterize the degree to which such motions are restricted or resisted.

One aspect of antibody structure in solution that is of particular interest in the context of our discussion of domain flexibility is the frequent disagreement between the molecular dimensions of antibodies and their fragments obtained by single-crystal diffraction and those found with small-angle scattering. Most small-angle scattering studies indicate that both intact immunoglobulins and their fragments have substantially larger average dimensions in solution than in the crystal.[54] The nature of the discrepancies is best illustrated by the small-angle X-ray scattering studies of the Kol myeloma protein[131] and its fragments,[130] as the crystal structure of the Kol Fab segment is known.[89,93,105] The radius of gyration (R_G) calculated from models based on the crystal structure of the Kol Fab was 28 Å, while the experimental R_G was 32 ± 2 Å; the differences between the crystallographic model and the best model derived from the small-angle scattering studies are shown in Figure 8, along with the agreement of the two models with the experimental scattering curve. In the absence of a crystallographic model for the entire Kol molecule, an end-to-end distance of 210 Å for the Fab arms of the molecule was estimated from the small-angle scattering data, which is considerably larger than the value of about 150 Å found in the crystal.[89] Similar discrepancies were found between the experimental R_G of the Kol Fc (32 ± 2 Å) and that calculated from models based on the crystal structure of the Fc fragment from pooled human serum[95] (29 Å). The shape of the Fc model that produced the best fit to the small-angle scattering data, two circular cylinders placed side-by-side,[130] is also considerably different from that found in the crystal, where the CH_2 domains are separated; a similar cylindrical Fc model was used to fit the small-angle scattering from the intact Kol molecule.[131]

More recent small-angle X-ray and neutron scattering studies of porcine antibodies[132] indicate that satisfactory fits to the scattering curves can be obtained by models more similar in shape to the crystalline fragments, although the overall dimensions of these models are also much larger than those obtained in crystallographic studies. A recent neutron scattering

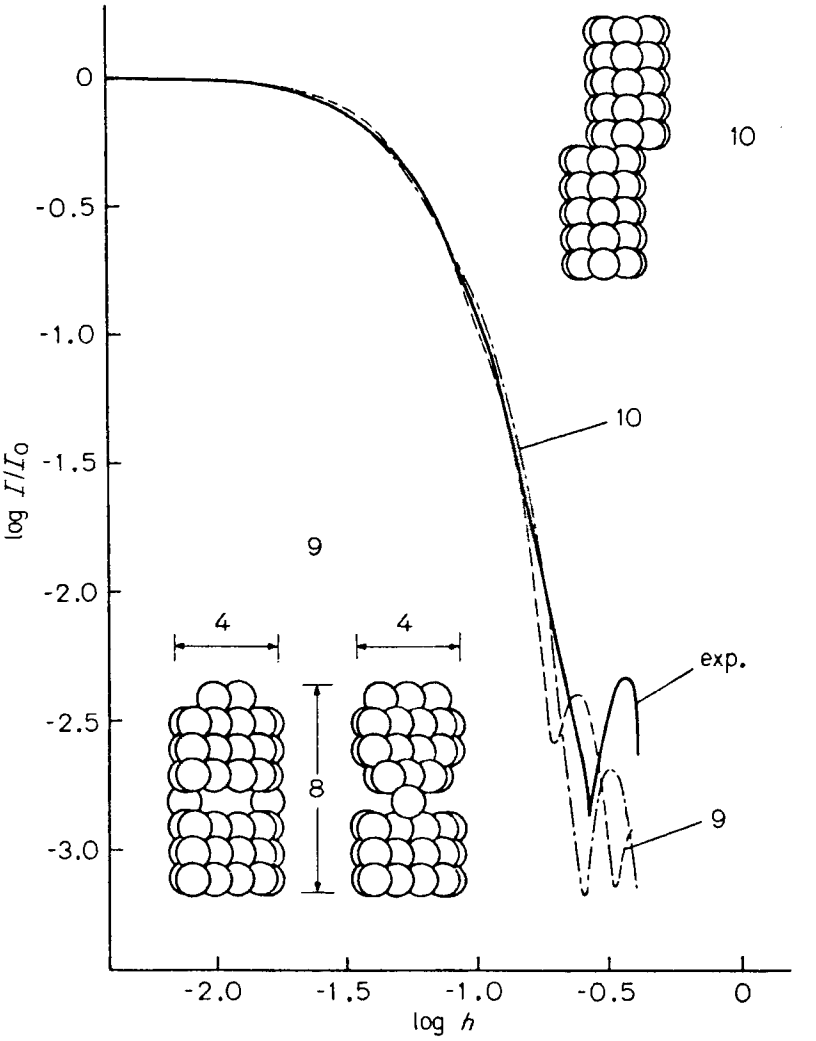


FIGURE 8. Comparison of models for the Kol Fab fragment derived from single-crystal diffraction studies (model 9) and small-angle X-ray scattering studies (model 10); the dimensions of the crystallographic model are given in nanometers. The experimental scattering curve for the Kol Fab is drawn as a solid line, and the scattering curves calculated from the models are drawn in broken lines. (From Ref. 130.)

study of the Mcg light-chain dimer by Schiffer et al.[133] provides the only example of reasonable agreement between small-angle scattering and single-crystal diffraction results. The experimental R_G in this case (24.0 ± 0.4 Å) is consistent with a model having the dimensions of the crystallographic Mcg structure but an elbow angle of 120 ± 6°; this range of elbow angles lies between the angles seen in the two crystal structures for this molecule (Table 1). Radii of gyration considerably smaller than those found earlier have been reported for another recent small-angle X-ray diffraction study of rabbit IgG.[420] The experimental R_G for the Fab fragment of these molecules was 22.6 ± 0.2 Å; from the elbow-angle calculations presented by Schiffer et al., one can estimate that an elbow angle of about 100° would be required to account for this low R_G value.

If the connecting segments between immunoglobulin domains are flexible, one would not expect the average conformation of the molecule in solution to correspond to any individual conformation that has been trapped in crystal lattice, so the fact that the dimensions derived

for immunoglobulins and their fragments from solution-scattering experiments are different from those found in the crystalline state is not in itself surprising. What is not clear is why the dimensions found in solution are almost always larger. One potential explanation that should be mentioned is that the samples used for small-angle X-ray scattering studies are polymerized by exposure to X-rays. It is unlikely that this process can account for the discrepancies discussed above, as the problem of sample polymerization is well known to workers engaged in small-angle scattering studies and would probably be detected.[55] Moreover, in at least one case, good agreement has been obtained from X-ray and neutron scattering studies of the same samples.[132] Since neutron radiation does not lead to sample polymerization; the similarity of the results obtained by these two methods argues against a significant contribution of radiation-induced sample polymerization to the discrepancies between the molecular dimensions found by small-angle scattering and single-crystal diffraction.

The question of whether these discrepancies result simply from hinge or elbow flexibility in the immunoglobulin molecule cannot be assessd so easily, however. A major difficulty encountered in the interpretation of small-angle scattering results is that there is no unique solution to the problem of fitting the experimental data. Reasonably strict limitations can be placed on possible models if one requires that the model predict both the average dimensions and the scattering curve observed experimentally, but the selection of a model can be a time-consuming, trial-and-error process.[130,132] For this reason, the small-angle scattering results on immunoglobulins have been interpreted in terms of a single equivalent structure; this approach has yielded models in good agreement with crystallographic results in a number of other systems.[54] Although it seems reasonable to expect that domain flexibility of the antibody molecule in solution accounts for some of the discrepancies between the small-angle scattering and crystallographic results, it may be difficult to describe a population of such molecules with a small enough number of parameters to test this idea experimentally.

It is generally accepted that domain or segmental flexibility in antibody molecules serves the function of allowing a single molecule to recognize pairs of antigenic determinants with different spatial separations and orientations with respect to one another. However, there is relatively little direct evidence for the involvement of flexibility in immunoglobulin function. The observations that IgG molecules whose hinge regions are deleted are defective in a number of biological functions[86] and that precipitating antibodies are more flexible than nonprecipitating antibodies[134] are consistent with this conclusion, however. In addition, a number of authors have suggested that antigen binding produces some conformational change in the antibody molecule which causes a change in the Fc segment, the region of the molecule recognized by other components of the immune system; these proposals are reviewed elsewhere.[120,121,135,163]

2. Protein A from Staphylococcus aureus

Protein A is a component of the cell wall of *Staphylococcus aureus* that binds to the Fc segment of IgG molecules. The extracellular portion of the protein released by lysostaphin digestion[136] is a highly elongated molecule[137] containing four mutually homologous Fc-binding regions[138] that can be separated by tryptic digestion.[139] The structure of one of these fragments (Fragment B) is known from crystallographic studies of the FB:Fc complex discussed earlier.[96,97] Fragment B has a compact structure consisting of two antiparallel α-helices and a poorly ordered strand of extended polypeptide; the termini of the fragment, which would correspond to the linking segments in the intact protein, are disordered in the crystal.

There is no direct evidence that the domains of protein A are flexibly linked, although hydrodynamic studies[157] have ruled out a compact, globular structure. The susceptibility of the polypeptide segments that join the domains of protein A and the other multidomain

proteins discussed below clearly suggest that these segments are exposed in the intact molecule but does not require that they have the same degree of flexibility as is found for the connecting segments of immunoglobulins. Although the observation of disordered chain termini in the crystal structure of isolated Fragment B is consistent with some flexibility in the linkages between domains of protein A, it is inconclusive; these segments could well be ordered in the intact molecule, where interactions with other regions of the molecule might stabilize a particular conformation.

3. Ovomucoid Protease Inhibitor Fragments

A number of protease inhibitors consist of several mutually homologous domains, which can often be separated by proteolytic digestion (reviewed by Laskowski and Kato).[140] The avian ovomucoid inhibitors, members of the Kazal family of inhibitors, consist of three such domains. The structures of two ovomucoid domains are known, the third domain of Japanese quail ovomucoid[141,142] and the third domain of turkey ovomucoid complexed with a bacterial protease.[143] The two homologous inhibitor fragments have very similar compact structures in spite of their different environments, indicating that they probably exist as independently folded domains in the intact inhibitors; the structure of a monomeric Kazal inhibitor complexed to trypsinogen has also been determined recently[148] and is similar to the ovomucoid fragments, although the primary sequence of this porcine pancreatic secretory inhibitor is substantially different from those of the ovomucoid fragments. The N-terminus of the turkey ovomucoid fragment is disordered in the crystalline complex,[143] and the N-termini from different molecules of the Japanese quail fragment interact in the crystal to form tetramers;[141] as the fragment does not aggregate in solution, this tetrameric association is probably a crystal packing artifact and does not reflect the conformation of the N-terminus of the fragment in solution. The latter observation, like the disordered N-terminus of the turkey ovomucoid fragment, is consistent with some flexibility in the linkages between domains of the intact ovomucoid but is similarly inconclusive.

As is the case for protein A, there is no direct evidence for flexibility in the intact ovomucoid inhibitors. The observation of repeated domains per se does not necessarily imply flexibility. Repeated structural segments are known to form essentially rigid, closed structures in several proteins; carp muscle calcium-binding protein[144] and rhodanese,[145] for example, have two similar segments in each chain, while wheat germ agglutinin[146] and γ-crystallin[147] have four segments in each chain. A stable, closed aggregation of the Kazal-type domains is unlikely, however, as members of the family are known to have from one to seven homologous domains.[140]

4. Other Proteins with Flexibly Linked Domains

Proteins with flexibly linked globular domains appear to be quite common, although no other example of this type of domain flexibility is as well characterized as the immunoglobulins. Evidence comparable to the early indications for segmental flexibility is available for several proteins; below we list a few examples characterized by stable fragments that are readily separated by limited proteolytic digestion and, in most cases, by hydrodynamic or electron-microscopic evidence consistent with domain flexibility. The list is not meant to be complete but is intended to illustrate other systems in which flexibly linked domains may serve a biological function. As with the immunoglobulins and the other proteins discussed above, a role for flexibility in these systems can be rationalized in terms of the need to bind multiple ligands with variable orientations.

Complement Component C1q — The C1q subcomponent of complement is a large protein complex consisting of 18 polypeptide chains with a total molecular weight of about 400,000 (reviewed by Porter and Reid).[149] In electron micrographs, the complex resembles a bouquet of tulips. The heads of the tulips can be identified with globular regions of the

molecule that bind the Fc segment of immunoglobulins, the first step in the activation of the complement system by the classical pathway. The stalks of the tulips correspond to collagenous sequences of the C1q complex. The globular and collagenous regions of the molecule can be separated by limited digestion with pepsin, but there is as yet no direct evidence that this joint is flexible. Small-angle scattering studies of C1q[150] have been interpreted as indicating that the collagenous regions of the complex are essentially rigid, however.

Spectrin — Spectrin is another large protein which is a major component of the "cytoskeleton" of erythrocytes (reviewed by Marchesi)[151] and other tissues.[152] Erythrocyte spectrin is found as a dimer of unlike subunits with a molecular weight of about 500,000 or as a tetramer consisting of two such dimers. A number of hydrodynamic studies suggest that spectrin has a highly elongated, flexible structure, and electron micrographs (reviewed by Cohen et al.)[153] are also consistent with this model. Recent studies[154,155] have shown that the spectrin molecule can be separated into several domains by proteolytic digestion, some of which can be associated with functional or antigenic properties of the native molecule.

Fibronectin and laminin — Fibronectin and laminin are large proteins found in the extracellular matrix of animal tissues; their molecular weights are about 450,000 and 900,000, respectively. Both proteins are composed of several disulfide-linked polypeptide chains and both can be separated into functional fragments by proteolysis (reviewed briefly by Engel et al.).[156] Electron-microscopic studies indicate that both proteins consist of elongated strands or "arms" (two for fibronectin and four for laminin) and that the joints between the arms have limited flexibilty.[156] The arms of fibronectin appear to bend preferentially in specific regions, while the arms of laminin seem to be relatively rigid. Recent physicochemical and thermodynamic studies[157] indicate that proteolytic fragments of fibronectin retain their structure and thermal stability when separated from the rest of the molecule.

B. Proteins with Domains Undergoing Order-Disorder Transitions

In this section we describe a rather different type of domain flexibility in which a protein may have a "domain" of disorder. We make a distinction here between a disordered region of a globular protein (or domain) and a globular domain or segment of a protein, such as the Fc segment of the crystalline Kol or Zie immunoglobulins discussed earlier, that is crystallographically disordered due to a flexible linkage with the rest of the protein. The problem of determining whether the observed disorder is static or dynamic is clearly the same for this type of disorder as for others.

1. Trypsinogen

The most extensive disordered region documented to date is found in trypsinogen, the inactive zymogen of trypsin. This region has been termed the "activation domain", as it is ordered in the active enzyme.[166] The activation domain consists of four segments of the polypeptide chain which form a contiguous patch on the surface of the molecule (Plate 1) upon activation or stabilization with strong, specific ligands such as the pancreatic trypsin inhibitor (PTI). Together these segments account for about 15% of the molecule and include, but are by no means limited to, the substrate binding site of the enzyme.

a. Evidence for Disorder

The brief description of the disordered domain of trypsinogen given so far is based on the structure solved in Munich by Bode et al.[167] and refined at 1.8 Å resolution by Fehlhammer et al.[166] A somewhat different view of the activation domain was obtained from a structure solved independently and partially refined by Kossiakoff et al.[168] in Pasadena. For this reason, some care has been taken to establish that the disorder observed in the Munich structure is genuine. We will briefly review this evidence in the course of a more detailed

description of the two trypsinogen models, as some confusion on this point still exists, even in recent reviews.[69]

The basic difference between the two models is in the extent of the disorder found in the activation domain. In the Munich structure, the ordered electron density terminates abruptly at the boundaries of the activation domain, and no interpretable electron density is found in the region that should be occupied by these segments of the polypeptide chain. Although it was not possible to distinguish between static and dynamic disorder, Bode et al.[167] were able to place a lower limit on the extent of the disorder. Isolated peaks of electron density, approximately one third as high as the density in well-ordered parts of the structure, could be found in or near the region where the activation domain should have been in their initial electron density map. This observation indicates that if the disorder were static, not more than a third of the molecules in the crystal could have the same conformation; thus there would be a minimum of three essentially resolvable conformations of the activation domain, assuming equal population of the conformations. In the Pasadena structure, most of the same segments of the polypeptide chain were characterized as loosely organized or having a large degree of libration.[168] Nonetheless, a single, predominant conformation was suggested, having for some segments about half the electron density as well-ordered regions of the molecule.

Four segments of the molecule identified as disordered in the Munich structure or loosely organized in the Pasadena structure. (Following the usual convention, the trypsinogen[169,170] is numbered here according to the homologous chymotrypsinogen sequence.[171]) The autolysis loop (residues 142-153), so named because it contains sites of autocatalytic cleavage in both trypsin[173] and chymotrypsin,[174] is also partly disordered in the refined structures of benzamidine-inhibited trypsin,[175] diisopropylfluorophosphate-inhibited trypsin[176] (DIP-trypsin), and the trypsin-PTI complex;[177] however, the weak electron density in this segment may result in part from the presence of varying amounts of α-trypsin, the cleaved form of the enzyme, in the different crystal forms.[176,178] The segment of the activation domain containing asp189 (residues 184-193) forms part of the specificity pocket in the active enzyme, and the segment containing cys220 (residues 217-223) is near the region of the substrate binding site (approximately residues 214 to 216) that forms a short intermolecular β-structure with the backbone of the substrate;[179-180] the latter segment is not described as loosely organized in the Pasadena model but has a conformation different from that found in DIP-trypsin. Flexibility in the segments of the activation domain forming the specificity pocket and the peptide binding site is particularly interesting because they are linked by an intramolecular disulfide bond between cys191 and cys220.[172] Disorder at the amino terminus of the molecule (which is val10 in the chymotrypsin numbering convention) is less surprising, as residues 10-15 are released from the molecule in the activation process and need not have a stable binding site on the enzyme.

The crystal data for the two trypsinogen structures are virtually identical, although the crystallization conditions are dissimilar: 1.5 *M* $MgSO_4$, pH 6.9 and 30% ethanol, pH 5-8, for the Munich and Pasadena structures, respectively. As the two crystal forms have the same trigonal space group and cell constants, one would normally assume that they are isomorphous and thus that the structure of the molecule should be the same in each form. A direct test of this assumption could be made by comparing diffraction data from the two crystal forms. This has not yet been done. However, both groups have used an isomorphous, trigonal crystal form of trypsin to test the validity of their structures. The activation domain appeared prominently in a difference map between trigonal trypsin and trypsinogen that was calculated with phase information from the Munich structure of trypsinogen (see Figure 2 of Reference 166); i.e., the two structures did not cancel out in this region as in the rest of the unit cell. This observation shows that the refined trypsinogen structure is essentially correct, as the phase information from this structure is clearly sufficient to locate the activation

domain when these segments of the molecule are ordered. This experiment also demonstrates unambiguously that the activation domain of the trypsinogen molecule in the Munich crystals is disordered relative to the trigonal trypsin structure. A similar experiment was performed by the Pasadena group and was found to be consistent with the predominant alternate conformation of the activation domain reported by Kossiakoff et al. The most convincing evidence for the Pasadena interpretation, however, is the observation that difference electron density for the alternate conformation appears in a difference map between the observed diffraction data and those calculated with a model from which parts of the activation domain were omitted. Difference maps of this type are a commonly used test of a protein model, as they allow the electron density of the omitted segments to be visualized without bias from the model. Similar calculations have been performed repeatedly for the Munich structure in the course of refinement with data in different solvents and at different temperatures[181] (discussed below); no density has been observed for the activation domain under any of the conditions examined. Thus, the idea that the two trypsinogen crystal forms are isomorphous is clearly misleading. There would seem to be little doubt that the two structures have been interpreted essentially correctly and differ in the degree of order of their activation domains.

As was outlined in the Introduction, the degree of disorder in a protein is best characterized by the B factors of a refined structure. Refinement of the Munich structure and its derivatives has been reported.[166,181] Refinement of the Pasadena structure is nearing completion, and the current model supports the original interpretation of Kossiakoff et al.[182] A more detailed comparison of the two models should be possible in the near future.

The reason for the differences between the two trypsinogen structures is unknown. The obvious possibility that the rather different properties of the crystallization media caused some change in the order of the activation domain was tested soon after the discrepancies between the two structures became known.[183] In these experiments, crystals grown in $MgSO_4$ were transferred to a polyethylene glycol solution; no significant difference in the structure of the protein in these two solvents was observed. Similar observations have been made recently using crystals grown in $MgSO_4$ and transferred to methanol solutions for low-temperature studies.[181] Another difference in the crystallization procedures is that the Munich group used pancreatic trypsin inhibitor (PTI) to prevent activation of the zymogen, while the Pasadena group used benzamidine. As PTI is relatively large, any molecules of trypsinogen complexed with this inhibitor would be excluded from the trypsinogen lattice, but a trypsinogen (or trypsin) molecule bound to benzamidine could fit in the lattice. This possibility is of interest because the binding of benzamidine might induce the zymogen to adopt a trypsin-like conformation at the active site, as p-guanidinobenzoate does (see below). No density is observed for benzamidine at the active site of the Pasadena structure, so this explanation is unlikely. The discrepancy could also be explained if the crystals used by the Pasadena group contained substantial amounts of trypsin in place of trypsinogen. Both groups took care to avoid activation of the samples, however.

Two other possible sources of discrepancy that should be mentioned involve the different procedures used in the two trypsinogen structure determinations. The Pasadena structure was solved independently with heavy-atom substitutions, while the Munich structure was solved by positioning a modified trypsin model in the trypsinogen unit cell with Patterson search methods.[167] The evidence that the Munich structure is correct, described in the previous paragraph, rules out the possibility that these procedural differences could account for the differences in the two trypsinogen structures. It has also been suggested[69] that the use of very low-resolution Fourier terms to compute electron-density maps of the Pasadena structure might affect the apparent order of the activation domain. This proposal has been tested recently by Walter et al.,[181] who found that the use of very low-resolution Fourier terms did not improve the interpretability of this region of the Munich structure.

The cause or causes of the differences in the activation domains of the two crystal structures

may well be quite subtle; there is general agreement on the location of the flexible segments in trypsinogen,[186] and the small discrepancies in the apparent extent of the flexible segments probably result from the different degrees of disorder in the two structures. The observations outlined above do not, for example, rule out an effect of the crystallization medium that is irreversible in the crystal; intra- or intermolecular contacts might be formed that are not broken when the solvent is changed. This possibility can be examined by a careful comparison of the refined trypsinogen models. More extensive comparisons of data and materials have been proposed, if necessary.[186]

The more interesting questions of the nature and degree of flexibility of the activation domain in solution will be even more difficult to answer. In discussing studies that address these questions in succeeding paragraphs, we will refer to the "disorder" in trypsinogen, as most of the crystallographic studies were performed on crystals grown initially under the same conditions as the Munich trypsinogen crystals. However, there is no evidence to suggest that one model or the other provides a better description of the structure of the protein in solution.

b. Transition to Order

There is general agreement that the reduced activity of trypsinogen and chymotrypsinogen results from their inability to bind substrates. Kinetic studies of the reduced activity of the zymogens of these serine proteases (reviewed by Neurath)[187] indicates that the zymogens are primarily defective in their ability to bind substrate; in addition, crystallographic studies of chymotrypsinogen[184,185] and trypsinogen[166,168] show that the catalytic residues of the active sites of the zymogens have conformations very similar to those found in the active proteases. In chymotrypsinogen, the substrate binding site appears to have a different conformation than in the active enzyme, while this region of trypsinogen is disordered to some degree, as we have seen. The function of the disorder in trypsinogen is thus to prevent the zymogen from functioning at inappropriate times. Studies of the transition to the ordered, active state are of interest both because of this involvement in the function of the enzyme and because they provide additional insight into the sources and long-range effects of the domain disorder.

The activation domain of trypsinogen can be ordered in two ways. One way is by activation of the zymogen to trypsin; upon cleavage of the peptide bond between arg15 and ile16 of the zymogen, the new N-terminus folds into the molecule to form an internal salt bridge with asp194. (A number of aspects of zymogen activation are reviewed in detail by Neurath.)[187] The other way to order the activation domain is to bind a strong ligand of the active enzyme to the zymogen. PTI, for example, binds trypsin and trypsinogen with dissociation constants of 6×10^{-14} M[188] and 2×10^{-6} M,[189] respectively; the difference in binding constants presumably reflects the energy required to order the activation domain of trypsinogen.[197]

Crystallographically, ordering of the activation domain by ligands is observed to occur in two steps. The structure of a complex between trypsinogen and PTI has been solved and refined at 1.9 Å resolution;[191] as these crystals are isomorphous to crystals of the trypsin-PTI complex,[177] a direct comparison of the two structures by the calculation of a difference electron-density map was possible. This analysis shows that two segments of the activation domain are ordered by the binding of PTI. Fixation of these residues forms a small cavity near asp194 which is filled by the N-terminus in trypsin but occupied by several solvent molecules in the trypsinogen-PTI complex. The N-terminal segment of trypsinogen remains disordered in this complex, and the autolysis loop, while partially ordered, remains considerably more disordered than in the trypsin-PTI complex; the latter segment also contributes to the binding cavity for the N-terminus in trypsin and its complex with PTI.

When the dipeptide ile-val, corresponding to the N-terminal residues of trypsin, is added to trypsinogen-PTI crystals at a concentration of 0.1 M, it is observed to bind to asp194,

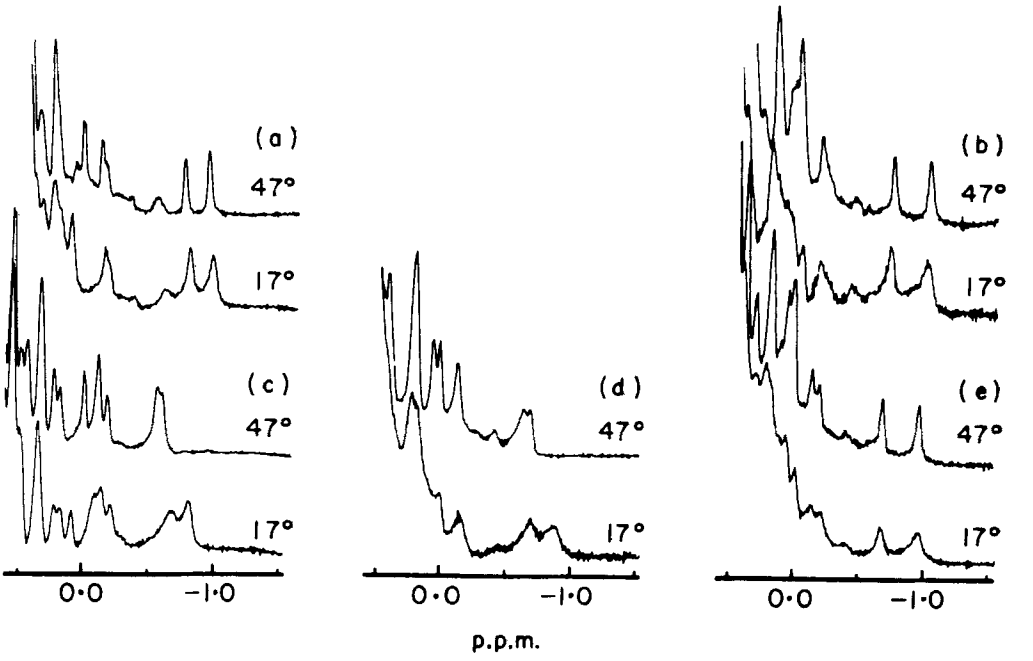


FIGURE 9. High-field proton NMR spectra of trypsin, trypsinogen, and their complexes with PTI in ^2H_2O at p^{2}H 8.0 and the indicated temperature. All experiments were performed in the presence of benzamidine and calcium; the protein components of the spectra are trypsin (a), trypsin and PTI (b), trypsinogen (c), trypsinogen and PTI (d), and trypsinogen, PTI and ile-val (e). (From Ref. 192.)

filling the adjacent cavity just as the N-terminus does in trypsin. In this ternary complex, the autolysis loop is ordered to almost the same degree as the corresponding region of the trypsin-PTI complex, making the two crystal structures essentially indistinguishable, except near the N-terminus. Because the differences in the structure of trypsinogen in these two complexes are relatively small, Bode et al.[191] suggested that trypsinogen is predominantly a two-state system, one state having a disordered activation domain as in the unliganded trypsinogen structure and the other having an essentially ordered, trypsin-like structure.

A similar picture is obtained from NMR studies of the trypsinogen-trypsin system in solution.[192] These experiments show that there are characteristic differences in the high-field spectra of trypsin and trypsinogen (Figure 9); the resonances in this region of the spectrum are assigned to methyl protons near aromatic sidechains, which are shifted to the high-field region by ring-current effects. Binding of PTI to trypsin produces very little change in the high-field spectrum of the enzyme, while the trypsinogen-PTI complex has a spectrum distinct from both trypsin (or the trypsin-PTI complex) and trypsinogen (Figure 9). The high-field spectrum of the ternary complex between trypsinogen, PTI, and the ile-val dipeptide is nearly identical to that of the trypsin-PTI complex, however.

It is interesting that none of the methyl protons giving rise to these high-field resonances are in the activation domain.[192] Thus the effects of PTI binding to trypsinogen observed by NMR, although qualitatively similar to the crystallographic results, pertain to a region of the molecule that is crystallographically indistinguishable from trypsin. Whether this is simply a reflection of the sensitivity of ring-current effects to small changes in structure, as suggested by detailed ring-current calculations,[192] or indicates that trypsinogen adopts a wider range of conformations in solution than the two-state crystallographic model would suggest is not clear.

It is clear, however, that the PTI-induced structure of trypsinogen is considerably less stable than those of either trypsin or the ternary complex of trypsinogen, PTI, and the ile-val dipeptide. The high-field NMR spectra of both trypsinogen and the trypsinogen-PTI complex show distinct dependencies on temperature between 17°C and 47°C, while those of trypsin and the trypsin-PTI complex are almost unchanged[192] (Figure 9); spectra of the ternary complex remain identical to the trypsin-PTI spectra over this temperature range. These observations again refer to regions of the molecule outside the activation domain.

Direct evidence that segments of the activation domain itself are easily deformed has been obtained in recent crystallographic studies of a complex between trypsinogen and the pancreatic secretory trypsin inhibitor (PSTI).[148] In these crystals, unlike the trypsinogen-PTI crystals, a section of the polypeptide chain near the activation domain, residues 21-23, form intermolecular contacts with the inhibitor moeity of a neighboring complex in the crystal lattice. These interactions force the mainchain of the zymogen from tyr20 to pro28 to adopt a conformation different from that found in the structures of trypsinogen and its complex with PTI. Presumably as a result of this distortion, neighboring segments of the molecule, including the autolysis loop, also have conformations differing substantially from those found in trypsin. The autolysis loop in particular is ordered except for residues 146 and 147, but is displaced by as much as 5 Å from its position in trypsin. This novel conformation allows lys156 to interact with asp194, which has the same conformation as in trypsin, and to partially fill the binding pocket occupied by the N-terminus in trypsin. The N-terminal segment of the zymogen is ordered in the trypsinogen-PSTI complex from residue 14 on, with residues 17 to 21 in a trypsin-like conformation. This feature of the trypsinogen-PSTI structure also results indirectly from the changes near residue 23, as the conformation of the N-terminus is stabilized in part by interactions with other regions of altered structure, including the autolysis loop (Figure 10). The other two segments of the activation domain adopt a trypsin-like conformation, possibly because they interact strongly with the inhibitor in a region where its structure is complementary to the structure of trypsin.

The implication of both the crystallographic and NMR results just described is that the structure of trypsinogen or inhibitor-stabilized trypsinogen is rather soft (i.e., easily deformable) in the absence of the proper contacts in the ile-val pocket, especially in the activation domain. In this regard, it is interesting that the addition of ile-val dipeptide to trypsinogen-PSTI crystals causes them to crack,[148,193] suggesting that the dipeptide displaces lys156 from the pocket and forces the trypsinogen component of the complex to adopt a more trypsin-like conformation that is incompatible with the crystal packing. Whether this interpretation is correct or not, it is clear that the stucture of trypsinogen in the crystalline complex with PSTI is influenced by crystal packing interactions, so a two-state model for the structure of trypsinogen cannot be ruled out on this basis. The relationship of ile-val binding to the stability of the activation domain and the substrate binding site also provides some confirmation of the function of the disorder in trypsinogen, as the integrity of the salt bridge between asp194 and ile16 is known to be essential for the activity of both trypsin and chymotrypsin (see Kumar et al.[194] and references therein).

Studies in solution using another method of trypsinogen-trypsin conversion show, however, that this process requires more than compensation of the charge of asp194; in addition, these experiments provide some information about the thermodynamics and kinetics of the transition. The reagent p-nitrophenyl-p′-guanidinobenzoate is hydrolyzed by trypsin and, more slowly, by trypsinogen, forming specific acyl adducts to ser195 at the active site of the protein.[195] Circular dichroism (CD) spectra of p-guanidinobenzoyl-trypsin (pGB-trypsin) display a characteristic negative ellipticity at about 270 nm which is not observed for pGB-trypsinogen. The CD spectrum of pGB-trypsinogen can be shifted to that of pGB-trypsin either by activation of the acyl-zymogen[195] or by addition of ile-val dipeptide.[196] The latter observation suggests that the dipeptide binds to pGB-trypsinogen, shifting the acyl-zymogen to a trypsin-like conformation.

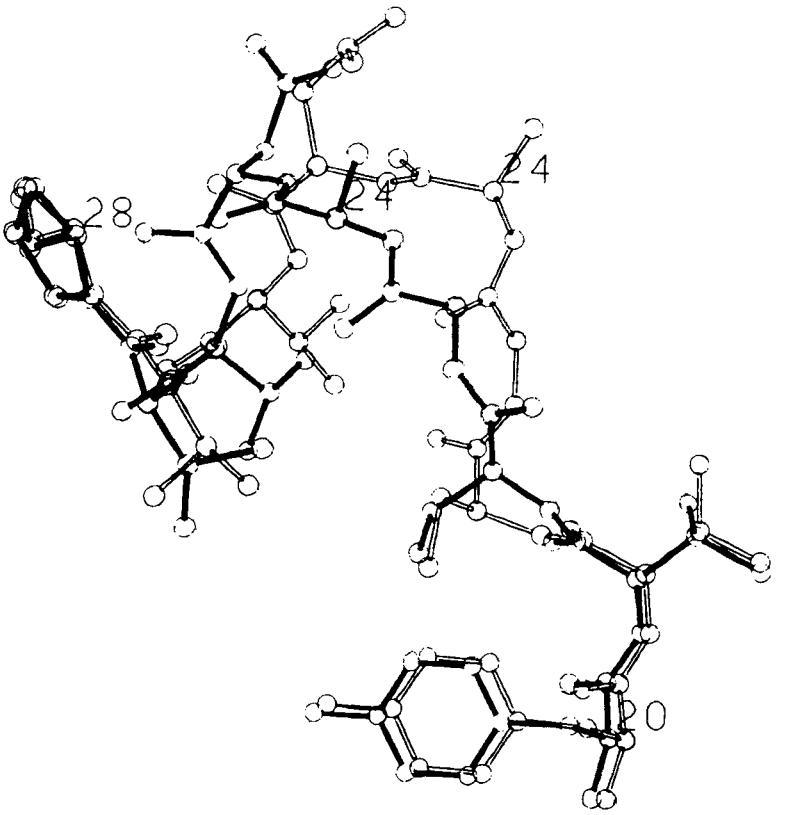


FIGURE 10. Structural differences between the trypsinogen-PSTI complex (Tg-PSTI, drawn with solid bonds) and the trypsin-PTI complex (T-PTI, drawn with open bonds) in the region between residues 20 and 28. (From Ref. 148.)

It is unclear how closely the complex between pGB-trypsin and ile-val resembles pGB-trypsin or the ternary complex of trypsinogen, PTI, and ile-val, however. The characteristic features of the CD spectrum of pGB-trypsin have been assigned to the pGB group[195] and thus are sensitive to the structure of the protein only in the vicinity of the active site. Neither the activity of trypsinogen toward small, synthetic substrates[196] nor the rate at which it is acylated by the pGB reagent[197] is affected by the addition of ile-val dipeptide; however, the deacylation rate of pGB-trypsinogen in the presence of 0.01 *M* ile-val is essentially the same as that of pGB-trypsin and some 60% slower than that of pGB-trypsinogen alone.[197] The latter observations can be interpreted as indicating that neither the covalently bound pGB group nor the ile-val dipeptide alone is capable of shifting trypsinogen to a trypsin-like conformation by itself, but that the cooperative effect of both ligands is. Direct crystallographic determination of the structure of the pGB-trypsinogen complex with ile-val may not be possible, as the half-life of the acyl complex, about 320 min at 25°C, is probably too short to allow it to be crystallized. In spite of this uncertainty about the structure of the pGB-trypsinogen complex outside the active site, the similarity in the CD spectra of pGB-trypsin and the complex clearly indicates that at least the conformation of the active site of the trypsinogen component of the complex is quite similar to that of trypsin.

Bode[197] has studied the ability of a number of peptides and other compounds to shift pGB-trypsinogen to a trypsin-like structure. By titration of pGB-trypsinogen with each compound, it could be established that binding was specific for a single site over the concentration range studied, and the affinity of the ligand for this site, expressed below as

the standard free energy of binding, could be determined. Neither calcium, which is known to stabilize the structures of trypsin and trypsinogen,[198] nor isoleucine alone has any appreciable affinity for the acyl zymogen ($\Delta G° > 0$ kJ mol^{-1}); the latter result was also obtained when the carboxyl group of isoleucine was neutralized by alkylation. The strongest affinity, -24.5 kJ mol^{-1}, was observed for the ile-val dipeptide. These observations demonstrate that specific, nonpolar solvation of the ile-val cavity is required to stabilize the active conformation of the enzyme in addition to compensation of the charge of asp194. The strong conservation of the N-terminal sequence ile16-val17 among serine proteases that are activated from a zymogen (reviewed by Bode)[197] had earlier suggested that this part of the protein would be involved in highly specific interactions. As might be expected from the sequencing studies, other dipeptides that bind strongly to pGB-trypsinogen, such as val-val (with an affinity of -15.9 kJ mol^{-1}), correspond to less frequently observed variants of the N-terminal sequences of this family of enzymes. The affinity of the tripeptide ile-val-gly, corresponding to the first three residues of trypsin, has the same affinity for pGB-trypsinogen as ile-val, indicating that gly18 makes little contribution to the binding; this is consistent with the X-ray structure of trypsin,[175] in which gly18 makes only a few van der Waals contacts with the rest of the protein.

The kinetics of the dipeptide-induced transition of pGB-trypsinogen to a trypsin-like conformation have been studied by Nolte and Neumann.[199] These authors demonstrated that the binding of ile-val to pGB-trypsinogen produces small changes in the UV absorption of the protein. The relaxation of absorption changes produced by a rapid temperature jump was measured to obtain kinetic constants. Identification of this relaxation process with the dipeptide-induced conformational changes associated with changes in the CD spectra of pGB-trypsinogen is supported by the observation that neither pGB-trypsinogen nor ile-val alone exhibits a relaxation under the same conditions. The relaxation of the complex is biphasic, consisting of a slower component, the concentration dependence of which is consistent with the conclusion that a single chemical relaxation process was observed over the range of experimental conditions employed, and a faster component whose time constant is insensitive to the concentrations of pGB-trypsinogen and ile-val. Nolte and Neumann attribute the rapid absorption change to dilution of the reaction mixture upon heating and analyze the slower component in terms of the induced-fit scheme given in Figure 11. The concentration dependence of the slow relaxation process indicated that the reaction proceeds predominantly by the substrate-guided pathway (i.e., by way of the "IT" state in Figure 11). The association rate of the complex was assumed to be diffusion controlled with an equilibrium constant, K_1, of about 2.3×10^{-3} *M*. Rate constants for the transition were found to be about 26 sec^{-1} and 0.6 sec^{-1} in the direction of the IT′ and IT complexes, respectively. As emphasized by Nolte and Neumann, the ordering of the activation domain must involve a large number of individual bond isomerizations that are not resolved by their measurements, so these rate constants cannot be interpreted in terms of molecular events. In particular, too little is known about the rates of such large-scale, cooperative transitions in proteins to allow one to say whether the observed rate constants are consistent with the crystallographically observed order-disorder transition.

The thermodynamic studies of Bode[197] provide two additional insights into the order-disorder transition in trypsinogen. One is that the active conformation of trypsin is only favored by an equilibrium constant of 10^2 over the inactive conformation at pH 8 and 20°C, in contrast to the apparent stability of trypsin from the point of view of X-ray diffraction and NMR measurements. This conclusion is based on the assumption that the loss of activity upon titration of trypsin to higher pH is due to protonation of ile16; presumably the N-terminus would be dissociated from its pocket in this state, and the structure would relax to a trypsinogen-like conformation. Similar estimates for the equilibrium between active and inactive conformations have been reported for homologous serine proteases (reviewed by Bode).[197]

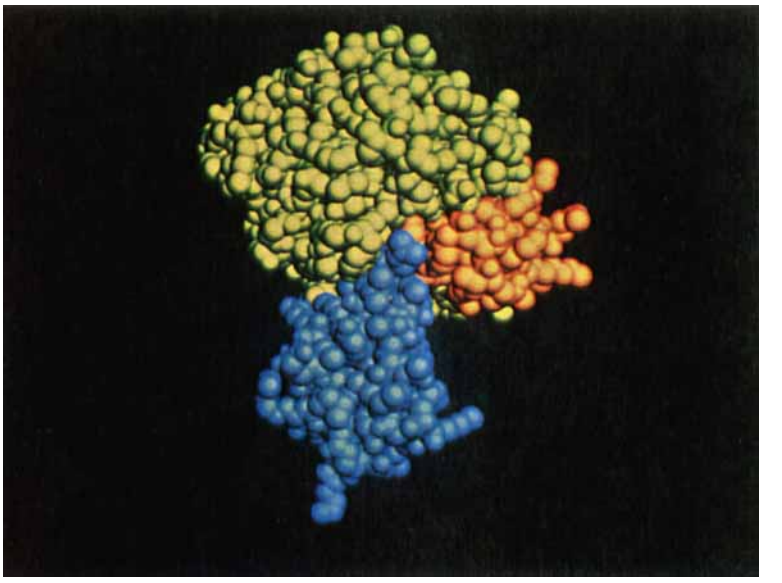


PLATE 1. Space-filling drawing of the trypsin-PTI complex.[179] Regions of trypsin that are ordered in both trypsin and trypsinogen[166] are drawn in yellow and the activation domain is drawn in orange. The location of the active center is indicated by the PTI binding site; the PTI molecule is drawn in blue.

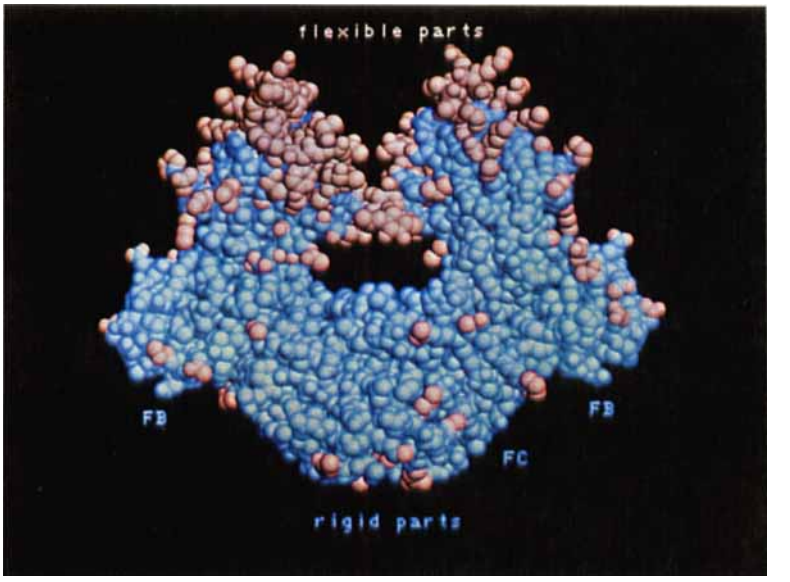
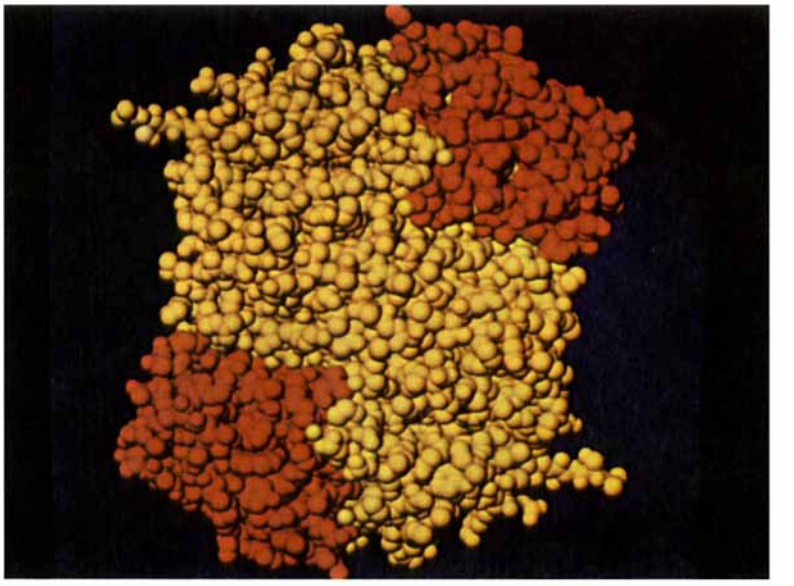


PLATE 2. Space-filling drawing of the FB:Fc complex.[96,97] Disordered atoms of the complex are drawn in red, and ordered atoms are drawn in blue. (Deisenhofer, J., unpublished drawing.)



(A)

(B)

PLATE 3. Space-filling drawings of the open (A) and closed (B) forms of citrate synthetase. The large domains are drawn in yellow and the small domains are drawn in red. (Remington, S., unpublished drawing, based on coordinates described in Reference 304.)

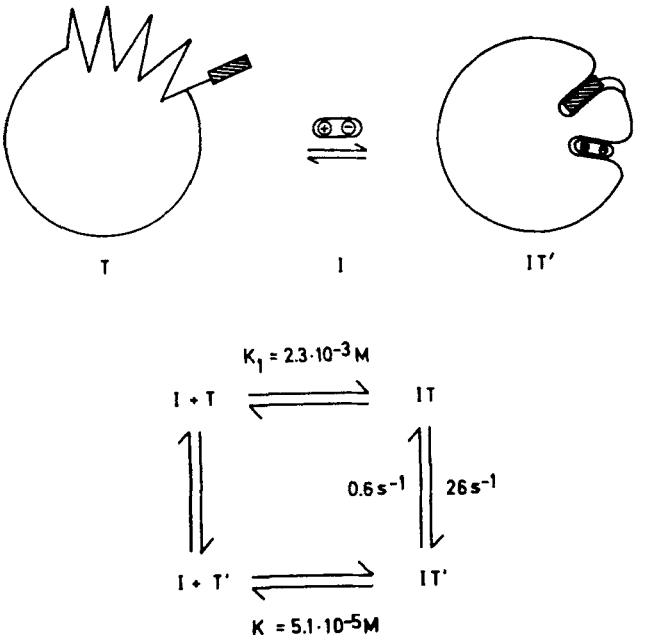


FIGURE 11. Kinetic scheme for the binding of ile-val dipeptide (I) to pBG—trypsinogen (T). States of the system that approximate crystallographically determined structures are illustrated schematically: in pGB-trypsinogen, the activation domain is disordered (crinkled line), and there is no binding site for the covalently bound pGB group (shaded rectangle); in the complex between pGB-trypsinogen and ile-val (IT'), the activation domain is ordered (smooth line), and the binding sites of both ile-val and the pGB group are formed. (Redrawn from Ref. 160; kinetic and equilibrium constants are from Ref. 199.)

A second interesting observation made by Bode is that the association constant for the binding of ile-val dipeptide to the preformed ile-val pocket in the trypsinogen-PTI complex is only about $10^5 M^{-1}$. With this parameter and an estimate of about $10^9 M^{-1}$ for the binding constant of the covalently attached ile-val group at the N-terminus of trypsin for the same pocket,[197] one can estimate the effective concentration of the covalently attached ile-val group as about $10^4 M$. This conclusion provides a convenient explanation for the failure of exogenous ile-val to noticeably stimulate the activity of trypsinogen or to bind to crystalline trypsinogen.[196] The requirement of a high effective concentration of ile-val to produce the order-disorder transition could provide the zymogen with an advantageous insensitivity to free peptides in its physiological environment.

c. Nature of the Disorder

Several recent experiments allow considerably more stringent limits to be placed on the degree of disorder in the activation domain of crystalline trypsinogen and provide some information on the question of whether the disorder is static or dynamic in nature. Walter et al.[181] report crystallographic studies of a chemically modified form of trypsinogen in which a mercury atom is inserted between the sulfur atoms of the disulfide bridge in the activation domain (cys191-cys220). As this mercury derivative of trypsinogen (Hg-trypsinogen) forms crystals isomorphous to those of native trypsinogen, the two forms of the enzyme could be compared directly. A difference electron density map at 2.3 Å resolution shows no significant differences between the two structures that can be attributed to the Hg atom, which was shown by chemical assay to be present at a molar ratio of 1.2:1 in the

Hg-trypsinogen compound. To demonstrate that the Hg atom was in fact inserted between cys191 and cys220, the activation domain was ordered by forming a complex between Hg-trypsinogen, PTI, and ile-val; this complex forms crystals that are isomorphous to crystals of the native complex. A difference map comparing these two complexes at 2.3 Å resolution shows strong difference density at the 191-220 cystine group, a large positive peak corresponding to the Hg atom and smaller negative peaks indicating small displacements of the adjacent sulfur atoms. Refinement of the structure at 2.1 Å resolution confirmed this interpretation and verified that there are no significant differences in other regions of the two structures.

The Hg peak in the difference map between the two complexes is elongated, indicating some disorder in the mercury. The Hg density can be modeled in terms of three slightly separated Hg positions of equal occupancy. This model is not unique, as B factors of 49 $Å^2$ for the central position and about 84 $Å^2$ for the external positions are required to account for the Hg density, suggesting substantial disorder in addition to the threefold static disorder assumed in the model. It is clear, however, that the Hg atom is in the expected position and thus that the absence of density in the comparison between Hg-trypsinogen and native trypsinogen demonstrates the existence of a considerably higher degree of disorder in the activation domain than could be documented in earlier studies. Walter et al. determined by empirical tests that a B factor of at least 200 $Å^2$ would be required to account for the complete lack of density for the Hg atom in their Hg-trypsinogen structures. This would correspond to motion with an r.m.s. displacement of over 1.6 Å if the disorder were dynamic or to at least 30 different conformations if the disorder were static, since Hg has over ten times as many electrons as carbon, nitrogen, or oxygen in a similar volume. The crystallographic experiments described so far cannot, of course, distinguish between these possibilities; moreover, the limits could be somewhat lower if the Hg insertion induces some disorder in the trypsinogen structure, as it does in the trypsinogen-PTI complex. Even when this factor is taken into consideration, however, it is clear that substantial disorder must exist in the activation domain of trypsinogen itself to account for the Hg-trypsinogen results.

Hg-trypsinogen has also been used by Butz et al.[200] in experiments designed to detect motion in the Hg atom on the nanosecond time scale. In these experiments, trypsinogen was labeled with the ^{199m}Hg isotope of mercury, which decays in a cascade of two γ photons with a known angular correlation. In the case of static disorder in the activation domain, one would expect the angular correlation of the γ-emissions to be preserved, while motion in the protein on the order of the halflife (2.35 nsec) of the state of the nucleus between emissions would lead to loss of this correlation. Due to the short lifetime of the ^{199m}Hg species, crystallization of the modified trypsinogen is not possible, so most samples were precipitated with ethanol to avoid obscuring intramolecular motions with tumbling in solution and to separate the protein from any Hg-EDTA remaining from the mercuration step. Comparison of the γ-emission anisotropy of Hg-trypsinogen to that of the complex between Hg-trypsinogen, PTI, and ile-val (Figure 12) indicates that there is loss of anisotropy in the unliganded trypsinogen derivative, corresponding to motions with a correlation time of about 11 nsec, while the Hg atom in the complex shows no wide-angle reorientation in the nsec time range. As they stand, these results cannot be interpreted in terms of static or dynamic disorder in Hg-trypsinogen, since the possibility exists that the recoil energy of the first γ-emission is responsible for reorienting the label;[200] the softness of the trypsinogen structure mentioned earlier is clearly consistent with this explanation. Butz et al. propose to address this question by studying the activation enthalpy of the transition. The experiments reported so far do demonstrate, however, that there are fundamental differences in the stability and order of the activation domains of free trypsinogen and the complex between trypsinogen, PTI, and ile-val.

An attempt was also made by Walter et al.[181] to determine the nature of the disorder in trypsinogen by studying its crystal structure at lower temperatures. One naively expects that

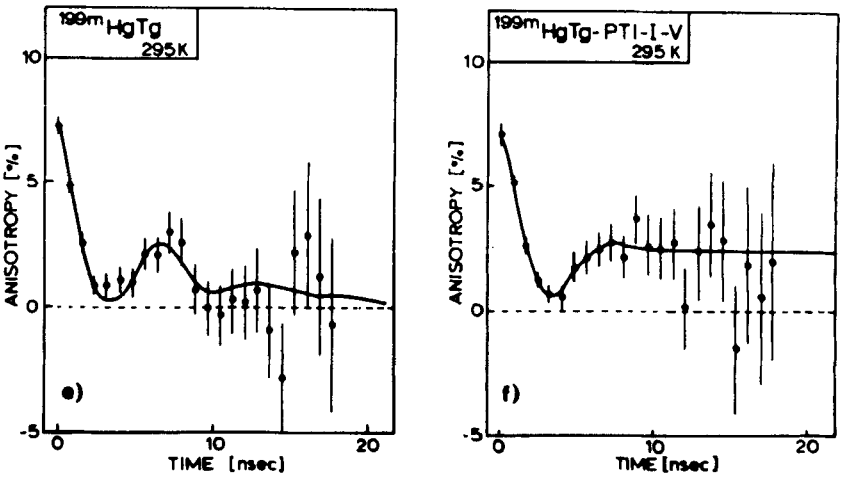


FIGURE 12. Decay of anisotropy of the gamma emissions from ^{199m}HG-trypsinogen (^{199m}HgTg; left panel) and from the complex of ^{199m}Hg-trypsinogen, PTI and ile-val dipeptide (^{199m}HgTg-PTI-I-V; right panel). (From Ref. 200.)

Table 3
LOW-TEMPERATURE STUDIES OF TRYPSINOGEN[181]

		Newly ordered atoms		B factors (Å²)	
Temperature	Solvent	Protein	Solvent	Overall	gly 18[a]
Room	$MgSO_4$	2	0	12.4	60.0
Room	50% CH_3OH	—	—	16.1	60.0
173 K	70% CH_3OH	11	31	11.6	37.7
103 K	70% CH_3OH	12	46	11.5	28.3

[a] An empirical maximum B factor of 60 Å² was imposed on all atoms, as trial calculations indicated that an oxygen atom with a higher B-factor would not be visible in the electron density map in any case.

dynamic disorder will be reduced at lower temperatures, while static disorder will not be affected by changes in temperature. In the experiments of Walter et al., trypsinogen crystals that had been grown from $MgSO_4$ solutions were transferred into 50% methanol to provide a reference structure at room temperature and into 70% methanol for study at 173 K and 103 K. The structure of trypsinogen was independently refined at 1.8 Å or better with data obtained at each of these temperatures. Although some atoms of the protein and a number of solvent molecules become ordered at lower temperatures (Table 3), none of these are in the internal segments of the activation domain. Near the N-terminus, however, gly18 is observed to become progressively more ordered at lower temperature (Table 3), and parts of val17 are visible at the lowest temperature studied. The observation that these residues "freeze out" at lower temperatures strongly suggests that this region of the N-terminal segment of trypsinogen is disordered due to thermal motion in the crystal; interestingly, the positions of these residues are different from those found in trypsin.

Earlier NMR studies of trypsinogen in solution provide direct evidence for dynamic disorder at and near the N-terminus of the molecule. Perkins and Wüthrich[192] observed that most of the NMR spectrum of trypsinogen is composed of broad resonances, with the

exception of two sharp doublets. These peaks could be assigned to val10, the N-terminus, on the basis of their titration behavior and the observation that they are retained upon dialysis of trypsinogen solutions but lost when "activated trypsinogen", a mixture of trypsin and free activation peptide, is dialysed. These results show clearly that the methyl groups of val10 have a unique freedom of motion with respect to the rest of the trypsinogen molecule. The crystallographic and NMR results taken together suggest that the entire N-terminal segment of trypsinogen is probably disordered in the crystal due to thermal motion. This conclusion is consistent with the accessibility of asp11-asp14 to chemical modification[201] and the similar rates at which the lys-ile bond in this segment of trypsinogen and in synthetic analogues of the activation peptide are cleaved.[202]

Although neither of the experiments just described provides information about large-scale flexibility in other regions of trypsinogen, neither the NMR results nor the low-temperature crystallographic studies can rule out dynamic disorder in the internal segments of the activation domain. Another experiment performed by Perkins and Wüthrich[192] to confirm the assignment of the val10 resonances illustrates this point for the NMR work. Knowing that the activation peptide (see inset) contains a unique cluster of four Asp residues

$$\overset{10}{NH_2\text{-val}} - \text{asp} - \text{asp} - \text{asp} - \text{asp} - \text{lys} - \overset{17}{\text{ile}} - \text{val}\ldots$$

that form a binding site for calcium ions,[201,202] these authors sought to resolve resonances from this region of the peptide by replacing calcium with lanthanide broadening reagents such as Gd(III). The difference spectrum between activated trypsinogen samples with and without Gd(III) (Figure 13) shows relatively sharp resonances near the chemical shift value expected for the β-methyl protons of aspartyl residues in a random coil; these resonances could also be assigned to the activation peptide on the basis of their dialysis behavior. The interesting observation from our point of view is that these resonances from the free activation peptide could not be resolved without the application of special techniques, even though the residues involved have considerably higher mobility than those in essentially rigid regions of the molecule. Failure to observe narrow NMR resonances in a protein spectrum clearly does not rule out the existence of substantial independent motion in some segments of the molecule.

Similarly, failure to observe ordering of the internal segments of the activation domain upon cooling trypsinogen crystals[181] cannot rule out dynamic disorder in this region of the molecule. The expectation that the thermal motion of an atom will be "frozen out" at some point by lowering the temperature is based on the assumption that the atom is subject to a simple potential energy function with a single minimum, such as a harmonic potential. Even if the extensive disorder found in trypsinogen were dynamic, it is unlikely that the potential functions of individual atoms within the activation domain can be this simple, as rather large r.m.s. displacements of bonded atoms are required to account for the observed disorder. Each of these atoms presumably interacts with a number of different atoms in the ordered region of the molecule as well as with a number of other atoms in the activation domain at different points within its range of motion.

The behavior of an atom subject to a potential function with multiple minima upon cooling is illustrated in Figure 14. Let us suppose that some atom of a protein in a crystalline lattice is subject to this potential function; x_1 and x_2 are imagined to be resolved positions of the atom. If the thermal energy of the system is at the level marked A, then this atom will exhibit a wide range of thermal motion, and the X-ray structure of the protein will be disordered due to thermal motion at this position. As the temperature of the system is lowered to level B, the corresponding atoms in different protein molecules of the lattice will distribute between the two potential minima centered at x_1 and x_2, and the X-ray structure will be

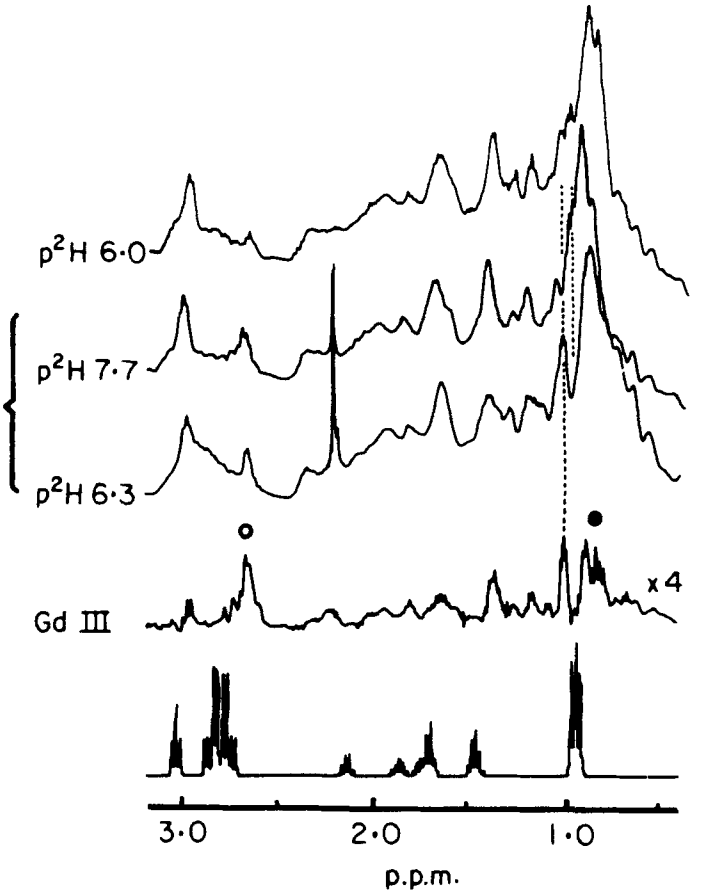


FIGURE 13. Proton NMR spectra of trypsin and trypsinogen showing resonances assigned to the activation peptide. The traces labeled "p²H 7.7" and "p²H 6.3" show the titration of a resonance at 0.9 to 1.0 ppm in the trypsinogen NMR spectrum assigned to methyl protons of val10; the sharp resonances near 2.2 ppm are from a reference compound. The trace labeled "Gd III" is a difference spectrum between two trypsinogen samples that were activated to trypsin and used prior to removing the free activation peptide; one sample contained calcium, and the other contained the broadening agent Gd(III). The open circle marks resonances assigned to methyl protons of the aspartyl residues of the activation peptide, and the filled circle marks resonances tentatively assigned to methyl protons of three valyl residues near the calcium binding site. The upper trace (p²H 6.0) shows a similar sample after dialysis to remove the activation peptide, and the lower trace is a calculated random-coil spectrum for the activation peptide alone. (From Ref. 192.)

disordered due to a combination of static and dynamic effects. Finally, when the temperature is lowered further to freeze out all thermal motion at level C, the X-ray structure will still be disordered at this atom, but the disorder will be static.

It is unlikely that this simple model applies to any of the atoms in the activation domain of trypsinogen, since as few as two resolvable conformations would probably be detectable at the resolution of the studies of Walter et al.[181] However, other observations made by Walter et al.[181] suggest deviations from a simple harmonic potential of an even more complex nature. Two serine O_γ atoms, in residues outside the activation domain, are distributed between two sites at 173 K but "freeze out" in only one of these at 103 K, suggesting a

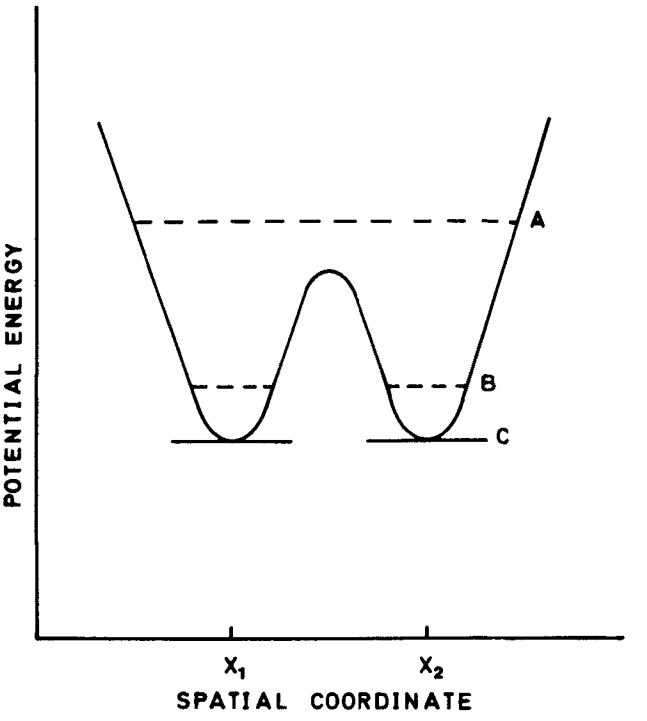


FIGURE 14. Hypothetical potential energy function for an atom of a protein. The symbols A, B, and C mark energy levels asociated with arbitrary temperatures described in the text.

potential function with two unequal minima. The average positions of a number of other atoms are observed to shift significantly as the crystals are cooled, and the B factors of several mainchain and sidechain groups of atoms seem to be significantly higher at lower temperatures than at room temperature. Both of these observations are consistent with changes in the potential functions affecting these atoms as the temperature is reduced; since the resolution of these structures was insufficient to warrant refinement of individual atomic temperature factors and occupancies, however, other explanations cannot be excluded. Some of these anomalies might, for example, result from changes in the crystalline environment due to freezing of the crystal surface at 103 K[181] or changes in the characteristics of the solvent.[203]

In addition to these considerations of factors affecting the behavior of individual atoms, it should be remembered that the ordering of the activation domain of trypsinogen is a concerted process. As is emphasized by the approximately two-state behavior of the system discussed earlier, this process probably exhibits the same general thermodynamic behavior as the folding of a globular protein or other cooperative conformational transitions, such as helix-coil transition of polypeptides. If this is the case, one would still expect lower temperatures to favor structure formation at equilibrium, as the enthalpy of these processes is usually negative in the direction of structure formation.[162] However, when considering the effects of cooling on the conformational transition itself, one cannot rule out *a priori* the possibilities that the kinetics of the transition will be sufficiently slowed at very low temperatures to prevent the system from attaining equilibrium in a time short compared to an X-ray diffraction experiment or that the cryosolvents used in such experiments will not have an unexpected effect on the thermodynamic properties of the transition at low temperatures. Either of these effects could provide an alternative explanation for the failure of the activation

domain of trypsinogen to become ordered at low temperatures, although there is no evidence to suggest that such factors are important in this system. In any case, it is clear that there are both theoretical and experimental complications to the low-temperature study of protein crystals and that failure of a disordered segment to become ordered at low temperatures cannot be taken as evidence of static disorder in the structure at physiological temperatures, even if a low enough temperature has been achieved to freeze out the thermal motion.

At this point in our discussion, some comment should be made on the relationship between the disorder observed in trypsinogen and the concept of "conformational substrates" used in some discussions of protein dynamics. Although it would appear on strictly semantic grounds that some of the phenomena observed by Walter et al.,[181] such as the serine O_γ atoms with multiple conformations, might be described by this phrase, it has unfortunately already been used in reference to two quite different observations. Austen et al.[426] introduced the phrase in their discussion of ligand rebinding to myoglobin, a process which presumably involves concerted movements of residues on the distal side of the heme pocket over a distance sufficient to provide a pathway through which the ligand can approach the heme.[257] The same phrase has been used by Frauenfelder et al.[427] and Hartmann et al.[428] in discussing the results of a series of low-temperature X-ray diffraction studies of myoglobin. These authors observed that the B factors for some atoms of the molecule (and thus the mean-square displacement, $<x^2>$, of the atoms) appeared to extrapolate to non-zero values at 0 K, which they took as evidence of small variations in the structure of different molecules in the crystal lattice at low temperature. As the maximum zero-point values of $<x^2>$ discussed by these authors was about 0.1 Å^2, it is not clear that the motions involved are large enough to correspond to those required to provide access to the ligand pocket of the heme; whether there is a connection between these two types of "conformational substates" thus remains to be established.

We believe that it would be inappropriate to further confuse the issue by attempting to apply this terminology to another distinct set of observations. In considering the low-temperature studies of trypsinogen, we are not concerned with the absolute magnitudes of $<x^2>$ (which are in any case not observable for the region of the molecule of interest) or with factoring the components of $<x^2>$ due to lattice effects from those due to conformational variation or thermal vibrations, as has been attempted in the studies of the myoglobin. The issue for trypsinogen is only whether one can order the activation domain by cooling and if not, whether any conclusion can be drawn about the nature of the disorder. As we have seen, the answer to both questions is "no" for the experimental conditions employed by Walter et al.[181]

d. Structural Basis of the Disorder

Although a number of questions remain about the extent and nature of disorder in trypsinogen, especially when the molecule is in solution, there is little doubt that the activation domain is considerably more flexible than the rest of the molecule. Fehlhammer et al.[166] have drawn attention to structural features of the activation domain that give some indication of why a large-scale order-disorder transition is observed in this region of trypsinogen but rarely in other proteins.

First, the residues of the activation domain make very few specific contacts with ordered regions of the molecule. As was shown earlier (Plate 1), the activation domain is on the surface of the molecule; although one disulfide bridge and a number of intramolecular hydrogen bonds are formed by residues in the activation domain, these interactions almost all involve other residues in the domain. Except for the order-disorder junctions of the mainchain, where segments of the activation domain are covalently joined to the rest of the molecule, the interactions between residues in the activation domain and the ordered regions common to trypsin and trypsinogen are almost entirely van der Waals contacts. Second, the

activation domain contains no aromatic residues; tyr151, tyr184, tyr141, and tyr215 are adjacent to order-disorder junctions.

Third, it is interesting that five of the seven junctions (the N-terminus is covalently attached to the molecule at only one end) are at glycyl residues which are strongly conserved among the vertebrate serine proteases. In two of these five instances, the junctions are immediately adjacent to a tryptophanyl residue in the ordered region. The sequence trp141-gly142 is very highly conserved in trypsin-related serine proteases and trp215 of the sequence trp215-gly216 is exchanged only for other aromatic residues.[204] These observations suggest that the relatively unhindered rotational freedom of the polypeptide at glycyl residues[205] contributes to the flexibility of the junctions and that a bulky aromatic group can aid in anchoring the polypeptide chain to a stable region of the protein; both of these hypotheses are useful in explaining the sharp order-disorder transitions at the boundaries of the activation domain.

The strong conservation found in the amino acid sequence near the transition points in trypsinogen and the overall similarities in the activation processes[187] of the vertebrate serine proteases suggests that disordered regions analogous to the activation domain of trypsinogen should be observed in the zymogens of other serine proteases. Chymotrypsinogen is the only other such zymogen whose crystal structure is known.[184] The structure of chymotrypsin has been solved at 2.0 Å resolution[206] and that of chymotrypsinogen[184] at 2.5 Å resolution. The largest differences between the zymogen and the active enzyme include regions of the polypeptide chain corresponding closely to the disordered segments in trypsinogen. However, none of the disordered segments of trypsinogen are characterized by Freer et al.[184] or Wright[185] as being ill-defined or disordered in chymotrypsinogen.

To examine this question more carefully, we have examined the crystal-packing contacts of chymotrypsingen using the coordinates of dataset 1CHG (revision of 07-APR-80) from the Protein Data Bank.[190] Two segments of the activation domain appear to make contact with adjacent molecules in the crystals studied by Freer et al., as judged by the occurrence of interatomic distances of less than 4 Å. Residues 13, 16, and 17 are involved in several such contacts with residues 239 to 244 of one neighboring molecule, and ser218 is in contact with thr110 of another. Since the chymotrypsinogen structure is not refined, a more detailed analysis of these interactions is probably not warranted at this time. The possibility clearly exists, however, that the conformation of the activation domain found in chymotrypsinogen is stabilized to some degree by the contacts listed. We have already encountered one example of the effects of lattice contacts on a disordered domain in the structure of the trypsinogen-PSTI complex;[148] further observations along these lines are made in the discussion of disorder in the Fc fragment below.

2. Fc Fragment

The evidence provided by the crystal structures of human Fc fragment[95,97] and its complex with fragment B of protein A from *Staphylococcus aureus*[96,97] for flexible linkage of immunoglobulin domains was discussed in the previous section. Of interest to our discussion of large-scale order-disorder transitions are the observations of Deisenhofer[97] that the CH_2 domains of the Fc fragment show different degrees of order in each of the crystallographically independent copies of the refined crystal structures.

Fc crystals contain two crystallographically independent but chemically identical heavy-chain fragments. Both chains are ordered, in the sense that there is electron density present for them in the crystal structure, except for 15 residues at the N-terminus and three at the C-terminus of each chain; the degree of order within the ordered region varies, however. The two CH_3 domains, which make extensive lateral interactions with one another, are well ordered; each has an average B factor of 19 $Å^2$. The CH_2 domains, which have no lateral interactions with one another because the appropriate contact areas are covered with carbohydrate, have average temperature factors of 25 $Å^2$ and 30 $Å^2$. As there are no systematic

variations of the B factors for different residues within the CH_2 domains, it is not clear whether these high average B factors reflect localized disorder within the domains or librational disorder of each domain in the crystal lattice; the latter would be consistent with the flexible linkage of the CH_3 and CH_2 domains discussed earlier. It is clear, however, that the observed differences in the degree of order of these domains must arise from differences in their crystalline environments.

In the FB:Fc complex, there is only one crystallographically independent heavy-chain fragment. The CH_2 domain of this chain has a domain of disorder consisting of three segments of the polypeptide chain. These segments form a contiguous region at one end of the domain (Plate 2) and account for about 20% of the residues in the Fc structure; four of the nine hexose units of the carbohydrate chain found in the Fc structure are also disordered. As the protein A fragment binds to the other end of the CH_2 domain, it is unlikely that this interaction is the cause of the disorder observed in the FB:Fc complex. Since the disordered region in the complex is not involved in crystal packing contacts, and since the CH_2 domains in the Fc crystal are involved in packing contacts in rough proportion to the degree of order they display, Deisenhofer et al.[96] suggest that the CH_2 domain may have a flexible structure in solution and that the effector functions of the immunoglobulin Fc segment[207] might thus be mediated by an order-disorder transition. At present there is no direct evidence that the disordered region of the crystalline CH_2 domain is dynamic in nature or that there is a physiological order-disorder transition in this segment of the immunoglobulin molecule, although spectroscopic[418] and calorimetric[420] studies have suggested that the CH_2 region of some immunoglobulins is less rigid than the rest of the molecule.

The principle implicit in the suggestion of Deisenhofer et al[96] that the degree of flexibility in part of a protein structure is inversely correlated with the amount of contact it makes with the rest of the protein or with other proteins deserves a brief comment. This idea derives from the common observation that a globular protein is best ordered in its densely packed interior regions and often poorly ordered in loops of polypeptide chain on its surface.[13] The effects of ligand binding to trypsinogen on the order of the activation domain clearly demonstrate how the binding energy of specific contacts can be used to reduce flexibility in parts of a protein. It seems likely that nonspecific interactions would have a similar effect, although they would be expected to provide much less stabilization energy than specific contacts. An interesting report has appeared recently, however, of an example in which an eight-residue segment of polypeptide chain on the surface of the Mcg Bence-Jones protein is disordered by crystal packing effects; the same segment is ordered in a crystallographically independent copy of the chain in which it makes no packing contacts.[115] Presumably other packing contacts made by the molecule are strong enough both to hold the molecule in the lattice and to distort the conformation of this short segment of the polypeptide chain. This effect is clearly a crystal-packing artifact, but the possibility of such lattice-induced distortions is clearly of interest in the interpretation of crystallographically observed disorder.

3. TMV Coat Protein

Tobacco Mosaic Virus (TMV) is a rod-shaped RNA virus consisting of a single RNA molecule with a molecular weight of about 2×10^6 and approximately 2100 identical protein subunits with molecular weights of 17,500 each.[208] The structure of an annular disk particle consisting of 17 protein subunits and no RNA, an intermediate in the assembly of the virus particle, was solved at 5 Å resolution by single-crystal diffraction techniques.[209] In this structure, a segment of about 30 residues (20% of the subunit) at the inner surface of the annulus was found to be disordered. In the structure of the intact virus, solved at 4 Å resolution by fiber diffraction techniques,[210] this region of the protein is ordered and interacts with the RNA. Although the possibility existed that the apparent disorder in the disk particle was an artifact of the averaging procedure required to solve this structure,[209] NMR studies[211]

indicate that this segment of the subunit is highly flexible in coat protein aggregates in solution. Narrow resonances assigned to the disordered region in part by comparison of mutant virus strains were observed in aggregates with average molecular weights from 52,000 to about 650,000, indicating that motion in the flexible segment is largely independent of the rest of the subunit. Only a few of the residues in the crystallographically disordered segment could be assigned to sharp peaks of the spectrum with certainty, but the area of these peaks was found to be consistent with the proposal that most of the segment is flexible in solution. Since this region of the protein subunit appears to contain a ten-residue α-helix in the intact virus, some unfolding of the secondary structure of the polypeptide chain would be involved in the observed disorder if the latter proposal is correct.

C. Proteins with Hinged, Globular Domains

In this section, we present examples of another type of domain flexibility in which essentially rigid, globular domains move relative to one another. In contrast to the proteins with "flexibly linked" domains discussed earlier, proteins with "hinged" domains seem to oscillate between two (or a few) stable equilibrium conformations. We use the term "hinged" to describe these proteins largely because the unfortunate phrase "hinge-bending enzyme" (implying that the enzyme catalyzes hinge bending) is often used in referring to them. As we will see, however, the flexible regions in "hinged" proteins are if anything less hinge-like than those connecting flexibly linked domains (see, e.g., Figure 6) or those at the order-disorder junctions of trypsinogen.

A number of authors have stressed the similarity between the changes in tertiary structure observed in hinged proteins and the changes in quarternary structure documented crystallographically a number of years ago by Perutz and his colleagues for the allosteric transition in hemoglobin. The only essential difference seems to be that the globular units of a hinged protein are covalently connected by one or more segments of polypeptide chain, and are thus called domains, while those of hemoglobin are not and are called subunits. The allosteric transition of hemoglobin and related topics have been reviewed recently by Perutz[212] and will not be discussed here.

1. Hexokinase

Yeast hexokinase, which catalyses the ATP-dependent phosphorylation of glucose and a number of structurally related sugars, occurs either as a monomeric enzyme with a molecular weight of about 50,000 or as a dimer of identical subunits, depending on the conditions under which it is studied.[213] Except where explicitly noted, we will only be concerned with the effects of glucose binding on the monomeric enzyme in the absence of nucleotide substrate.

The first evidence for domain flexibility in this enzyme came from a comparison of the crystal structures of the two yeast hexokinase isozymes. The structure of the B isozyme (HKB) is known from two crystal forms. Form BII, which contains a dimer of crystallographically independent subunits, has been solved at 3.5 Å resolution,[214] and form BIII, which contains a monomer, has been solved[214,215] and refined[216] at 2.1 Å resolution. The structures of the three crystallographically independent copies of the B isozyme monomer in these crystals are almost identical and are unchanged in the presence of o-toluoyl glucosamine (OTG), a competitive inhibitor of glucose for this enzyme. The monomer is bilobal, and the two lobes are separated by a distinct cleft (Figure 15a); the bindng site for glucose in the crystalline B isozyme is at the bottom of the cleft.[217]

The structure of the complex between the A isozyme and glucose has been solved and refined at 3.5 Å resolution.[218,219] Although the tertiary structures of each of the lobes of the A isozyme are essentially the same as in the B isozyme, the relative orientations of the lobes are quite different. In the hexokinase A-glucose (HKA-glucose) complex, the small lobe,

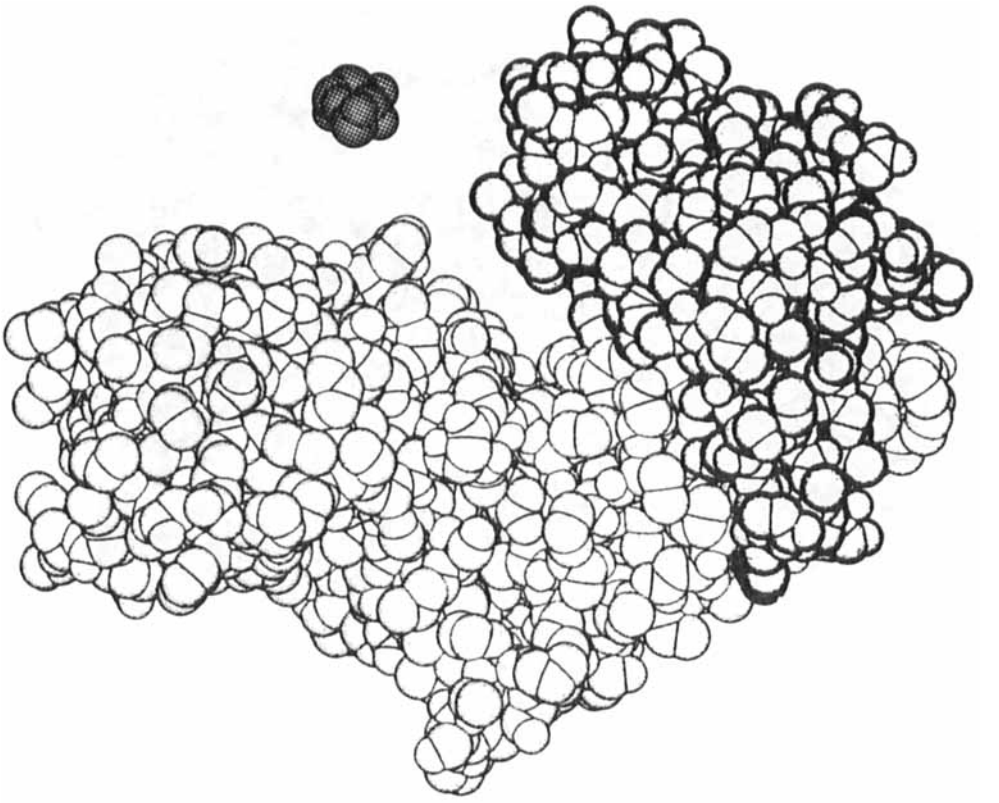


FIGURE 15. Space-filling drawings of (a) the unliganded form of hexokinase (crystal form BIII) and free glucose, (b) the complex between hexokinase A and glucose. In these panels, the large lobe of the enzyme is lightly shaded, the small lobe is darkly shaded, and the glucose molecule is cross-hatched. (From Ref. 220) Panel c is a stereo drawing of the HKA-glucose complex which shows the depth of the glucose binding site in the cleft; both lobes of the enzyme are lightly shaded in this panel, and the glucose molecule is more heavily shaded. (From References 251 and 254.)

about one third of the subunit, is rotated 12° relative to its position in the B isozyme. (The term "rotation" is used here only to describe the differences in these two structures; how the enzyme gets from one conformation to the other is of course unknown.) The cleft is closed in the complex (Figure 15b), and the glucose binding site is all but buried between the lobes.[218,220]

Although the principal conformational change produced by glucose is a rigid-body rotation of the lobes of the enzyme, this movement is accompanied by small changes in the conformation of segments of polypeptide on the surface of the protein.[220] Some of these smaller rearrangements probably result from differences in the crystalline environments of the two hexokinase isozymes, as they are found near crystal packing contacts; changes in the conformation of segments near the active site of the enzyme could have some functional importance, however. As we will see, these general features of the glucose-induced conformational change in hexokinase are common to all examples of hinged enzymes that have been studied in detail; in each case a rigid-body rotation of two compact globular domains (or lobes, in the case of hexokinase) is coupled with small changes in the conformation of polypeptide loops near the active site.

Inspection of the hexokinase models shows that two polypeptide chains join the lobes of the monomer (Figure 16). One of these is an extended segment of polypeptide chain that connects a short β-strand following the two N-terminal α-helices with the first β-strand of

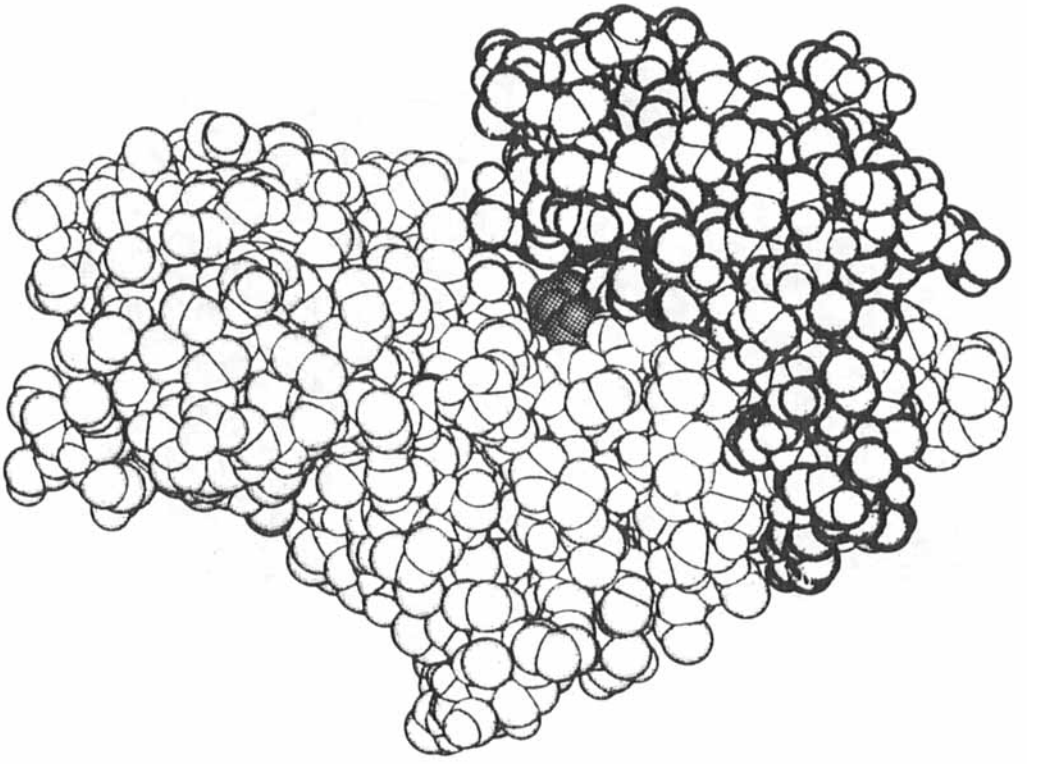


FIGURE 15B.

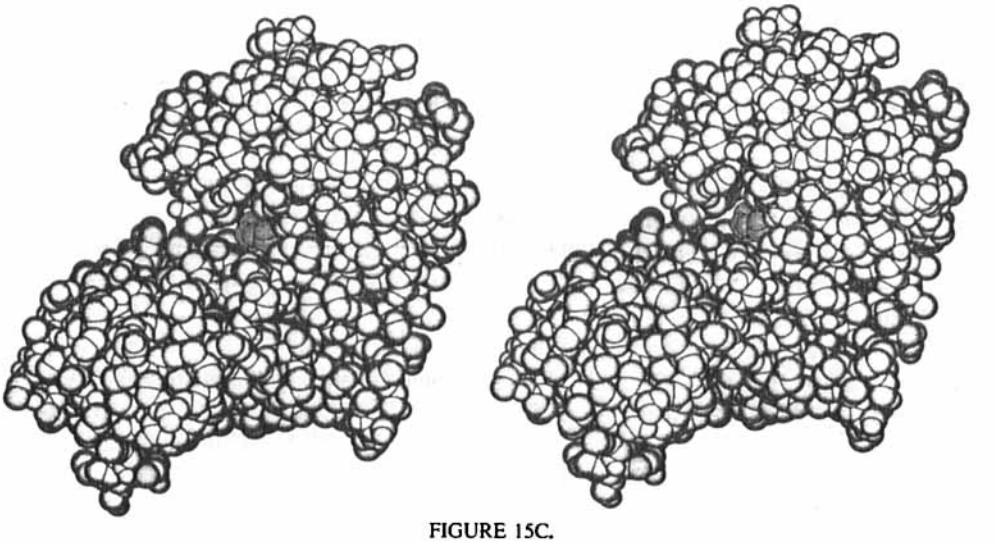


FIGURE 15C.

the small lobe. (The small lobe is formed by the β-sheet and the two α-helices at the back of the molecule in Figure 16.) As this loop is about 10 residues long and on the surface of the monomer, it probably need not contain a distinct hinge point to accommodate the different orientations of the two lobes.

The second connecting strand is a short segment of four residues between the last β-strand of the small lobe and a helix in the large lobe; this strand is at the back of the cleft

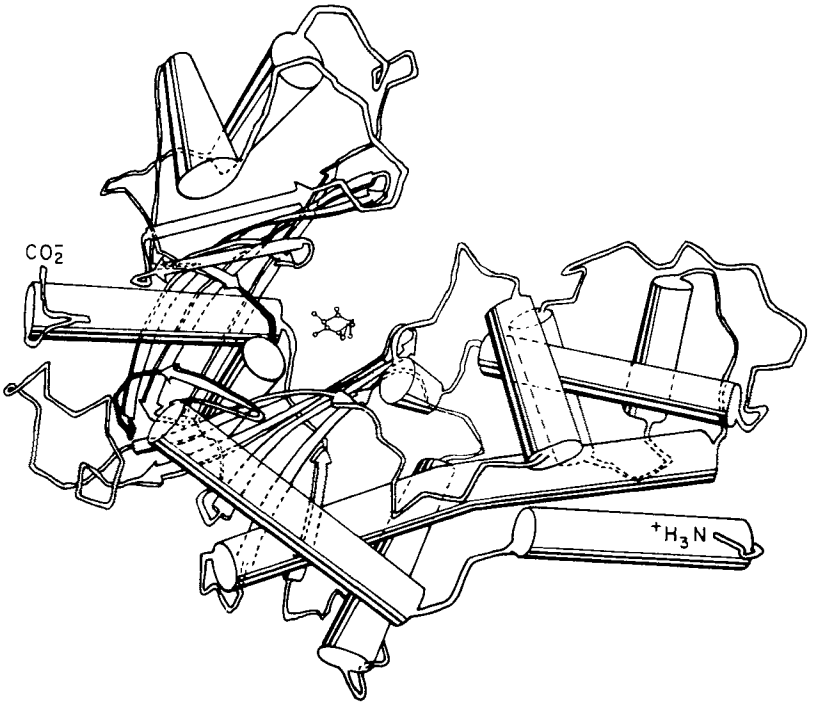


FIGURE 16. Schematic diagram of the folding of the hexokinase monomer in the open conformation crystal form BIII). Cylinders represent α-helices and arrows represent strands of β-sheet. The connecting strands between the small lobe (to the rear in this figure) and the large lobe are shaded. (Adapted from Reference 215.)

and contributes to the glucose binding site (Figure 16). No significant differences in the mainchain conformational angles of this segment could be detected in the two hexokinase models;[220] thus there is no obvious hinge point in hexokinase, such as was seen in earlier examples of domain flexibility. However, one cannot rule out the possibility that hinge points exist. The absence of an independently determined amino-acid sequence limited the degree to which these structures could be refined, especially the lower-resolution HKA-glucose complex; if a hinge point were located near the axis of the rotation that describes the conformational change (as is the case for citrate synthetase, see below), more precise atomic coordinates would be required to detect the small differences in conformational angles at the hinge than are presently available.

The A and B isozymes of the yeast enzyme are genetically distinct;[221] their amino-acid sequences must be quite similar, however, as peptide maps indicate that about two thirds of the peptides resulting from complete digestion by trypsin are common to both isozymes.[222] As we will show in the following paragraphs, it is reasonably certain that the conformations of the hexokinase isozymes are the same or very similar in solution and that the major difference in the conformations of the crystalline isozymes results from glucose binding; i.e., both isozymes adopt the open conformation of the crystalline B isozyme when free (unliganded) in solution, and the binding of glucose induces a change in either isozyme to the conformation of the crystalline HKA-glucose complex (Figure 17).

Although the nature and magnitude of the crystallographically determined conformational change were surprising, some glucose-induced isomerization in hexokinase had been expected on the basis of earlier theoretical considerations and kinetic studies. Koshland had proposed the induced-fit theory in part to explain why water is not a good substrate in

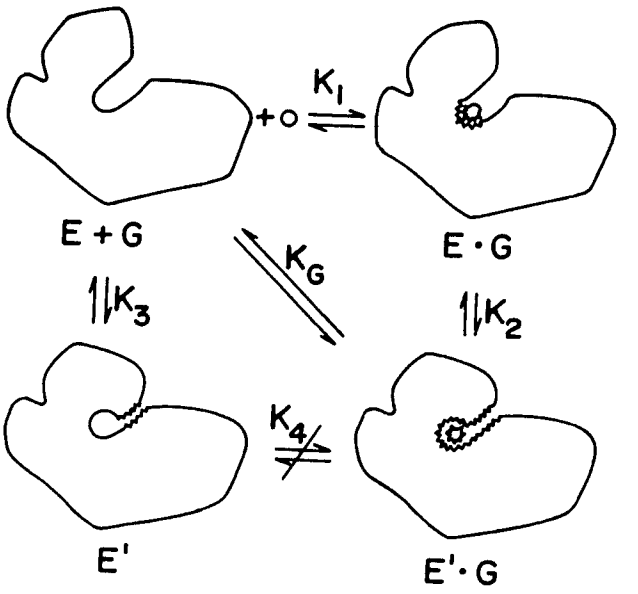


FIGURE 17. Equilibrium scheme for the binding of glucose to hexokinase. The open conformation of the enzyme is labeled E, the closed form is labeled E′, and the glucose molecule (small circle) is labeled G. Only the E and E′·G structures have been determined crystallographically. Regions of the protein or glucose that are inaccessible to solvent are indicated by a crinkled line. (From Ref. 218.)

reactions involving a hydroxyl group on a larger molecule,[223] such as the 6-hydroxyl group of glucose. Experimental evidence for induced fit in hexokinase was first provided by the observations that the enzyme has an ATPase activity in the absence of a sugar substrate and that the Km for ATP in this reaction is considerably higher than in the kinase reaction with glucose as the sugar substrate;[224] the ATPase reaction presumably involves the equilibrium K_3 in Figure 17. The substrate synergism indicated by these observations is consistent with a glucose-induced conformational change that increases the affinity of the enzyme for ATP. However, the induced-fit hypothesis holds that substrate binding produces a change in the active site of the enzyme from an inactive configuration of catalytic groups to the active form.[223] Direct evidence that sugar substrates stimulate the activity of hexokinase in this way was provided by Sols and collaborators,[225,226] who showed that the ATPase activity of the enzyme is stimulated by xylose or lyxose. These five-carbon sugars are analogous to hexose substrates of hexokinase, but lack the 6-hydroxyl group that is normally phosphorylated.

a. Glucose Binding or Isozyme Difference

In view of the evidence for induced fit in hexokinase, it is obviously tempting to associate the crystallographically observed conformational change with the expected isomerization of the enzyme from an inactive to an active conformation. One encounters such temptations frequently in the study of hinged enzymes, but the relationship of the conformation of a hinged enzyme in solution to any crystallographically determined structures is quite difficult to establish. This question has been of particular interest in the hexokinase system, as the crystal structures of the native enzyme and the glucose complex involve different isozymes; this fact clearly complicates the interpretation of the crystallographic results. As the correspondence between the crystallographically determined conformations and the properties of the enzyme in solution is best established for hexokinase, we will discuss the evidence

bearing on this question in detail. Similar problems are encountered for all enzymes with hinged domains.

On inspection of the crystal structures (Figure 15), it is evident that the entry and exit of glucose from the active site must be considerably restricted in the HKA-glucose complex relative to the HKB structure. This subjective impression is supported by solvent accessibility calculations[218,220] and recently has been verified by direct measurement of the glucose binding characteristics of the crystalline isozymes. Wilkinson and Rose[227] demonstrated that HKA-glucose crystals could be washed free of excess glucose in the crystallization medium, leaving a single glucose molecule tightly bound to each protein molecule; in the washing process, the crystals are fragmented to microcrystals with maximum dimensions of about 1 μm, so that diffusion of glucose through the solvent channels in the crystal lattice is not likely to have a significant effect on these results. By transferring washed crystals containing labeled glucose into solutions containing unlabeled glucose, exchange of bound and free glucose could be measured; the release of glucose was observed to be a single-exponential process, indicating that a single glucose binding site was involved and that the crystals are homogeneous in this respect. The rate at which glucose is released from the crystalline complex (0.7 min^{-1}) and the dissociation constant of the complex ($<10^{-6}$ M) could be determined from the exchange data. The binding of glucose to the crystalline hexokinase B monomer (crystal form BIII) is probably quite weak,[228] however, with a dissociation constant of about 50 m*M*.[229] These differences in the properties of the crystalline isozymes are clearly consistent with the X-ray structures.

In contrast, the binding of glucose to the hexokinase isozymes in solution is quite similar. The dissociation constants for glucose binding differ at most by a factor of two over a wide range of conditions.[230,231] For the monomeric A and B isozymes in particular, dissociation constants of 0.2 and 0.3 m*M*, respectively, have been measured recently;[232] these values are clearly intermediate between those found for the crystalline isozymes, as would be expected if the conformation of each isozyme in solution were an equilibrium between the conformations found in the crystal.

Even more striking is the agreement of the association rates for the formation of glucose complexes of the two isozymes. A value of 2×10^6 M^{-1} sec^{-1} has been measured for both isozymes in studies of the relaxation of intrinsic protein fluorescence following a temperature-jump perturbation of the complex.[230] These results agree quite well with association rates derived from isotope-trapping studies, which provide a measure of the dissociation rate of the complex.[235] For the A isozyme, values of 1.7×10^6 and 2.5×10^6 M^{-1} sec^{-1} were estimated for k_{on} from k_{cat}/Km and from the dissociation constant of glucose, respectively,[233] while a value of 2.7×10^6 M^{-1} sec^{-1} has recently been obtained for the B isozyme.[234] The similarity of these rates is clearly inconsistent with the idea that the structure of each isozyme in solution is static and the same as it is in the crystal, as the access of glucose to the active site must be quite different for the two crystallographic conformations (Figure 15).

The kinetics of glucose binding to hexokinase have themselves been taken as evidence for a substrate-induced conformational change in the enzyme. Both Hogget and Kellet[230] and Rose at al.[233] suggested that the low association rates for glucose and hexokinase resulted from an isomerization of the enzyme, as the observed rates are lower than those expected for a simple diffusion-controlled encounter, estimated as 10^9 M^{-1} sec^{-1} or more.[236] This argument alone is not particularly compelling for hexokinase. Eigen and Hammes[236] point out that association rates on the range of 1 to 10×10^6 M^{-1} sec^{-1} are not uncommon for enzymes and suggest factors that might account for such observations; an obvious possibility that would be difficult to rule out for a markedly bilobal enzyme such as hexokinase is that there is some degree of static restriction of the access of substrate to the active site.

The temperature-jump studies of Hogget and Kellet[230] also provide some additional information about the nature of the glucose-induced conformational change in hexokinase. A

Table 4
EFFECT OF GLUCOSE ON THE RADIUS OF GYRATION OF HEXOKINASE B IN SOLUTION[237]

	Radii of gyration (Å)		
	Predicted	**Observed**	**Difference**
Free HKB	23.7[a]	24.7 ± 0.2	1.0
HKB + glucose	22.8[b]	23.8 ± 0.1	1.0
Decrease	0.9	0.9 ± 0.2	

[a] Calculated from the BIII crystal structure. [216]
[b] Calculated from the KHA-glucose crystal structure. [219]

single relaxation process consistent with a simple bimolecular reaction was observed for both hexokinase-glucose complexes, and no relaxation process was found for the enzyme alone. Because there was earlier kinetic evidence for a glucose-induced conformational change, however, the relaxation data were correctly interpreted in terms of an induced-fit mechanism (Figure 17). This analysis indicates that glucose binding follows a substrate-guided pathway (via the E · G complex in Figure 17) for both hexokinase isozymes.

The most direct evidence that glucose induces a conformational change in hexokinase analogous to that inferred from the crystal structures comes from small-angle X-ray scattering studies. McDonald et al.[237] measured the radius of gyration (R_G) of monomeric hexokinase B in the presence and absence of saturating concentrations of glucose and found that R_G is reduced significantly in the presence of the sugar (Table 4). Moreover, the observed decrease of 0.9 Å in R_G corresponds to the difference in the radii of gyration calculated from the crystallographic models, assuming that the hexokinase B monomer in the BIII crystal form represents the conformation of the unliganded enzyme and that the hexokinase A monomer in the crystalline HKA-glucose complex represents the conformation of the HKB-glucose complex in solution. These studies clearly show that the B isozyme, which appears only in the open conformation in existing crystal forms, can adopt a more compact conformation upon binding glucose in solution; the corresponding experiment unfortunately could not be performed on the A isozyme, as this protein tends to aggregate at the high concentrations required for small-angle scattering studies[238]

One difficulty with the small-angle scattering results on hexokinase is that they leave some doubt about the degree to which the structure of hexokinase in solution resembles either of the crystal structures. The observed and predicted radii of gyration for both the free enzyme and the glucose complex are significantly different; in both cases, the magnitude of the difference is essentially the same as the change in R_G produced by glucose binding (Table 4), with the observed R_G greater than the calculated R_G. McDonald et al. suggested that the difference might be due to the absence of some atoms from the crystallographic models used for the calculation of R_G, but subsequent model calculation on hexokinase showed that the calculated R_G is little changed when all sidechain atoms beyond the β-carbon are omitted from the calculation.[239] Although other sources of error, such as neglect of the contrast between protein and solvent in the calculation of R_G[237] or interparticle interference effects,[240] may contribute to the discrepancy, it is noteworthy that observed and calculated radii of gyration obtained more recently in the same laboratory using similar experimental condition and computational techniques agree quite well.[239,241,242]

The agreement of the observed and calculated values of R_G for dimeric hexokinase B[241] is particularly interesting in light of fluorescence-quenching studies[231,243,244] which suggest

that the cleft of unliganded hexokinase may be less open at pH 5.5 than at pH 8.3. As the small-angle scattering studies on the hexokinase dimer were performed only at pH 5.5 and those on the monomer at pH 8.5, an effect of pH on the measured R_G may also contribute to the discrepancy between the observed and calculated radii of gyration of the monomer. Unfortunately, although the hexokinase crystals are all grown from solutions at pH 7.0,[219,245,246] potential effects on the conformation of the cleft due to the high salt concentrations needed to stabilize the crystals[231] or to lattice forces in the crystal further complicate attempts to compare observed and calculated radii of gyration.

While the source of the discrepancies between the observed and calculated radii of gyration for hexokinase remains unresolved, it is unlikely that this difficulty affects the conclusion of McDonald et al.[237] that glucose induces a conformational change in the molecule. As noted by these authors, the similarity of differences in observed and calculated values of R_G for both liganded and unliganded proteins clearly indicates that both comparisons are affected by the same factors: thus the differences between the two measures of R_G in the free and ligated states can be validly compared.

b. Lattice Constraints on Domain Flexibility

Three other types of observations concerning the crystal structures of hexokinase are also consistent with the conclusion that glucose induces a conformational change in the monomeric isozymes. Although these observations provide considerably less information on this question than the small-angle scattering studies just discussed, analogous results are often encountered in discussions of hinged proteins, and some comment on their interpretation may be useful.

One such observation deals with conformational changes produced in the protein by diffusing substrate into the crystal. The addition of low concentrations (20 m*M* or less) of glucose to crystals of the B isozyme of hexokinase, for example, appears to produce small conformational changes throughout the smaller lobe of the molecule;[217] the changes are similar to the effects produced by placing the crystals in a saturated solution (<0.5 m*M*) of the inhibitor OTG. In retrospect, it is clear that these "soaking" experiments are quite misleading. To a good approximation, the glucose-induced conformational change is a rigid-body rotation of the small lobe relative to the large lobe.[220] The many small conformational changes seen in the soaking experiments with the HKB crystals probably result from the onset of this rotation,[218] which is prevented by crystal packing constraints from proceeding; crystals of the B isozyme are in fact observed to crack and dissolve upon prolonged exposure to solutions with higher glucose concentrations[218] or to other sugars.[269] OTG, on the other hand, is now known to produce only small changes in the structure, leaving the molecule in the open conformation;[247] this is demonstrated most effectively by the observation that electron density from the BIII monomer, normally grown and studied in the presence of OTG,[246] could be averaged with that from the native BII dimer to improve the quality of both structures.[248,249]

In view of these observations on hexokinase, it is clear that if one wishes to study ligand binding crystallographically, it is important to attempt to crystallize the protein-ligand complex directly, rather than rely on soaking experiments.[218,250] The open form of hexokinase found in the BIII crystal form, for example, is obtained by crystallization of either the unliganded enzyme or the HKB-OTG complex under the same conditions,[246] indicating again that the structure of the protein in solution is probably little changed by OTG.

More recent studies of hexokinase demonstrate, however, that co-crystallization of a protein and its ligand is not sufficient to guarantee that the desired complex will be crystallized. Shoham and Steitz[251,252] have grown crystals from a solution containing hexokinase B, xylose, and ADP (crystal form BIV) which are in an entirely different space group than other hexokinase crystals; as an earlier crystal form is obtained under the same conditions but without xylose or ADP, it was reasonable to assume that binding of one or both of these

ligands altered the structure of the enzyme in some way to favor new crystal contacts. The low-resolution structure of this crystal shows, however, that the protein is in an open conformation indistinguishable (at 7 Å resolution) from earlier B isozyme structures. Another example of the failure of co-crystallization to circumvent the effects of crystal packing forces is found in the case of aspartate aminotransferase, below.

A second type of observation suggestive of a ligand-induced conformational change in a crystalline protein is that the crystals crack or dissolve upon adding (or removing) the ligand. We have already noted that the BII and BIII crystals, which contain the isozyme in the open conformation, crack upon adding sufficiently high concentrations of glucose. In contrast, crystals of the HKA-glucose complex rapidly disintegrate when transferred to solutions without glucose.[218] Although these observations are clearly consistent with a glucose-induced conformational change that is too large to be accommodated in the crystal lattice, it is important to recognize that destruction of the crystal would also result under these conditions if a bound glucose molecule interfered with (or participated in) crystal packing contacts. This explanation is unlikely in the case of hexokinase, as the single glucose binding site per monomer expected from solution studies[213] is well removed from crystal packing contacts in all crystal forms of both isozymes examined so far.

There is a second way that crystallographic studies of ligand binding to a hinged protein can be misleading, however. We have just seen that in the case of hexokinase, for example, low concentrations of glucose produce changes in the structure of the protein in the BII and BIII crystals.[217] Although it was clear from the difference electron density maps comparing the enzyme with and without glucose that glucose binds predominantly in the cleft between the lobes of the monomer, a number of smaller difference electron density peaks were found at other points on the molecules; some of these could be attributed to protein conformational changes, but the overall effect of glucose binding was to produce a rather "noisy" difference map. In this situation, one cannot rule out the possibility that one of the "noise" peaks near a lattice contact is a secondary ligand binding site that is responsible for disruption of the crystal. Such intermolecular binding sites may themselves be artifacts of the crystalline environment. The copper-binding sites of crystalline thioredoxin provide an example of this phenomenon.[253] Copper ions are required for crystallization of this protein and form contacts between neighboring molecules in the lattice; no physiological role for copper binding to this protein known, however. An effect analogous to that produced by glucose binding to crystalline hexokinase is probably responsible for the difficulties encountered in locating the binding site for 3-phosphoglycerate on phosphoglycerate kinase; the changes produced in the structure of the enzyme (or in the crystal packing) by this ligand are so extensive that the ligand peak cannot be distinguished from noise peaks in the resulting difference electron density map.[342]

The third type of crystallographic argument that indicates a requirement for flexibility in a protein is the absence of a clear pathway allowing access of a ligand to an interior binding site. For hexokinase, the restricted access of glucose to its binding site can be seen in Figure 15. (The depth of the glucose binding site in the cleft is particularly striking in the stereo version of this figure.) Although there is no direct evidence that glucose binds in the HKA-glucose complex as shown in the figure,[218,220] the conclusion that glucose binds to the proposed site in the binary complex is supported indirectly by the observation that a glucose molecule placed at this position of the HKA-glucose structure is enclosed in a cavity between the two lobes of the monomer with only its 6-hydroxyl group readily accessible to solvent[218,220] (Table 5); it seems unlikely that this arrangement would be stable if the cavity were not occupied by a sugar molecule.[218]

While such observations certainly suggest that some movement of the protein is probably needed to allow binding and release of the ligand, they do not require the type of large-scale motion found for hexokinase. Similar observations of restrictions to ligand binding

Table 5
SOLVENT ACCESSIBILITY OF GLUCOSE BOUND TO HEXOKINASE[220]

Glucose atom	Contact area (Å²)		
	Free glucose	Open complex	Closed complex
C1	9.6	3.7	0.4
O1	7.4	0.0	0.0
C2	6.2	5.9	0.0
O2	8.5	4.3	0.8
C3	4.7	0.0	0.0
O3	8.8	1.2	0.2
C4	3.6	0.2	0.0
O4	7.9	0.0	0.0
C5	3.1	0.0	0.0
O5	2.1	0.9	0.7
C6	15.5	0.2	0.9
O6	8.2	4.4	2.9
Total	85.6	20.8	5.9

have also been made for essentially rigid proteins; the highly refined structures of metmyoglobin[255] and oxymyoglobin,[256] for example, confirmed the earlier low-resolution observations[257] that there is no open pathway through the protein by which a ligand could approach the heme in either myoglobin or hemoglobin. Presumably, small fluctuations in the structure open a transient pathway in the molecule;[257] the plausibility of such localized conformational changes has been verified by model calculations.[258]

c. Uniqueness of the Crystal Structures

The experimental results discussed so far indicate that both hexokinase isozymes exhibit a glucose-induced conformational change in solution, going from a more open cleft in the absence of glucose to a more closed cleft when glucose is bound. The question remains whether the conformations observed in the different crystal structures correspond to conformations the enzyme adopts in solution. If the domains of hexokinase were flexibly linked like those of an immunoglobulin, for example, the conformations found in the hexokinase crystals would represent just two of many possible orientations of the lobes. We have already seen that this is not the case for the open conformation of hexokinase, as essentially the same structure is found in the three crystallographically independent copies of the HKB monomer known to reasonably high resolution from studies of the BII and BIII crystal forms.[249] It is largely for this reason that we have classified hexokinase as a hinged protein.

The closed conformation of the HKA-glucose complex has only been observed in one crystal structure. One might expect that steric restrictions would limit the number of compact conformations of the molecule, however, as both lobes appear to make specific, intimate contact with a bound glucose molecule.[220] More direct evidence on this point is presented in our discussion of the kinetic role of the complex below.

d. Functional Roles of the Crystal Structures

If the glucose-induced conformational change in hexokinase is an example of induced fit, we would expect that the closed conformation of the enzyme is active. The situation for hexokinase is somewhat more complex than this, however. Anderson et al.[247] concluded from a study of the binding of the inhibitor OTG to the refined BIII structure that the enzyme

in this complex remains in the open conformation because the toluoyl group of the inhibitor sterically blocks the conformational change. Since the interactions of the glucosyl moeity of the inhibitor with the enzyme are identical to those made by glucose, these authors suggested that the glucose-induced conformational change is essential for the activity of the enzyme; i.e., OTG is an inhibitor because it blocks the conformational change. This conclusion is consistent with earlier observations showing that a number of glucose derivatives with large substituents at the 2-hydroxyl group are inhibitors of both the kinase[259] and ATPase[224] activities of the enzyme.

Although it was not possible to identify any essential catalytic groups in the HKA-glucose structure, Bennett and Steitz[220] suggested that any such groups were likely to be in one of two segments of the polypeptide chain that are brought into the active site by the glucose-induced conformational change. The sequences of these two segments are now known;[263] the segment near residue 71 (gly-gly-ala) contains no potentially reactive groups, but the sequence near residue 157 contains at least one lysine residue, for which a plausible function can be suggested.[262] The precise role of this residue is unclear, however, as chemical modification studies do not implicate an essential amino group for this enzyme.[264]

The glucose-induced conformational change of hexokinase may be necessary for activity, but Wilkinson and Rose[227] have shown that the crystallographically described conformational change is not sufficient to catalyze phosphate exchange. These authors demonstrated that the enzyme is not active in the HKA-glucose crystals. It was shown previously that neither BII nor BIII crystals are active.[217] The inactivity of crystalline HKA-glucose complex is not due to the high-salt environment of the crystals, as Wilkinson and Rose have produced active crystals by crystallizing a ternary complex of hexokinase A, glucose, and ADP under essentially the same conditions.

The role of the crystalline HKA-glucose structure in the sequence of events leading to the active ternary complex has been established by a novel isotope-trapping experiment in which the crystalline complex of enzyme and labeled glucose is rapidly dissolved in a reaction mixture containing excess unlabeled glucose. With this technique, Wilkinson and Rose[228] showed that almost all of the glucose in the crystalline complex (80%) was converted to glucose-6-phosphate before it could dissociate from the enzyme; since the HK-glucose crystals are inactive, product formation must have occurred after the crystal fragments dissolved. The implication of this observation is that once the restraints of the crystal lattice are removed, the crystalline HKA-glucose complex can either (1) react directly with nucleotide to form a catalytically competent ternary complex or (2) convert rapidly to a species that can bind nucleotide before glucose exchange occurs. In either case, it seems clear that the crystalline complex is kinetically competent upon rapid dissolution of the crystals.

The similarity of the kinetic properties of binary hexokinase-glucose complexes in the crystal and in solution indicates that their structures are the same or very similar; together with the crystallographic evidence cited above for a unique "open" conformation of the enzyme, this observation implies that the glucose-induced conformational change in solution must be very similar to that inferred from the crystallographic studies.

e. A Two-State Process?

Although the biochemical and crystallographic studies discussed so far are all consistent with a simple two-state structural model for glucose binding to hexokinase, the calorimetric studies of Takahashi et al.[265] show that the process is more complicated. The results obtained by these authors with isothermal calorimetry are formally consistent with a two-state process, as the calorimetric enthalpy change for the binding reaction is the same as the van't Hoff enthalpy change.[266] However the thermal denaturation profiles obtained by differential scanning calorimetry (Figure 18) show that the binding of glucose improves the thermal stability of the protein considerably, increasing the enthalpy of unfolding by a factor

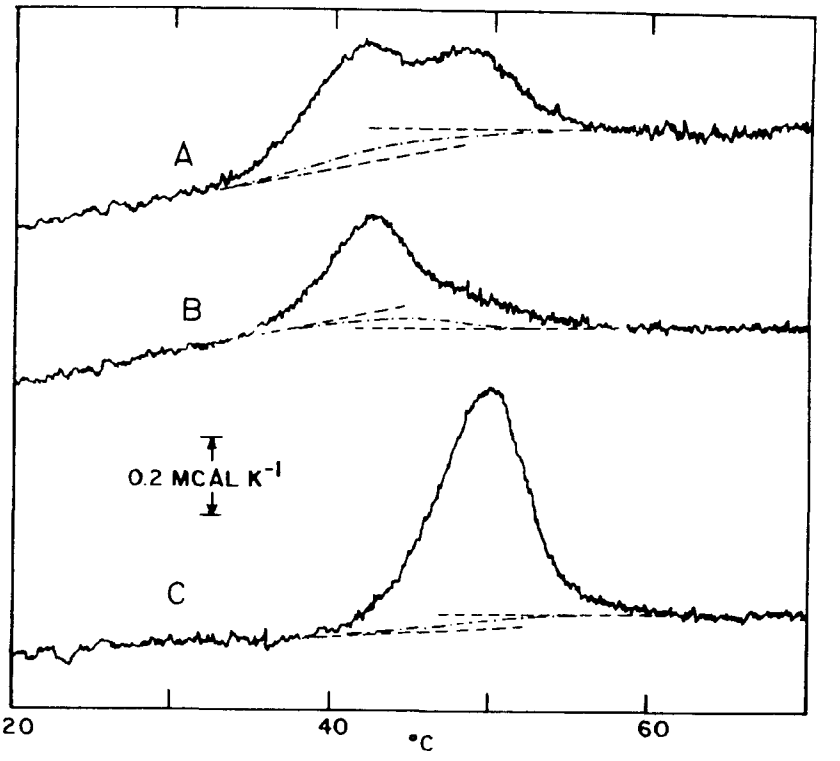


FIGURE 18. Differential scanning calorimetry traces showing the thermal denaturation of hexokinase in the presence (C) and absence (A) of glucose. The middle trace (B) shows the effect of salt on the denaturation of the protein. (From Ref. 265.)

of two. Moreover, the thermal stability of the two lobes of the enzyme seem to be affected to different degrees.

In contrast, the overall binding reaction shows no enthalpy change in the range from 10°C to 30°C, indicating that the binding is "entropically driven". As this enthalpy change was measured calorimetrically, the heat capacity change of the reaction must also be zero. This result is unusual for a protein-ligand interaction[267] and is difficult to reconcile with the observation that glucose produces a large increase in the thermal stability of the protein; the latter observation suggests some change in the vibrational modes of the protein, which should be accompanied by a change in heat capacity.[267]

That the vibrational spectrum of hexokinase does change upon glucose binding is suggested by preliminary inelastic neutron-scattering studies;[268] the nature of the changes in the inelastic scattering profile is consistent with the calorimetrically observed stabilization of the protein by glucose. Jacrot et al.[268] suggest that the apparent discrepancy between their results and the absence of a net heat capacity change upon glucose binding might be explained if a charged group on the protein were buried when glucose is bound; this would presumably compensate for the change in heat capacity due to changes in the vibrational modes of the protein upon glucose binding. Whatever the explanation for the complex thermodynamic behavior of the hexokinase-glucose system, it is unlikely that it is a two-state system in the thermodynamic sense.

2. Alcohol Dehydrogenase

Alcohol dehydrogenase is a zinc-containing metalloenzyme that catalyzes the NAD^+-dependent oxidation of a variety of alcohols to aldehydes.[270,271] In this review, we will be

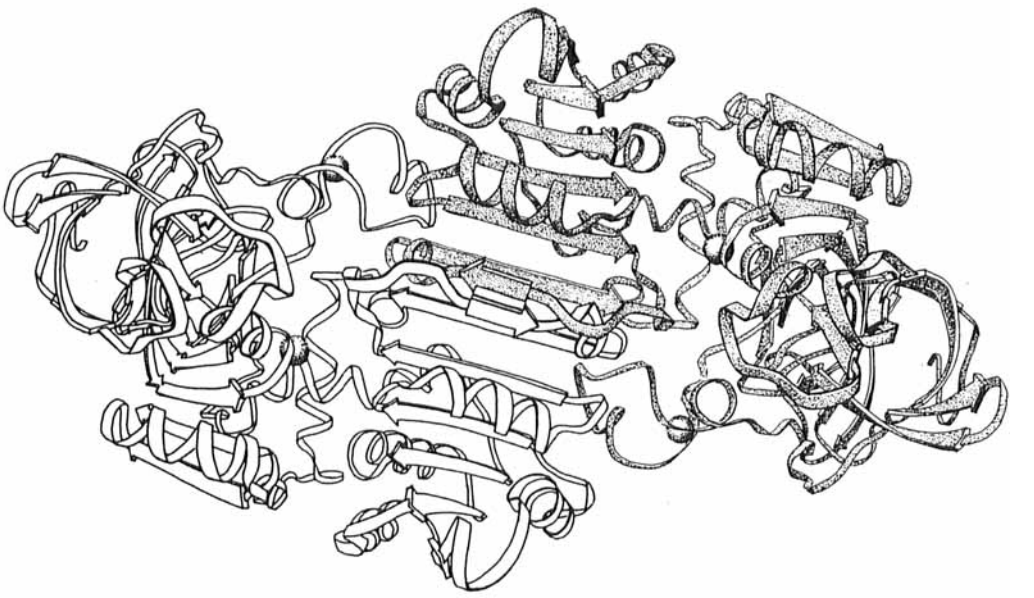


FIGURE 19. Schematic diagram of the folding of the LADH dimer; one subunit is shaded and the other unshaded. The coenzyme-binding domains associate to form the core of the dimer, and the catalytic domains lie to the outside of the molecule. The large, shaded circles mark the locations of zinc atoms in the structure. (From Ref. 274.)

concerned primarily with the mammalian enzyme from horse liver (LADH), for which domain flexibility has been demonstrated by crystallographic studies. LADH is a dimer with a total molecular weight of about 80,000. The structure of the native enzyme has been solved at 2.4 Å resolution.[272] (The unliganded species of the enzyme that we refer to as "native" is often called the apoenzyme in the literature, even when both zinc atoms per subunit are fully occupied.)

The architecture of native LADH is illustrated in Figure 19. The chemically identical subunits of the molecule are related by an exact crystallographic twofold axis in crystals of the native molecule; thus there is only one crystallographically independent subunit. The subunit consists of two domains, a nucleotide-binding domain comprising nearly 40% of the subunit and a catalytic domain. The nucleotide binding domains of two subunits interact extensively to form the core of the dimer, but the catalytic domains, which lie on opposite sides of the core, have no interaction with one another (Figure 19). Binding sites for nucleotide and substrate lie in the clefts separating the catalytic domains from the core.

Domain flexibility in LADH was established by comparing the structure of the native enzyme to that of a ternary complex of LADH, NADH, and dimethyl sulfoxide (DMSO), an inhibitor of the enzyme.[273,274,286] The structure of the ternary complex was solved independently at 2.9 Å resolution.[274] In these crystals, the catalytic domains of the two crystallographically independent subunits have moved relative to the core of the dimer, narrowing the clefts (Figure 20). The conformational change can be described as an essentially rigid-body rotation of the catalytic domains, although there are small changes in a segment of the catalytic domain that make new interactions with the core in the ternary complex. A rotation of 7.5° was reported by Eklund et al.,[274] but an analysis of partially refined models of both structures[277] using different techniques indicates that the reorientation of the catalytic domains involved a rotation of about 10° (Reference 285); the size of the conformational change in LADH is thus quite comparable to that found for the glucose-induced isomerization of hexokinase.

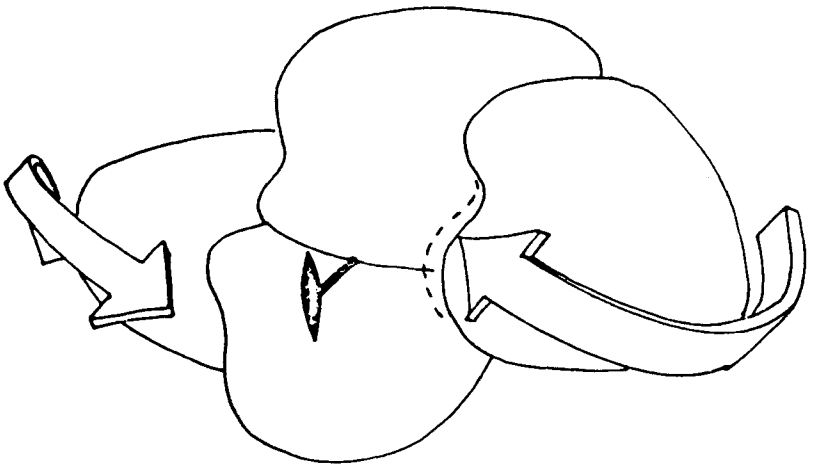


FIGURE 20. Schematic drawing of the coenzyme-induced conformational change in LADH. As in Figure 22, the coenzyme-binding domains are at the center of the dimer; the heavy line shows the position of the twofold axis of the molecule. the catalytic domains rotate towards the core of the dimer upon coenzyme binding, as indicated by the arrows. (From Ref. 273.)

Eklund et al. do not identify a particular segment of the polypeptide change as a "hinge" for LADH but suggest instead that a rolling contact between two α-helices at the domain interface serves this function (see also Brändén and Eklund).[286] One can see in the schematic drawing of the molecule (Figure 19) that those helices are also the segments of the polypeptide chain that link the two domains; one of them provides ligands for the reactive zinc atom (the zinc atom nearer the center of the cleft in Figure 19), and the other forms the back of the cleft. Whether there are abrupt, hinge-like changes in the conformation of residues in the loops flanking these helices will become clear when the LADH structures are more highly refined.

The same isozyme of LADH is used in all of the crystallographic experiments, so there is no question that the conformational differences in the native enzyme and the ternary complex are the consequences of ligand binding. The binding of the cofactor to the enzyme is apparently required to produce the conformational change, as the binary LADH-NADH complex[278] and a number of ternary complexes.[274-276,278-280] crystallize either in the triclinic crystal form descibed above or in a monoclinic crystal form. Although the structure of the enzyme in the monoclinic crystal form has not been characterized, packing arguments based on a comparison of this crystal form to the related native crystals[281] suggest that the conformation of the molecule in the monoclinic crystals is also different from that of the native molecule.

In contrast, binary complexes with alcohol or aldehyde and complexes involving incomplete coenzyme analogues invariably form crystals that are isomorphous to crystals of the native enzyme;[274,275,278,282-284] the carboxyamide group on the nicotinamide ring of the coenzyme seems to be particularly important for positioning the ring so that the conformational change can occur.[283] The conformation of the enzyme is also sensitive to the environment of the catalytic zinc atom, however; one ternary complex involving an inactve coenzyme analogue and an aldehyde substrate can be crystallized either in the open conformation of the native enzyme or in the closed conformation of the LADH-NADH-DMSO complex, depending on the concentration of the substrate.[276]

The existence of coenzyme-induced isomerizations of the enzyme in solution is indicated by a number of kinetic, spectroscopic, and NMR experiments,[270] but the relation of these

processes to the crystallographically determined conformational change is less well established. This situation is due mainly to the lack of direct evidence on the magnitude of the coenzyme-dependent isomerization in solution, such as is provided by small-angle X-ray scattering studies in the hexokinase system. In addition, the number of crystallographically independent views of LADH in the structures solved so far is relatively small. However, there is sufficient indirect evidence suggesting a close correspondence between the conformations of the enzyme in the crystal and in solution for us to regard the LADH domains as hinged rather than flexibly linked.

The correspondence of the closed conformation found in the triclinic crystal form to the active form of the enzyme in solution is relatively well established. As we have seen, the triclinic crystals contain two crystallographically independent subunits; the observation that the two subunits are essentially identical in several ternary complexes[274-276,280] is one indication that this conformation of the enzyme is probably stable. Direct microspectrophotometric studies of crystals containing a ternary complex of LADH, an inactive coenzyme analogue, and chromophoric substrate[276] indicate that the environments of both coenzyme and substrate are the same as they are in solution. Moreover, similar microspectrophotometric studies had earlier shown that the coenzyme in crystalline ternary complexes could be reversibly oxidized and reduced by diffusing aldehyde or alcohol into the crystals.[287] These observations would be more conclusive evidence that the active site conformation of the triclinic ternary complex is the same as in solution, except that the microspectrophotometric studies were performed at 23°C, where the crystals are known to be unstable;[278] thus one cannot rule out the possibility that the enzyme in the destabilized crystals on which the microspectrophotometric studies were performed has a somewhat different conformation from that determined by X-ray diffraction studies at 4°C.

Although only one crystallographically independent subunit of LADH in the open conformation has been studied, this form of the enzyme is probably stable as well. Native crystals, like crystals of ternary complexes, are relatively fragile and must be grown and maintained at 4°C, since they dissolve at room temperature;[274,278] crystals of the native enzyme also dissolve rapidly in the presence of NADH.[288] As we have seen, however, complexes with substrates or incomplete coenzyme fragments will crystallize in the same crystal form or can be diffused into native crystals without disrupting them. These observations suggest that the open conformation of the enzyme is stable and that the fragility of the native crystals is due to weak packing contacts which are unlikely to have much influence on the structure of the protein. The same argument can be applied to the closed conformation of the enzyme but is less convincing, as ternary complexes of LADH can crystallize in other crystal forms which have not yet been solved.[274]

In contrast to the simple bimolecular association observed for hexokinase and glucose, the binding of coenzyme to LADH is relatively complex. Because of the similarity of the gross features of the ligand-induced conformational changes in these enzymes, it is interesting to compare some of their properties in solution.

An ordered mechanism of substrate addition with coenzyme binding first[289] has been established for LADH by a number of studies,[270,271,296] and Eklund et al.[274] have suggested that the coenzyme-induced conformational change provides a molecular explanation for this phenomenon. Ordered binding is not a necessary consequence of domain flexibility, however, as there is no obligatory order of substrate addition for hexokinase.[234,260,290] The differences in the kinetic schemes of these enzymes are clearly a reflection of the quite different topologies of their active sites; the binding sites for both coenzyme and alcohol are found in the extended cleft of LADH (Figure 19) and are both affected by the conformational change,[274] while the nucleotide binding site on hexokinase lies on the surface of the molecule at the edge of the cleft[217,261] and is affected relatively little by the glucose-induced conformational change.[220]

More recent studies of coenzyme binding to LADH provide direct information about the rate of the conformational change itself. Studies of the pH dependence of LADH fluorescence

in the absence of substrate indicate that the enzyme undergoes a pH-dependent conformational change which results in fluorescence quenching.[292] The pK_a of the isomeration involved in this process is about 9.8 and is linked to coenzyme binding, which was shown previously to result in the quenching of protein fluorescence. In studies of the relaxation of the process responsible for fluorescence quenching following a pressure jump,[293] reciprocal relaxation times of 2500 sec^{-1} to 7000 sec^{-1} were observed over the pH range from 7.5 to 10.0 in the absence of coenzyme.

Interestingly, considerably slower reciprocal relaxation times were obtained in the presence of coenzyme (60 to 300 sec^{-1}), corresponding to a rate of about 300 sec^{-1} for the closing of the cleft at pH 7.7. Whether this observation reflects a change in the rate of the underlying molecular process, due perhaps to a change in low-frequency vibrational modes of the protein upon coenzyme binding,[267] or results simply from a kinetic complication in which the binding of coenzyme interferes in some way with the quenching process is not known; an alternate binding site observed for the nicotinamide ring of the coenzyme[283,284] might contribute to such kinetic complications.

The pressure relaxation studies of Coates et al.[293] also indicate that the rate at which the cleft of LADH opens in the presence of NADH is quite slow, having a value of about 60 sec^{-1} at pH 7.7. It is not clear, however, that the conformational change per se is the rate-limiting factor in coenzyme release, as initial rate studies indicate that the dissociation rate of NADH from the enzyme is about 3 sec^{-1} near this pH.[294] Interestingly, the release of NADH is the rate-limiting step for the oxidation of alcohols by LADH over a wide range of conditions,[271,272,294] and the release of ADP, not glucose, is rate limiting for hexokinase in the forward direction.[234] Thus the rate of the conformational change per se would seem to be less a limiting factor for these enzymes than other factors, such as the size or charge of the ligand whose release is rate limiting. It is also interesting in this regard that NMR studies of NADH binding to LADH[291] indicate that the coenzyme is freed of constraints placed by the LADH-NADH complex on its motion at a rate of about 100 sec^{-1}, which is much faster than the rate at which it dissociates from the complex; the process monitored by the NMR studies has not been identified, however.

There is a remarkable similarity in the thermodynamics of binding for the ligand associated with domain flexibility in both LADH and hexokinase. As we have already seen is the case for hexokinase, calorimetric studies of the binding of NADH to LADH show that this process is "entropically driven", in the sense that the net enthalpy of binding is essentially zero.[299] In view of the poorly understood complexities revealed by the calorimetric studies of glucose binding to hexokinase, it is probably premature to associate this behavior with the large-scale conformational changes in these two enzymes. Such a correlation is suggested, however, by the observation that the binding to LADH of ADP-ribose, a coenzyme analogue that does not produce the conformational change,[282] has a nonzero enthalpy of binding.[299]

In contrast, the binding of NADH to yeast alcohol dehydrogenase (YADH) is accompanied by a substantial enthalpy change. The yeast enzyme, a tetramer, is homologous to LADH;[300] although its structure is unknown, a plausible model for the YADH monomer can be constructed by incorporating the YADH amino-acid sequence into the LADH structure.[301] It is unclear whether YADH undergoes a coenzyme-induced conformational change; however, the observation that coenzyme binding and release are rapid and not rate-limiting steps for the yeast enzyme[271] suggests that there are substantial differences in the binding of coenzyme to these enzymes.

The most interesting aspect of domain flexibility in LADH is the question of what function it serves. Eklund et al.[274] describe a region of the monomer near the axis of rotation where the conformational change has little effect on the positions of the atoms. As the reactive zinc atom and its ligands are near this region, the coenzyme-induced conformational change does not produce any major changes in the active site of the enzyme. The protein ligands

of the reactive zinc atom are unchanged and, in contrast to hexokinase, no new residues are brought into the active site by the conformational change. Eklund et al. suggest that one possible function of the conformational change would be to shield the active site from solvent; subsequent studies have shown that the conformational change does displace a number of ordered solvent molecules from the cleft.[276] It is interesting that an analogous region of the active site of lactate dehydrogenase[295] is shielded from solvent by a quite different type of ligand-induced conformational change[274,286] (see below); the mechanisms of these two enzymes differ in a number of respects, however, even for common steps such as proton release.[296]

Eklund et al.[274] also suggest that subtle changes in the environment of the reactive zinc atom of LADH, presumably too small to be detected in the existing crystal structures, might change the catalytic properties of the metal. The binding of coenzyme in solution is known to affect the environment of the metal atom; the reactive zinc atom can be replaced with a Co(II) atom,[297] which shows distinct changes in its visible spectrum when coenzyme is bound to the protein.[298] Whether the same changes are observed in the different crystalline conformations of LADH is not yet known, however.

These observations suggest that domain flexibility in LADH has relatively subtle effects on the catalytic efficiency of the enzyme, in contrast to the apparently obligatory induced fit seen in hexokinase. It clearly would be interesting to know whether the native conformation is catalytically active. In principle, one could answer this question by studying the activity of native crystals of LADH, in which the enzyme is "trapped" in the open conformation. Unfortunately, the instability of native LADH crystals in the presence of coenzyme has made such experiments impractical.

3. Citrate Synthetase

Citrate synthetase catalyzes the formation of citrate from oxaloacetate and acetyl-coenzyme A (acetyl-CoA). The overall reaction probably consists of three steps: enolization of acetyl-CoA, condensation of enolic acetyl-CoA and oxaloacetate to citryl-CoA, and hydrolyss of the latter intermediate to citrate and CoA;[302,303] the evidence that these three activities occur as distinct steps in the enzymatic reaction and that citryl-CoA is a true intermediate is not conclusive, but this approximate description of the reaction is adequate for our purposes. The enzyme from eucariotes is a dimer of identical subunits with a total molecular weight of about 100,000.

Two highly refined crystal structures of citrate synthetase have been reported.[304] A tetragonal crystal form, obtained with the enzyme from pig heart in the absence of substrates, has been refined at 2.7 Å resolution. This crystal contains one crystallographically independent monomer, i.e., the subunits of the dimer are related by an exact crystallographic twofold axis. The molecule is unusual for a protein of this size in that it consists almost entirely of α-helices. Each subunit consists of two domains, the larger of which interacts with the corresponding domain of the other subunit to form the core of the dimer; the smaller domains, which account for about a quarter of the subunit, are located on opposite sides of the core in an orientation that leaves a deep cleft between the small domain of one subunit and the large domain of the other (Plate 3A). This form of the molecule can be crystallized from phosphate or from high concentrations of the product citrate. A binding site for citrate is observed in the cleft between the domains.

A monoclinic crystal form, obtained with citrate synthetase from chicken heart in the presence of coenzyme, has been refined at 1.7 Å resolution. These crystals also contain a single crystallographically independent subunit. The same crystal form can be obtained with the pig heart enzyme either in the presence or absence of coenzyme, but the crystals grown from the chicken heart enzyme were used for the high-resolution crystallographic analysis as they are better ordered. The conformation of the protein in the monoclinic crystals is

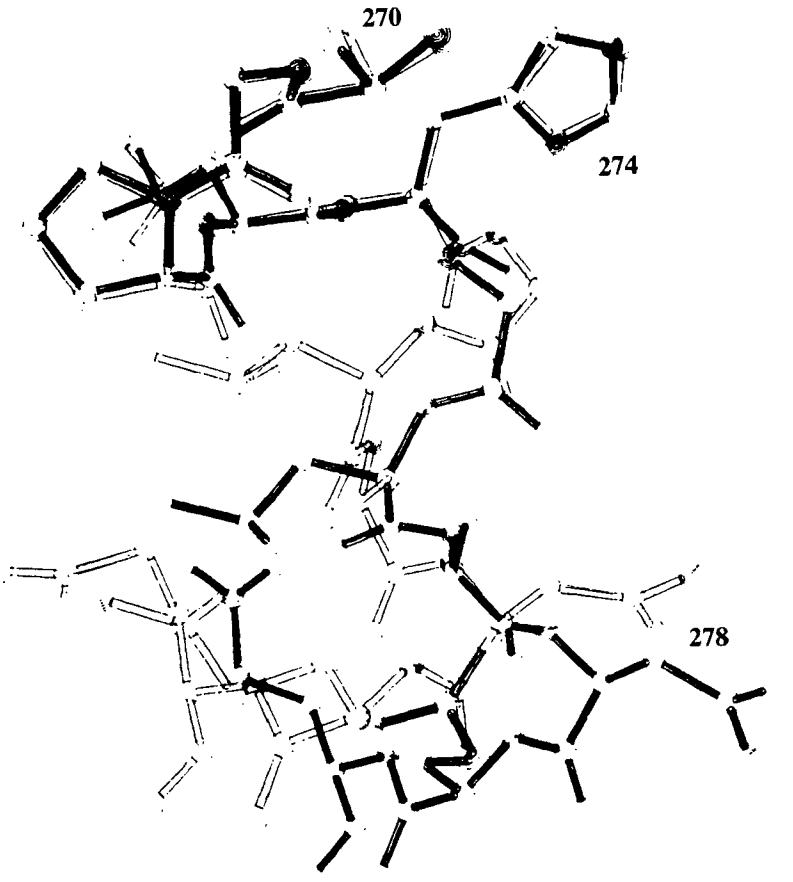


FIGURE 21. Drawing illustrating the hinge point at his274 of citrate synthetase. For this figure, the large domains (approximately residues 1-274 and 381-437) of the open and closed forms of the enzyme were superimposed. One form is drawn with open bonds and the other with solid bonds; nitrogen atoms are shaded. (From Ref. 160, based on coordinates described in Ref. 304.)

always the same, regardless of whether they were grown in the presence or absence of the coenzymes. Compared to their positions in the open conformation of the molecule, the small domains are rotated about 18° towards the core of the dimer, closing off the cleft (Plate 3B). As is the case for hexokinase and LADH, however, there are significant local changes in the conformation of segments involved in different contacts in the two forms of the molecule.

In the closed conformation, the citrate binding site is considerably altered. Compared to its contacts in the open conformation, a bound citrate molecule interacts with several additional residues on both domains of one subunit and with a residue from the large domain of the other subunit of the dimer; as a result, all of its oxygen atoms interact with groups on the protein, and it is almost completely buried in the cleft. The bound coenzyme interacts with several residues from each subunit in the closed conformation. As the regions of the molecule involved in these interactions are well separated in the open conformation, an intact coenzyme binding site exists only in the closed form of the molecule.

The citrate synthetase structures are the most highly refined among the proteins showing hinged domains. In contrast to the situation in hexokinase and LADH, one can see well-defined hinge points in both of the segments of the polypeptide chain linking the domains of the citrate synthetase monomer (Figure 21). The differences in the main chain conformation

of the two models are relatively small, however, as the hinge points are near the axis of the rotation that describes the conformational change. Both hinge points are near the citrate binding site, and one, near his274, contributes directly to the formation of this site.

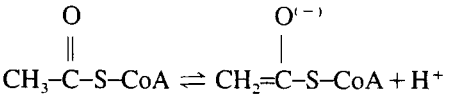
These observations highlight a few interesting common features in the organization of the hinged enzymes whose structure is known in detail. In all three proteins, the globular regions that move relative to one another are linked by two segments of the polypeptide chain. In contrast, there is only one connecting strand between the domains of the flexibly linked proteins discussed earlier. In this respect, it is interesting that the single-stranded connections in the hinge regions of immunoglobulins seem to be more flexible than the connections within the Fab or Fc segments, which are double-stranded as a consequence of the pairwise lateral association of domains. In the hinged enzymes, moreover, at least one of the connecting strands is directly involved in forming the binding site for the ligand that induces the conformation change. Whether these interactions contribute in some way to triggering the conformational change is an intriguing question on which we have no information. A feature common to both LADH and citrate synthetase is that the large domain motions appear to be coupled with small changes in the quaternary interactions of the two domains that form the core of the dimer;[285,308] this behavior may be compared with that of aspartate carbamoyltransferase,[425] in which small changes in the relative orientations of the domains of each subunit are coupled with large changes in the quaternary structure of the molecule. These observations presumably indicate that even the more stable interactions between globular domains or subunits of the proteins involved do not result in strictly "rigid" associations.

The binding of oxaloacetate, the smaller substrate of citrate synthetase, is probably the factor that induces the conformational change in the enzyme, although this distinction cannot be made as clearly for citrate synthetase as it can for hexokinase or LADH. An ordered binding mechanism with oxaloacetate binding first is kinetically favored for citrate synthetase;[305] the crystallographic observations that oxaloacetate cracks crystals of the open form of the enzyme and that the binding site for the coenzyme is completely formed only in the closed conformation[304] are consistent with this mechanism and suggest that oxaloacetate induces the conformational change.

On the other hand, citrate synthetase is subject to substrate inhibition at higher concentrations of acetyl-CoA. Johansson and Petterson[306] attribute this inhibition to population of the alternate substrate-binding pathway, in which acetyl-CoA binds first; assuming an ordered mechanism in each case, these authors calculate that binding of the coenzyme reduces the association rate of oxaloacetate from about $70 \times 10^6\ M^{-1}\ sec^{-1}$ to about $30 \times 10^6\ M^{-1}\ sec^{-1}$. This interpretation is consistent with the observation that crystals of the closed form of the enzyme are formed in the presence of the coenzyme.[304] Taken together, the kinetic and crystallographic studies of citrate synthetase indicate that either substrate can shift the conformational equilibrium of the enzyme towards the closed structure, but that oxaloacetate is more effective in this role at normal substrate concentrations.

In contrast to the situation found with hexokinase or LADH, the binding and release of substrates is not rate limiting for citrate synthetase; the rate-limiting step for this enzyme is probably the enolization of acetyl-CoA.[307] Interestingly, the turnover number for citrate synthetase, about 6000 sec^{-1}, is considerably faster than that of either hexokinase or LADH, even though the magnitude of the effective "rotation" involved in the conformational change of citrate synthetase is larger. It should be noted, however, that there is presently little evidence that citrate synthetase adopts only the two crystallographically observed conformations in solution. Although there is evidence to suggest that there is essentially only one closed form of the enzyme (see below), one cannot rule out a more flexible open conformation; we have tentatively classified citrate synthetase as a hinged enzyme on the basis of the strong similarities between this enzyme and the examples discussed earlier.

The function of the conformational change in citrate synthetase appears to be more analogous to that found for hexokinase than to that proposed for LADH, in that the conformational change is probably required for activity. As was mentioned earlier, enolization of acetyl-CoA (inset)



is thought to be a step in the reaction catalyzed by citrate synthetase. No exchange of methyl protons with solvent is observed when the enzyme is incubated with acetyl-CoA, as would be expected if the enzyme catalyzed the partial reaction shown, but addition of L-malate, an unreactive analogue of oxaloacetate, to the incubation mixture results in exchange.[307] This observation clearly suggests that an induced-fit process occurs in citrate synthetase.

As a specific catalytic mechanism has not been proposed for this enzyme, one cannot say whether the crystallographically observed conformational change brings any critical functional groups into the active site or whether the effect of the conformational change is confined to alteration of the substrate binding sites. Neither crystal form of citrate synthetase described so far is active;[304] there are a number of possible explanations for the inactivity of enzyme crystals, however, and it is not known why these citrate synthetase crystals are inactive. As is the case for hexokinase and LADH, the conformational change in citrate synthetase serves to shield the active site from solvent in the closed form of the enzyme.

A third crystal form of citrate synthetase has recently been obtained by crystallization of the enzyme in the presence of oxaloacetate.[308] These crystals contain two crystallographically independent copies of the citrate synthetase monomer in a closed conformation similar to that found in the monoclinic crystals described earlier; it is thus likely that the crystallographically observed closed conformation of citrate synthetase is a stable conformation of the molecule in solution and is little influenced by crystal packing effects. The interesting feature of the oxaloacetate complex is that the conformation of the small lobe may be different from that found in the native molecule and its complex with coenzyme. A preliminary comparison of the three citrate synthetase structures (Table 6) indicates that the small domains of the dimer in the oxaloacetate complex do not superimpose well on the small domains of the other two structures; the large domains of all four crystallographically independent monomers superimpose on one another quite well by comparison.

It is unlikely that these differences in the small domains, which involve localized shifts in a few segments of the polypeptide chain, are due to crystal packing effects, since the two crystallographically independent copies of this domain in crystals of the oxaloacetate complex superimpose well on one another. An interesting possibility suggested by the apparent correlation between the structure of the small domain and the nature of its ligands is that the different conformations of this part of the molecule are related to the different activities catalyzed by citrate synthase.

4. Glyceraldehyde-3-Phosphate Dehydrogenase

Glyceraladehyde-3-phosphate dehydrogenase (GPDH) catalyzes the oxidation and phosphorylation of glyceraldehyde-3-phosphate to 1,3-diphosphoglycerate.[309] The enzyme invariably occurs as a tetramer with a total molecular weight of about 145,000. The enzyme from rabbit muscle has been the subject of a number of kinetic and physicochemical studies, as it displays negative cooperativity in the binding of coenzyme, NAD^+.

The evidence for domain flexibility in GPDH comes from crystallographic studies of the enzyme from the thermophilic procaryote *Bacillus stearothermophilus*. The structure of the complex between this enzyme and NAD^+ has been solved at 2.7 Å resolution.[310] This crystal form contains the holoenzyme; i.e., an NAD^+ molecule is bound to each subunit of the tetramer. The structure of the bacterial enzyme is very similar to that of the holoenzyme

Table 6
COMPARISON OF CITRATE SYNTHETASE STRUCTURES

	C2	P4	C4/1	C4/2
C2	—	0.72 (2299)	0.56 (2336)	0.54 (2320)
P4	1.35 (748)	—	0.77 (2341)	0.75 (2338)
C4/1	1.00 (697)	1.87 (662)	—	0.29 (2360)
C4/2	0.99 (697)	1.86 (662)	0.32 (666)	—

Note: The table gives the rms difference (in Ångstroms) between C_α coordinates after optimal superposition of individual domains of the different citrate synthetase structures; the number of C_α atoms contributing to the comparison is given in parentheses below the rms difference. Comparisons of the large domains lie above the diagonal, and comparisons of the small domains lie below the diagonal. The closed and open structures described by Remington et al.[304] are denoted C2 and P4, respectively; the two crystallographically independent subunits of the second closed structure[308] are identified as C4/1 and C4/2. (From Ref. 160.)

from lobster, which was determined earlier.[311] Each subunit of the enzyme consists of two domains; a coenzyme-binding domain and a catalytic domain. The coenzyme-binding domain is similar in structure to those found in a number of other dehydrogenases,[312] including LADH. In GPDH, however, the catalytic domains form the core of the tetramer, and the coenzyme-binding domains are on the outside of the molecule.

Preliminary reports of the structure of the unliganded molecule (the apoenzyme) indicate that the coenzyme-binding domains have moved away from the core of the tetramer in this form of the enzyme, exposing the coenzyme-binding site to solvent.[313-315] The structural details of this conformational change are not yet available, although movement appears to involve a rigid-body rotation of the domains,[313,316] as is the case for the other examples of hinged enzymes.

The interesting question about the conformational change in GPDH is whether it contributes in some way to the negative cooperativity of coenzyme binding. The structure of the enzyme indicates that this is possible. The coenzyme binding site of one subunit of the holoenzyme is formed in part by an irregular segment of the polypeptide chain (the "S-loop") that extends from the catalytic domain of another subunit; as the environment of the S-loop is altered by the conformational change, it could be responsible for the allosteric interactions among the coenzyme binding sites.[310,313] However, the possibility that a large conformational change is not required to produce the negative cooperativity observed for GPDH is suggested by the observation that GPDHs from two other species, the Atlantic lobster *(Homarus americanus)* and the Mediterranean lobster *(Palinurus vulgaris)*, appear not to undergo a conformational change upon binding coenzyme,[318,319] even though both bind coenzyme cooperatively.[317,318] Interestingly, crystals of Mediterranean lobster GPDH holoenzyme are catalytically active.[320]

These crystallographic results on GPDH from the two species of lobster were unexpected, as a number of earlier investigations had shown that coenzyme binding produces a large conformational change in GPDH in solution; these included studies of sedimentation velocity,[321-323] small-angle X-ray scattering,[324,325] and of an intramolecular acyl-transfer reaction[326,327] that could not take place in the holoenzyme without some change in its conformation.[311] However, these physiochemical studies were all performed on GPDH from yeast or mammalian muscle; there is no direct evidence that the lobster or *B. stearothermophilus* enzymes share these properties. Although crystallographic studies of the bacterial enzyme[310,313,314] indicate that it probably exhibits similar behavior in solution, it has been shown that the Mediterranean lobster enzyme does not undergo the acyl-transfer reaction.[320] It is thus unclear whether the differences in domain flexibility suggested by the various GPDH crystal structures reflect genuine differences in the flexibility of the enzyme from different species, are artifacts of crystallization, or result from subtler differences in the conditions under which the proteins were studied.

5. Aspartate Aminotransferase

Aspartate aminotransferase catalyzes the pyridoxal phosphate-dependent transfer of an amino group from aspartate or glutamate to an α-keto acid.[328] The enzyme from animal tissue is a dimer with a total molecular weight of about 90,000. Distinct isozymes of aspartate aminotransferase are found in the cytosolic and mitochondrial fractions of animal tissue.

The structure of cytosolic aspartate aminotransferase from chicken heart has been determined at 3.2 Å resolution,[329,429] and the structure of the mitochondrial enzyme from the same source has been determined at 2.8 Å resolution,[330] and a preliminary report of the structure of the cytosolic enzyme from pig heart at 2.7 Å resolution has also been presented.[331] We will confine our attention to the latter two structures for which domain flexibility has been demonstrated; these two molecules are very similar, even though the amino-acid sequences of the mitochondrial and cytosolic isozymes are identical in only half of their residues.[331,332] Both isozymes were crystallized in the presence of the coenzyme, which is covalently bound to the protein in the pyridoxal phosphate form. In its pyridoxamine phosphate form, the coenzyme can be removed from the protein by dialysis;[333] the resulting apoenzyme of the mitochondrial enzyme forms crystals isomorphous to holoenzyme crystals.

When α-methylaspartate, an inhibitor that competes with aspartate, is diffused into crystals of cytosolic aspartate aminotransferase, extensive changes are seen in the small lobe of one of the two crystallographically independent subunits of the molecule.[331] The distribution of difference electron density in a comparison of the holoenzyme and its complex with the inhibitor suggest that the small lobe makes a small, concerted movement towards the core of the dimer when the inhibitor is bound; the conformational change seen by this technique is probably prevented from going to completion by crystal packing constraints, since the crystals crack and dissolve in the presence of some substrates.[334] As the active sites of the dimer are equivalent in solution, the asymmetry that this soaking experiment reveals in the crystalline molecule is presumably an artifact of the crystal packing.[331]

Remarkably, the same result is obtained when the cytosolic enzyme is crystallized in the presence of α-methylaspartate; this observation suggests that the species of the dimer in solution which has only one substrate site occupied is selectively crystallized under the conditions of this experiment.[331] If this interpretation is correct, these orthorhombic crystals of cytosolic aspartate aminotransferase provide a quite striking example of the stabilization of a specific conformation of a protein by crystal packing forces.

In contrast to the cytosolic enzyme, crystallization of the mitochondrial isozyme in the presence of α-methylaspartate produces two new crystal forms, one of which has recently been solved at low resolution.[332,335] The structure of this form of the enzyme indicates that the small lobe has probably rotated as a rigid body towards the core of the molecule;[335]

a similar movement is suggested in one subunit of the molecule when N-(5′-phosphopyridoxyl)-L-aspartate is diffused into crystals of the mitochondrial enzyme.[333] Given the strong similarities between the aspartate aminotransferases and the hinged enzymes discussed earlier, it seems likely that this crystallographically determined conformational change is a reasonable model for the behavior of the enzyme in solution, although it is also clear that there are complex packing effects in the best-studied crystal forms of this enzyme. Chemical-modification studies of aspartate aminotransferase are consistent with the occurrence of some substrate-induced conformational change in solution,[47] but the crystallographic studies of this enzyme are not sufficiently far advanced to attempt to correlate the results of these two methods.

As is the case for several other hinged enzymes, it is uncertain what functional role of domain flexibility might serve in aspartate aminotransferase. Microspectrophotometric studies of holoenzyme crystals of both isozymes[334,336] have shown that the crystalline enzyme is at least partially active towards substrate diffused into the crystal lattice. Whether these observations indicate that the substrate-induced conformational change is not necessary for activity or simply that some fraction of the molecules (or subunits) in the crystal can adopt an active conformation without disrupting the crystal lattice is not yet known.

6. Kinases Other Than Hexokinase

Anderson et al.[254] have compared the gross structures of yeast hexokinase, phosphoglycerate kinase from horse muscle,[337] and adenylate kinase from pig muscle[338] by inspection of space-filling drawings. On the basis of the observations that all three molecules are bilobal to some extent and that substrates bind in the cleft separating the lobes, these authors proposed that a substrate-induced conformational change analogous to that found in hexokinase would be a common property of kinases. Blake and Evans[337] had earlier suggested that the bilobal nature of these kinases might be indicative of a common mechanism. Neither pyruvate kinase[339] nor phosphofructokinase[340] shows the pronounced cleft found in the other kinases; as the latter enzyme is crystallized in the presence of one of its substrates; however, Anderson et al. suggest that it might be in a closed conformation.

a. Phosphoglycerate Kinase

A large change in the conformation of phosphoglycerate kinase upon binding substrates was first observed in low-angle X-ray scattering studies[239] of the enzyme from yeast; these results were subsequently confirmed by hydrodynamic studies.[421] A substrate-induced conformational change in this enzyme was also suggested independently by Banks et al.[341] on the basis of the structure of equine muscle phosphoglycerate kinase at 2.5 Å resolution. Although there is no evidence for a conformational change of the muscle enzyme in solution, the structures of the yeast and muscle enzymes are known from earlier crystallographic studies to be quite similar.[422,423]

Banks et al. proposed binding sites for phosphoglycerate and ATP on opposite sides of the cleft of the enzyme, which placed the substrates too far apart (about 10 Å) to interact; this situation arises because the "cleft" in phosphoglycerate kinase is much wider than that found in hexokinase or the other hinged enzymes studied in detail. Banks et al. thus suggested that a change in the orientation of the two domains of the enzyme that form the cleft would be required to bring the substrates together. Although the binding site proposed by these authors for phosphoglycerate was based only on model-building studies and therefore quite tentative, recent crystallographic studies of the yeast enzyme[424] have confirmed the general features of this substrate-binding model. The proposed isomerization is consistent with the observation from small-angle scattering and hydrodynamic studies that both substrates are required to induce a conformational change in the yeast enzyme.

The binding of substrates to the crystalline phosphoglycerate kinases has complex effects on the integrity of the crystals and the degree of order in the bound ligand, depending on

whether sulfate ion is present and on the pH of the experiment.[342,424] A particularly interesting contrast between the two crystalline enzymes is that the binding of 3-phosphoglycerate to the muscle enzyme causes such extensive changes in the molecule that the bound substrate cannot be located with confidence,[342] while the yeast enzyme was apparently crystallized inadvertently in the presence of this substrate.[424] As both substrates are required to produce the large conformational change in solution, the relationship of the changes produced by 3-phosphoglycerate in the muscle enzyme to the structural changes documented by the small-angle scattering and hydrodynamic studies is unclear. Although the solution studies demonstrate that phosphoglycerate kinase exhibits some type of large-scale flexibility, there is no direct evidence that the enzyme is "hinged" in the sense that we have defined. As Banks et al.[341] have argued, however, the pronounced domain structure of the enzyme makes this proposal quite plausible. A rolling-contact "hinge" analogous to that found in LADH has been proposed recently for phosphoglycerate kinase by Blake and Rice,[342] while Watson et al.[424] have drawn attention to the existence of several gly-gly-gly sequences in the yeast enzyme and have proposed a specific "trigger" mechanism for the conformational change.

b. Adenylate Kinase

The analogy proposed by Anderson et al.[254] between the structure of adenylate kinase and the other kinases mentioned above is somewhat strained. Adenylate kinase has a molecular weight of only 22,000, which is about the size of one of the lobes of hexokinase or phosphoglycerate kinase. The cleft in adenylate kinase is formed by two helical protrusions which are also connecting segments between strands of the β-sheet at the core of the molecule.[338]

There are three crystal forms of native adenylate kinase; all are in the same space group, but their unit cell constants are different.[343] Two of these crystal forms, designated A and B, are quite similar, differing by only 2 Å in one of the unit cell dimensions. The A form has been solved at 3 Å resolution,[338] and the less stable B form has been solved at 4.7 Å resolution.[343] Comparison of these structures[343] shows that a glycine-rich loop of about seven residues located along one side of the cleft moves by as much as 6 Å, and smaller motions are observed in some of the helices forming the cleft.

A reversible transition between these crystal forms is induced by changes in pH or by the binding of some heavy-atom compounds. It is difficult to establish whether these factors influence the structure of the molecule directly or cause some change in the crystal packing contacts which indirectly influence the molecule; Sachsenheimer and Schulz[343] present arguments in favor of the former alternative. Neither of the adenylate kinase structures has been refined, however, and the resolution obtainable with the less stable B crystal form is insufficient to allow a detailed comparison of the structures. In addition, the binding sites of ATP and AMP, the substrates of adenylate kinase, are not yet well established.[344]

c. Pyruvate Kinase

Unlike the other kinases discussed so far, pyruvate kinase is a tetramer; each of the identical subunits of the molecule is folded into three domains,[339] and the active site is situated in a crevice between the large central domain and the smallest of the domains, which accounts for about 20% of the subunit. Stammers and Muirhead[345] have suggested, on the basis of low-resolution studies, that this domain might be flexibly connected to the rest of the molecule, as it is poorly ordered in the crystal. The details of this disorder are unclear, however. Low-resolution studies of substrate binding[345] show only localized changes in the structure of the protein.

d. Phosphofructokinase

Phosphofructokinase is also a tetramer of identical subunits, each of which consists of two domains.[340] The enzyme is allosteric, showing a sigmoidal dependence of the reaction

velocity on the concentration of the sugar substrate, fructose-6-phosphate. The activity of the enzyme is apparently regulated by variations in its affinity for this substrate; i.e., phosphofructokinase is a "K system" with respect to fructose-6-phosphate in the notation of Monod et al.[409] The active site of the enzyme is in a crevice between the domains, as is the case for a number of enzymes;[410] however, recent studies at 2.4 Å resolution[346] have shown that fructose-6-phosphate is at the surface of the subunit and interacts with residues from a second subunit of the tetramer. For this reason, Evans et al.[346] suggest that a change in the quaternary structure of the enzyme can account for its allosteric properties and that a large conformational change within the subunits is unlikely. A study of the enzyme crystallized in the presence of an allosteric inhibitor is in progress[346] and should resolve this question.

7. Transport Proteins

a. Arabinose-Binding Protein

The arabinose-binding protein (ABP) of *Escherichia coli* is one of several proteins in the periplasmic space of the bacterium that participates in the active transport of metabolites into the cell. The structure of ABP has been solved at 2.4 Å resolution.[347] Although arabinose was not added to the crystallization medium, the protein apparently retained one tightly bound arabinose molecule throughout its purification and crystallization.[348] The arabinose molecule is found buried deep in a cleft between the two domains of the molecule, where it is inaccessible to solvent.

Entry and exit of the sugar from its binding site in solution probably involves a large conformational change in ABP, as small-angle scattering studies[242] show a significant reduction in the radius of gyration of the protein in the presence of sugar. The details of this conformational change are not yet known; the change in radius of gyration is consistent with a rigid-body rotation of the two domains through an angle of about 18°. A study of the crystal structure of ABP in the absence of sugar is in progress[348] and should provide a test for this model.

Interestingly, the identification of the buried arabinose molecule could be confirmed by soaking ABP crystals in solutions containing galactose, which displaced the bound sugar.[348] As is also the case for the exchange of glucose from the HKA-glucose complex,[227] the mechanism by which the buried substrate is exchanged without disrupting the crystals is not known.

b. Riboflavin-Binding Protein

Riboflavin-binding protein (RBP) is a transport protein found in the whites of avian eggs. The crystal structure of this protein is not known, but there is evidence from small-angle X-ray scattering studies[349] to suggest that the binding of riboflavin by RBP is accompanied by a large conformational change in the protein. This work is of interest because the authors make some effort to study the nature of the conformational change by exploiting the capabilities of small-angle scattering in combination with hydrodynamic and spectroscopic data. As the radius of gyration, surface area, and hydration of the protein all appear to increase in the absence of riboflavin with little change in its secondary structure, Kumosinski et al.[349] suggest that RBP is, in our terminology, a hinged protein.

D. Other Examples of Flexibility

A few examples of domain flexibility have been documented which do not fit in the categories we have described so far. This results, of course, in part from the arbitrary nature of the categories we have defined. Other systems discussed in this section are not sufficiently well characterized as yet to define the nature of the flexibility, and some do not quite fit our equally arbitrary definition of domain flexibility. The sample of systems discussed was chosen largely to illustrate selected points and is not meant to be comprehensive.

1. Muscle Proteins

Contractile proteins in general and those of muscle in particular are perhaps the most obvious place to look for examples of large-scale flexibility in biological systems. The concept of a flexible connection between the globular and fibrous regions of the myosin molecule has been a central feature in most theories of the role of cross-bridges in the sliding-filament model of contraction, both in the early ideas of Huxley and his collaborators[350] and in the refinements and extensions of this model since.[351-353] As yet, however, the molecular details of the flexibility in his system remain unclear, so there is little basis for comparison to the examples discussed in this review. Two recent reviews discuss experimental studies of flexibility in myosin[354] and other contractile proteins.[355] A particularly interesting development in this field is the introduction of time-resolved fiber diffraction studies of intact muscle using the very high intensity X-rays available from a synchrotron.[356,357]

The only component of muscle that has been studied crystallographically is tropomyosin, a fibrous protein consisting of a "coiled coil" of two α-helices. Tropomyosin crystals have a very high solvent content and are relatively poorly ordered,[358] which limits the resolution to which the structure can be determined. Departures from a regular coiled-coil structure observed in studies of these crystals at 20 Å resolution[358] suggest, however, that there may be localized regions of flexibility even in this fibrous molecule.

2. Tomato Bushy Stunt Virus

One of the earliest crystallographically documented examples of domain flexibility in proteins was found in the coat protein of tomato bushy stunt virus (TBSV). This protein consists of two globular domains which adopt one of two different orientations with respect to one another in the intact virus, depending on the location of the subunit with respect to the icosahedral symmetry elements of the capsid.[359] It was originally believed that there were only two major conformational states of the subunit,[360] but the recent structure at 8 Å resolution of the "expanded state" of the virus,[361] which is observed at high pH in the absence of divalent ions, indicates that substantial rotations of the domains of some subunits are involved in the transition to this form of the virus. The latter observation suggests that the domains of the TBSV coat protein may be flexibly linked but "trapped" in a small number of conformations in the virus. The observation that one domain of subunits at particular locations of the expanded particle is more disordered than the rest of the structure[361] is consistent with a flexible linkage of the domains.

The TBSV coat protein illustrates a difficulty with the classification scheme we have used in earlier sections of this review. One important function of domain flexibility in the TBSV coat protein is to allow the chemically identical subunits of the viral capsid to adapt to the different chemical environments found at "quasi-equivalent" positions of the icosahedral lattice of the particle; in this respect the subunit behaves functionally as a "hinged" protein, as it is only required to assume two different conformations in the capsid. On the other hand, the structure of the expanded form of the virus suggests that more extensive flexibility is involved in transitions between different states of the molecule; one can imagine, for example, that flexible linkage of the domains might be important in assembly of the virus. Thus the distinction we have drawn earlier between "hinged" and "flexibly linked" domains may be inappropriate for TBSV and other self-assembly systems in which there are different functional requirements for flexibility in different physiological states of the system.

The TBSV subunit also provides the clearest example of a protein that exhibits two distinct types of functional domain flexibility. In addition to the differences in the relative orientation of their globular domains, subunits at quasi-equivalent positions of the icosahedral lattice show substantially different degrees of order in their N-terminal regions.[359] The degree of order in this "domain" correlates with the conformation of the globular domains of the subunit; in one conformation about 25% of the subunit is crystallographicaly disordered,

and in the other about a third of these residues are ordered and interact with like segments from two other subunits of this type. It is not known whether the disorder in these region is static or dynamic, but the former seems more likely because the N-termini of the coat protein subunits are all on the inside of the virus particle, where the RNA is located. As the RNA does not possess the 60-fold redundancy imposed on the arrangement of coat-protein subunits by the icosahedral symmetry of the capsid, at least part of it would be disordered in any crystallographic study, along with any regions of the coat protein forced to depart from capsid symmetry by interactions with the RNA. In TBSV, it appears that the RNA is completely disordered, and the N-terminal regions of the coat-protein subunit are presumably disordered because they interact with it.

3. Aminoacyl-tRNA Synthetases

Aminoacyl-tRNA synthetases play a critical role in protein synthesis, the specific linkage of an amino acid with the appropriate transfer RNA.[362] The crystal structures of two of these enzymes are known. The structure of the tyrosyl-tRNA sythetase from *B. stearothermophilus* has been solved at 2.7 Å resolution,[363] and the structure of a catalytically active fragment of the methionyl-tRNA synthetase from *E. coli* has been solved at 2.5 Å resolution.[364] A substantial region of the molecule is disordered in crystals of the tyrosyl-tRNA synthetase, where no electron density is observed for the C-terminal quarter of the enzyme;[365] although this structure has not yet been refined, the disorder has persisted throughout the application of a phase-extension procedure that improved the quality of the ordered part of the structure considerably.[366]

The interactions of a number of tRNA synthetases with tRNA molecules in solution have been studied by small-angle neutron scattering. With this technique, it is possible to examine the behavior of the protein component of the system alone by adjusting the mixture of ^1H_2O and ^2H_2O in the solvent to match the scattering density of the tRNA component. This approach has been used to demonstrate the existence of large-scale flexibility in valyl-tRNA synthetase from yeast, for example; tRNA binding induces a large change in the radius of gyration of the monomeric form of this enzyme.[367] Recent small-angle neutron scattering studies of tyrosyl-tRNA synthetase from *E. coli*, which shows strong homology to the enzyme from *B. stearothermophilus*, indicate that tRNA binding produces little change in the shape or radius of gyration of this molecule, however.[397] Although the nature of the crystallographically observed disorder in this molecule remains to be established, the small-angle scattering results would seem to argue against a large rearrangement of hinged domains. Whether the flexibility implied by the observed disorder has any function is also not known.

It is unclear whether similar large-scale disorder occurs in the methionyl-tRNA synthetase. One can easily estimate that the 480 residues in the model reported by Risler et al.[364] account for little more than 80% of the protein in the crystal, which has a molecular weight of about 65,000.[368,369] However, Risler et al. do not explicitly discuss disorder in the structure, which is also unrefined.

Small-angle neutron scattering experiments indicate that tRNA binding reduces the radius of gyration of the intact methionyl-tRNA synthetase[370] but has little effect on the proteolytically modified molecule.[371] The latter form of the molecule is the same active fragment found in the crystals studed by Risler et al., which is produced by digestion of the native molecule with trypsin.[369] This treatment removes about 25% of mass of the native subunit from the C-terminus of the polypeptide chain.[368] The role of this C-terminal segment in the tRNA-induced change in the radius of gyration of the intact molecule is not known. It is also uncertain whether this change in radius of gyration results from a change in the quaternary structure of the molecule or from some type of domain flexibility, although Dessen et al.[370] prefer the former explanation.

4. Sequence-Specific DNA-Binding Proteins

There is evidence for domain flexibility in several sequence-specific DNA-binding proteins (i.e., repressors and gene-activator proteins), although the exact nature of the flexibility is as yet uncertain. The clearest example of domain flexibility among these proteins is found in the catabolite gene activator protein (CAP) from *E. coli*.[372,373]

Crystals of CAP contain one crystallographically independent dimer with a total molecular weight of 45,000, the chemically identical subunits of which consist of two domains each. Model-building studies[372] suggest that the smaller C-terminal domain, about a third of the subunit, is involved in DNA binding, while the N-terminal domain forms the intersubunit contacts of the dimer and binds the effector, cyclic AMP.[373] The orientations of the two C-terminal domains relative to their respective N-terminal domains is quite different. The N-terminal domains of the dimer are related by a simple twofold rotation within experimental error; when the N-terminal domains are superimposed, however, a further rigid-body rotation of nearly 30° is required to superimpose the C-terminal domains.[372]

The structures of two repressor proteins are known, both from the *E. coli* bacteriophage lambda. The structure of the cro repressor has been solved at 2.8 Å resolution.[374] Cro crystals contain four crystallographically independent copies of the protein arranged with approximate 222 symmetry, but unlike the CAP subunits, the four copies of cro show no major differences in structure. The cro protein is only as large as the C-terminal fragment of CAP, however, and is not folded into distinct domains that might move relative to one another.

The structure of a DNA-binding fragment of the c*I* repressor (the "lambda repressor" responsible for maintaining lysogeny) has been solved at 3.2 Å resolution.[375] In contrast to cro, the crystalline fragment of the c*I* repressor is probably an independent N-terminal domain of this protein;[419] it accounts for nearly 40% of the c*I* subunit and is about 1.5 times as large as the presumed DNA-binding domain of CAP. The three crystallographically independent copies of the c*I* fragment have essentially the same structure, providing further evidence that the structure of the domain is stable in solution. It is clearly possible that the connecting peptide between the domains of the c*I* repressor is flexible to some degree, but there is no direct evidence of this.

NMR studies of the *lac* repressor from *E. coli*[376] suggest that members of this group of proteins also display some type of large-scale flexibility in solution. The *lac* repressor is a tetramer with a total molecular weight of about 150,000. As is the case for the c*I* repressor of lambda, an N-terminal DNA-binding domain can be separated from each subunit of this protein by limited proteolytic digestion. The N-terminal domain of *lac* repressor (the "headpiece") is about the same size as the C-terminal domain of CAP; isolated headpiece is monomeric, while the C-terminal domains of the molecule remain aggregated as a tetrameric "core" particle after digestion. Comparison of the resolution-enhanced proton NMR spectra of intact *lac* repressor, headpiece, and core shows that almost all of the sharp resonances present in the intact molecule are associated with the headpiece. It is not clear whether these resonances arise from global motion of the entire headpiece or from a group of highly mobile surface residues localized to the headpiece, although Wade-Jardetzky et al.[376] prefer the former explanation.

Steitz et al.[262] speculate that in multidomain regulatory proteins, such as CAP, lambda c*I* repressor, or *lac* repressor, fluctuation of the DNA-binding domain between two or more conformations might allow the protein to "sample" potential binding sites during one-dimensional diffusion along a DNA molecule. It remains to be established, however, that all such molecules are capable of the same types of motions. At present, too little is known about the nature or extent of the flexibility in any one of these proteins to describe its motions in detail or to classify it as flexibly linked or hinged.

5. Phosphorylase

Glycogen phosphorylase is a large enzyme subject to complex regulatory controls. In addition to the well-known interconversion of active and inactive forms of the enzyme by phosphorylation and dephosphorylation,[377] respectively, phosphorylase *b*, the less active form, is allosterically activated by AMP and allosterically inhibited by ATP and other metabolites.[378] Phosphorylase *a*, the more active form, is also subject to some allosteric control but is much less sensitive to effectors than phosphorylase *b*. Both forms of phosphorylase are found as tetramers of identical subunits at high protein concentrations, but the predominant form of the enzyme under physiological conditions is probably a dimer with a total molecular weight of about 195,000.

Crystallographic studies of the phosphorylated form of the enzyme, phosphorylase *a*, (reviewed by Fletterick and Madsen)[379] suggest that this form of the enzyme may display a type of large-scale flexibility distinct from any of the categories of domain flexibility discussed earlier. The phosphorylase *a* dimer forms crystals with a single crystallographically independent subunit;[380] this structure has been solved at 2.5 Å resolution.[381] Low-resolution studies of the binding of maltoheptose (an oligosaccharide substrate) and glucose-1-phosphate to phosphorylase *a*[382] indicate that several contiguous segments of the polypeptide chain, together accounting for nearly 20% of the subunit, undergo small shifts in position when both of these ligands are diffused into cross-linked crystals of the enzyme. Three of the shifted segments are located near the subunit interface of the dimer, where they form a broad belt around the center of the molecule.[383]

The occurrence of small, concerted movements over a large, contiguous region of a protein is by no means unreasonable. However, the existence of such motions is likely to be more difficult to establish crystallographically than has been the case for domain flexibility. As we have seen, results similar to those described for phosphorylase *a* are obtained when substrates are diffused into crystals of a hinged enzyme. However, the hinged enzymes are so defined because the structure of the enzyme-substrate complex is known, and one can conclude that crystal-packing interactions prevent the substrate-induced conformational change in crystals of the unliganded enzyme.

For phosphorylase *a*, two factors complicate interpretation of the crystallographic results. First, the crystals must be cross-linked to prevent them from shattering and dissolving in the presence of glucose-1-phosphate and maltoheptose.[382,388] Thus one cannot rule out the possibility that the enzyme undergoes a much larger substrate-induced conformational change in the absence of crystal-packing constraints. On the other hand, it is known that oligosaccharide substrates bind to a site on the enzyme distinct from the active site, the "glycogen storage site".[387] As this site is near a crystal-packing contact, it is possible that the cracking of phosphorylase *a* crystals in the presence of substrates is due less to the substrate-induced conformational changes than to direct interference with the crystal packing.

In spite of the uncertainty about the magnitude of the substrate-induced conformational change in phosphorylase *a*, it is quite plausible that the segments of the polypeptide chain observed to move upon substrate binding[382] are involved to some degree in the allosteric response of the enzyme. One of these segments may provide an allosteric link between the active site of the subunit and the dimer interface,[382-384] and a second contains the residue at which the enzyme is phosphorylated by phosphorylase kinase, ser14. The allosteric effector site, at which both AMP and ATP bind,[385,386] is near these two segments and the dimer interface. Another of the shifted segments seems to link the active site with the glycogen storage site.[382,383]

Crystals of phosphorylase *b* also crack in the presence of glucose-1-phosphate and oligosaccharide,[388,393] but the conformational changes responsible for this phenomenon have not been studied in cross-linked crystals. Phosphorylase *b* forms crystals that are isomorphous to the phosphorylase *a* crystals described above;[382,389,390] the structure of this form of the enzyme has been solved at 3 Å resolution[391] and partially refined.[392,393]

In spite of the similar behavior of phosphorylase crystals in the presences of substrates and the gross structural similarity of the two forms of the enzyme implied by the isomorphism of their crystals, there are substantial differences in the properties of these molecules in the crystalline state. Phosphorylase *a* crystals are stable in the presence of AMP,[385] for example, but phosphorylase *b* crystals are not.[386] Glucose-1-phosphate produces conformational changes in phosphorylase *a* that are less extensive but qualitatively similar to those observed in the presence of both substrates,[382,385] but only localized changes are seen in the structure of phosphorylase *b* in the presence of this substrate.[391]

The most striking difference between the crystalline phosphorylase molecules is found in a comparison of their catalytic activities.[388] Cross-linked crystals of both phosphorylase *a* and phosphorylase *b* are active, and both have the same affinities for substrates, assessed by Km values, in the crystal as in solution; the latter observation suggests that both forms of the enzyme are in the less active "T" allosteric state in the crystal.[379,392] Interestingly, phosphorylase *a* is as active in the crystal as phosphorylase *b* is in solution, even though the maximal velocities of both forms of the enzyme are reduced 10- to 100-fold by crystallization. It is thus unlikely that the isomorphous crystal lattices have "trapped" the two forms of phosphorylase in the same conformation.

Direct comparison of phosphorylases *a* and *b* at low resolution by difference Fourier techniques[380] showed that the main difference in the two structures is at the N-termini of the subunits.[394] It is now known that the first 19 residues of phosphorylase *b* are at least partially disordered in the crystal,[391] while the same region of phosphorylase *a* is at least partially ordered.[394] However, as neither structure is completely refined, there is still some question about the degree of order in both forms of the enzyme.

The N-terminal segment of phosphorylase *a* is predominantly but not completely, ordered.[379,384] A higher degree of flexibility at the N-terminus of phosphorylase *b* is likely, as the observed disorder in this segment has persisted during partial refinement of the structure.[393] On the other hand, the observation of paired positive and negative difference electron-density features in this region of the difference Fourier comparing the phosphorylases[380] suggests that there is partial order in phosphorylase *b*. On the basis of these observations, it would appear that the differences in the activities of the crystalline phosphorylases are associated with rather subtle structural differences. However, some caution is required in comparing the structures of the native enzymes with the properties of cross-linked crystals in the presence of substrates; in view of the effects of substrates on such crystals of phosphorylase *a* described earlier, it is unclear to what degree the active molecules might be "distorted" from the native structures. Regardless of the actual magnitude of the substrate-induced conformational changes in phosphorylase, it is clear that they are considerably more extensive than the localized differences in the *a* and *b* forms of the molecule. The regulation of this enzyme thus seems to involve a number of types of flexibility involving structural units of different size.

6. Phospholipase

Phospholipase catalyzes the hydrolysis of the C_2 ester bond in 3-phosphoglycerides.[395] Bovine pancreatic phospholipase A2 is a single polypeptide chain with a molecular weight of 14,000; in the pancreas, the enzyme occurs as a zymogen (prophospholipase) that can be activated by proteolytic removal of its N-terminal heptapeptide. Comparison of the crystal structures and catalytic activities of phospholipase and prophospholipase reveals an interesting parallel between the activation process of this enzyme and that of trypsin.

The structure of the activated enzyme is highly refined at 1.7 Å resolution,[395] and the structure of the prophospholipase has been solved and refined recently at 3.0 Å resolution.[396] Two regions of the zymogen are disordered in the crystal. A segment at the N-terminus of the molecule, including the residues that are removed upon activation, is poorly ordered,

with no density visible at all for the first 10 residues of the refined structure; a nearby loop, residues 61 to 74, is also essentially completely disordered. These segments, which account for nearly 20% of the zymogen, form one side of a pocket that contains the active site of the molecule. Prophospholipase is otherwise quite similar to the active enzyme.

Dijkstra et al.[396] suggest two factors that might be responsible for the observed disorder of the zymogen. First, the N-terminus of the activated molecule forms a hydrogen bond with asn71 of the loop; this interaction is clearly not possible in prophospholipase. Second, it is likely that the additional residues at the N-terminus of the zymogen sterically prevent the disordered loop from assuming the configuration found in the activated molecule.

The manner in which phospholipase binds phospholipid substrates in aggregates such as micelles is not known, but it is reasonably certain that one or both of the segments that are disordered in the prophospholipase but ordered in the activated molecule contribute to this binding. Presumably because their catalytically active residues are in the same configuration, both the zymogen and the activated enzyme are active towards monomeric phosphoglycerides. The zymogen is defective only in the hydrolysis of aggregated substrates, against which it is two to three orders of magnitude less active than the activated enzyme.

As we will see in subsequent paragraphs, some degree of crystallographic disorder in isolated polypeptide segments is not particularly uncommon; however, sharp transitions from complete disorder to essentially complete order have been documented only for trypsinogen and prophospholipase. Although the two disordered segments of prophospholipase together account for a substantial fraction of this small protein, it is difficult to describe the single 14-residue loop and the few residues at the N-terminus of the molecule that become ordered when the enzyme is activated as a domain, even under the rather broad definition of the term used earlier in this review. Thus the striking similarity in the catalytic properties and the structural changes accompanying activation of prophospholipase and trypsinogen clearly illustrates the absence of any sharp division between domain flexibility and small-scale flexibility on either structural or functional grounds.

7. Lactate Dehydrogenase

Lactate dehydrogenase (LDH), which catalyzes the NAD^+-dependent oxidation of lactate to pyruvate, is a tetrameric molecule with a total molecular weight of about 140,000. The regulation of LDH activity in different tissues by the formation of hybrid tetramers of subunits from its heart (H) and muscle (M) isozymes is well known.[398] However, unlike GPDH, the other tetrameric dehydrogenase we have encountered, LDH does not bind coenzyme or substrate cooperatively.

LDH does not display domain flexibility per se but is of interest because the small-scale conformational changes observed when LDH binds coenzyme and substrates may have some functions in common with the coenzyme-induced conformational change in LADH.[274,286] All but the most recent crystallographic studies of LDH are reviewed by Holbrook et al.[398] These authors and White et al.[295] describe the differences between the crystal structures of unliganded LDH (i.e., apo-LDH) and an abortive ternary complex of LDH, NAD^+, and pyruvate in detail; both structures involve the M_4 isozyme of dogfish LDH, but the crystals are not isomorphous. The structures have been solved independently at 2.0 Å and 3.0 Å, respectively.

The largest conformational change observed upon going from the apoenzyme to the ternary complex is in residues 98 to 120 on the surface of the molecule; the first half of this segment is an extended loop of polypeptide, and the remainder consists of a short α-helix (αD) and part of a second α-helix (αE). In the ternary complex, the loop is folded over the active site, presumably shielding it from solvent, while in the apoenzyme, the loop is in a more open conformation. Although neither structure is refined, the loop appears to be poorly ordered in both the apoenzyme and the complex. Shifts of up to 10 Å are observed in

mainchain atoms of the loop, and shifts of nearly 5 Å are found in the adjacent C-terminal α-helix, αH. A number of smaller changes are also observed in nearby residues, all in the immediate vicinity of the active site.

The conformational change in LDH is also of interest because some information about its rate is available. The motion of the loop has been studied with a derivative of the H_4 isozyme of porcine LDH in which tyr237 was specifically nitrated;[399,400] this residue is on the surface of the native molecule near the position occupied by the loop in the ternary complex. Although the structure of the nitrated protein has not been determined directly, changes observed in the optical absorption of the nitrotyrosyl group when a ternary complex is formed are consistent with the changes in its environment predicted by the crystallographically observed conformational change of the loop.

Studies of the relaxation in absorption of the nitrotyrosyl group following a temperature jump[400] indicate that the loop closes at a rate of about 3000 sec^{-1} and opens at a rate of about 600 sec^{-1}. Not surprisingly, these rates are about an order of magnitude faster than those observed for the movement of the hinged domains of LADH in the presence of coenzyme. Proton NMR studies of nitrotyrosyl-LDH[399] are consistent with these results, in that no narrow resonances indicative of rapid motion on the NMR time scale (about 10^{-8} sec) are observed. On the other hand, saturation-transfer ESR studies of complexes of spin-labeled NAD^+ with LDH and different substrates[401] have been interpreted in terms of loop motions with correlation times between 10^{-5} and 10^{-6} sec. Whether the differences in the rates observed by these techniques indicate that different parts of the loop exhibit motions with different rates[400] or that one or the other of the measurements is sensitive to motions other than those in the loop is not clear.

As we outlined in the introduction, the interpretation of crystallographic evidence for flexibility in short segments of polypeptide is complicated by the increased probability that the conformation of such a peptide will be influenced by crystal-packing effects. We have already discussed one example in which small-scale disorder is apparently caused by the crystal packing in the Mcg Bence-Jones protein.[115] A more likely complication is that the conformation of a poorly ordered or flexible loop of polypeptide will be stabilized by crystal-packing contacts; several such examples are mentioned briefly below.

For LDH in particular, it is known that the loop which is folded over the active site in the ternary complex participates in intermolecular contacts in crystals of the dogfish M_4 apoenzyme.[402] In crystals of the murine C_4 apoenzyme, however, this loop is in a predominantly closed conformation. Whether the differences in the conformation of the loop region of these proteins are the cause or the result of the differences in the crystal packing is not known.

It has been suggested on the basis of these two apoenzyme structures that the loop region of LDH fluctuates between open and closed configurations in solution;[401,403] although it is clear that a thermodynamic equilibrium must exist among the conformations available to the loop, there is some doubt that the conformations of the loop region observed in the crystal are stable in solution. Both the partial disorder of the loop in crystals of the dogfish M_4 isozyme[295] and the crystal structure of a complex between porcine H_4 LDH and the covalent substrate analog 3-S-[5-(3-carboxy-3-hydroxypropyl)]NAD^+ suggest that the loop is relatively flexible, even in the closed conformation.[403] In the latter complex, the loop is closed over the active site, but the detailed conformation of the loop differs from that observed in the LDH-NAD^+-pyruvate ternary complex. Here again, however, it is uncertain whether these differences in conformation result from differences in the amino-acid sequences of the proteins involved or from differences in the nature of the ligands.

Although some questions remain about the details of the ligand-induced conformational changes in LDH, it is clear that the loop restricts access to the active site in the closed conformation. As suggested by Brändén and Eklund[286] and Eklund et al.,[274] the loop move-

ment in LDH, like the larger conformational changes in LADH and other hinged enzymes, may serve to exclude water from the active site of the enzyme during catalysis. Unlike that of LADH, however, the conformational change in LDH brings several residues into contact with the substrates and is coupled with observable changes in the orientation of catalytic groups in essentially rigid regions of the enzyme.[295,398]

8. Other Examples of Small-Scale Flexibility

a. Penicillopepsin

A well-documented example of small-scale flexibility in which the conformation of a segment of polypeptide is stabilized by crystal-packing interactions is provided by a comparison of the highly refined structure of penicillopepsin at 1.8 Å resolution[404] with that of an isomorphous complex of this enzyme and a tripeptide fragment of the inhibitor pepstatin. A difference Fourier between these structures at 1.8 Å resolution shows that a 13-residue "flap" adjacent to the active site of the enzyme moves up to 2.2 Å from its position in the unliganded molecule to interact with the inhibitor.[405] This segment is well-defined in crystals of the native molecule, presumably because it is involved in a strong intermolecular packing contact;[406] the degree of flexibility of this segment in solution is not known. Some movement of the flap had been predicted earlier on steric grounds.[406]

b. Triose Phosphate Isomerase

Triose phosphate isomerase (TIM) provides a similar example in which a relatively flexible segment of the molecule is stabilized by the crystal packing. In nonisomorphous crystals of the enzymes from chicken muscle and yeast, an external loop of about eight residues in one of the two crystallographically independent subunits is poorly ordered.[407] In the other subunit of both crystals, the same loop is ordered, presumably due to the more extensive intermolecular contacts it makes. The relatve degree of disorder in the different copies of the loop is uncertain, as neither structure is refined. Some flexibility within the loop is possible, as the conformations of the ordered loops in the two crystals appear to be different; no firm conclusion on this point is possible, however, because different proteins are being compared.

The addition of the substrate dihydroxyacetone phosphate (DHAP) to crystals of chicken TIM stabilized by suitable concentrations of salt results in a shift of the less ordered copy of the loop toward the active site, where it may interact with the bound substrate; the copy of the loop involved in packing contacts does not move. When DHAP is diffused into crystals of the yeast enzyme, however, both copies of the loop undergo a similar conformational change. As crystals of both enzymes show increased disorder in the presence of substrate, it is possible that TIM exhibits more extensive substrate-induced conformational changes in solution.

c. Phosphoglycerate Mutase

Another enzyme in which small-scale crystallographic disorder may be associated with more extensive substrate-induced conformational changes in solution is phosphoglycerate mutase. In the unrefined crystal structure of the yeast enzyme, the ten C-terminal residues of the molecule, at least some of which are essential for activity, are not observed.[408] Interestingly, the location of the last ordered residue of the polypeptide chain is such that the disordered segment could fold over the entrance to the active site. Crystals of phosphoglycerate mutase are unstable in the presence of the cofactor 2,3-diphosphoglycerate; as the enzyme is a tetramer, however, it is unclear whether this instability is related to changes in quaternary structure, changes in tertiary structure at some level, or to more direct interference with the crystal packing.

III. SUMMARY AND PERSPECTIVES

The examples we have presented establish that there are several criteria by which one can recognize domain flexibility and distinguish it from the small-scale motions that many biophysical and biochemical techniques indicate occur universally in proteins.[1,2,18,22] An obvious consequence of the existence of domain flexibility is that regions of proteins displaying domain flexibility do not have a unique conformation in solution, even if one ignores short-range fluctuations and isolated flexible loops of polypeptide on the surface of a molecule. The existing examples of domain flexibility suggest, however, that there are a limited number of ways in which conformational variability can occur in these proteins. We have tentatively identified three main classes of domain flexibility:

1. Proteins of one class consist of essentially rigid, globular domains that are flexibly linked. The domains of these proteins tend to make relatively few interactions with one another and seem to have a relatively wide range of motion. The more flexible of these linkages, such as the hinge segments connecting the Fab arms of an immunoglobulin to the Fc segment, seem to involve only a single polypeptide chain.
2. Proteins of another class also consist of essentially rigid, globular domains connected by a flexible hinge, but the domains of these proteins can adopt only a few orientations with respect to one another. There are extensive interdomain contacts in all such orientations, and in the best studied examples, there are two polypeptide chains linking the domains.
3. Proteins of the third class have a domain of disorder which folds into a contiguous region on the surface of the protein under some conditions. The polypeptide segments involved in these domains tend to have few specific contacts with ordered regions of the protein. Disordered segments begin abruptly, usually at a glycyl residue, and are often anchored to the ordered part of the protein by a nearby aromatic residue.

There are relatively few well-characterized examples of domain flexibility at present and fewer still for which function has been convincingly established. There is at least one such example in each major class of domain flexibility, however, and it seems clear that there is no common function among them. The function of flexibility in flexibly linked proteins, such as immunoglobulins, is almost certainly to allow the protein to bind multiple ligands that have no fixed orientation with respect to one another, but the functions of flexibility in the other two classes seem to be more varied.

If we confine our attention to enzymes, it would appear that a domain of disorder, such as is found in trypsinogen or prophospholipase, may serve to allow regulation of the activity of the enzyme toward macromolecular substrates and to provide a means of irreversibly switching the enzyme from the inactive form to the active form. Bode[197] has suggested that a similar mechanism involving an equilibrium constant between disordered and ordered states closer to unity might account for the partial activity seen in the zymogens of serine proteases in the blood-clotting[411] and complement[412] systems. Whether these functions are common to other enzymes in this class is unclear, however, as only the two examples listed above have been structurally characterized.

Domain flexibility in at least one hinged enzyme regulates its activity in a quite different manner; the opening and closing of the domains of hexokinase seems to allow the enzyme to switch between inactive and active forms in each catalytic cycle in order to minimize its activity in the absence of specific sugar substrates. However, it is not yet certain whether domain flexibility is required at all for the activity of the other two well-characterized hinged enzymes, alcohol dehydrogenase and citrate synthetase. The only common feature to the domain flexibility found in the hinged enzymes is that all have a "closed" conformation in

which solvent is excluded from the active site. Except for hexokinase, it is not clear that this is an important factor for the quite different chemical reactions catalyzed by these enzymes.

Two general developments can be expected in the study of domain flexibility in the near future. As we have seen, a number of flexible proteins are still incompletely characterized; further crystallographic study of these systems will undoubtably lead to a more complete description of the types of large-scale motions that occur in proteins. Perhaps more important, it is likely that more critical studies of proteins known to exhibit domain flexibility by a variety of experimental techniques will provide a clearer picture of the functions of this flexibility; the examples discussed earlier illustrate that most of our ideas on this topic are based on rationalization rather than experiment. Other areas in which much work remains to be done are the kinetic and thermodynamic characterization of domain motions.

One important lesson learned in the study of domain flexibility is that crystal-packing interactions can have a significant effect on the structure of a protein. In many discussions of protein structure, the question of whether the structure of a protein is the same in the crystal as in solution has been given a single answer.[62,63] It is obvious from the examples presented earlier in this review, however, that the effects of crystal packing and the relationship of the crystal structure of a protein to its properties in solution must be established experimentally for each crystal form.

It is evident that the only way to determine the effects of crystal packing on the structure of a protein is to determine the structure of the same or closely related proteins in a number of different crystalline environments. The best techniques for comparing the crystal and solution structures of a protein are clearly those that can measure the same properties of the protein in the crystal and in solution. Small-angle scattering techniques have proved to be particularly useful in this regard, as the parameters measured by this technique can be calculated from the crystal structure of a protein. The use of single-crystal microspectrophotometry to compare the spectral properties of a crystalline protein with those observed in solution has been illustrated by some of the examples discussed earlier,[287,320,334,336] and it should be possible to study a number of properties of crystalline proteins by similar methods; optical studies of suspensions of microcrystals have been used to measure the ligand-binding kinetics of crystalline heme proteins,[413] for example. A number of ESR experiments have been performed on protein crystals,[414] and the recently developed techniques of solid-state NMR have also been applied to crystalline proteins and peptides.[415,416]

An interesting by-product of the discovery of domain flexibility that may prove quite useful in the field of enzymology is the development of techniques for the study of the activity of such proteins in the crystalline state. Although techniques for assessing the activity of crystalline proteins are well known,[62,63] Rose[235] recognized that a flexible enzyme that is "trapped" in one conformation by crystal-packing interactions might represent an important step on the reaction pathway, even if it is unable to convert substrates to products. As discussed in detail earlier, Wilkinson and Rose have used isotope-trapping studies on rapidly dissolved crystals of hexokinase to establish that one crystal form of the enzyme has just these properties;[228] the same authors have employed an isotope-exchange technique to show that another form of hexokinase is catalytically active, even though it cannot perform a full turnover.[227]

The implication of these experiments is that the opportunity of trapping a flexible enzyme in one conformation may allow one to separate and study different steps of an enzymatic reaction in more detail than would otherwise be possible. A related application of crystallization as a tool for manipulating a flexible enzyme has been reported by Maret et al.[297] These authors used crystal-packing interactions to stabilize the structure of LADH in the absence of some or all of its zinc atoms; this procedure permitted the selective exchange of the zinc for cobalt, which had not been possible in solution. The use of such techniques to

examine the properties of flexible enzymes trapped in different conformations will clearly provide considerable insight into the functional roles of domain flexibility. Other uses of the potential to "synchronize" a sample of an enzyme that displays domain flexibility at different points in the reaction remain to be explored.

Since our manuscript was submitted, several articles that deal directly with topics discussed by us in detail have appeared; a review on domains in proteins that also addresses the topic of domain motions,[430] an electron-microscopic study demonstrating Fab twisting and elbow bending,[431] a detailed presentation of the intact Mcg structure,[432] a study of the conformational change in LADH lacking the active-site zinc atom,[433] an open conformation of a binding protein similar to arabinose-binding protein,[434] and two solution studies of the structure of aspartate aminotransferase[435,436] have been reported.

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REFERENCES

1. **Careri, G., Fasella, P., and Gratton, E.,** Statistical time events in enzymes: a physical assessment, *Crit. Rev. Biochem.*, 3, 141, 1975.
2. **Gurd, F. R. N. and Rothgeb, T. M.,** Motions in proteins, *Adv. Prot. Chem.*, 33, 73, 1979.
3. **Campbell, I. D., Dobson, C. M., and Williams, R. J. P.,** Proton magnetic resonance studies of the tyrosine residues of hen lysozyme — assignment and detection of conformational mobility, *Proc. R. Soc. London B*, 189, 503, 1975.
4. **Hull, W. E. and Sykes, B. D.,** Fluorotyrosine alkaline phosphatase: internal mobility of individual tyrosines and the role of chemical shift anisotropy as a ^{19}F nuclear spin relaxation mechanism in proteins, *J. Mol. Biol.*, 98, 121, 1975.
5. **Wagner, G. and Wüthrich, K.,** Proton NMR studies of aromatic residues in the basic pancreatic trypsin inhibitor (BPTI), *J. Magn. Reson.*, 20, 435, 1975.
6. **Lakowicz, J. R. and Weber, G.,** Quenching of protein fluorescence by oxygen. Detection of structural fluctuations in proteins on the nanosecond time scale, *Biochemistry*, 12, 4171, 1973.
7. **Hvidt, A. and Nielsen, S. O.,** Hydrogen exchange in proteins, *Adv. Prot. Chem.*, 21, 287, 1966.
8. **Barksdale, A. D. and Rosenberg, A.,** Acquisition and interpretation of hydrogen exchange data from peptides, polymers, and proteins, *Meth. Biochem. Anal.*, 28, 1, 1982.
9. **Richarz, R., Sehr, P., Wagner, G., and Wüthrich, K.,** Kinetics of the exchange of individual amide protons in the basic pancreatic trypsin inhibitor, *J. Mol. Biol.*, 130, 19, 1979.
10. **Hilton, B. D. and Woodward, C. K.,** Nuclear magnetic resonance measurement of hydrogen exchange kinetics of single protons in basic pancreatic trypsin inhibitor, *Biochemistry*, 17, 3325, 1978.
11. **Hetzel, R., Wüthrich, K., Deisenhofer, J., and Huber, R.,** Dynamics of the aromatic amino acid residues in the globular conformation of the basic pancreatic trypsin inhibitor (BPTI), *Biophys. Struct. Mech.*, 2, 159, 1976.
12. **Gelin, B. D. and Karplus, M.,** Sidechain torsional potentials and motion of amino acids in proteins: bovine pancreatic trypsin inhibitor, *Proc. Natl. Acad. Sci. USA*, 72, 2002, 1975.
13. **Richards, F. M.,** Packing defects, cavities, volume fluctuations, and access to the interior of proteins, *Carlsb. Res. Commun.*, 44, 47, 1979.
14. **Richards, F. M.,** Areas, volumes, packing and protein structure, *Ann. Rev. Biophys. Bioeng.*, 6, 151, 1977.
15. **Norvell, J. C., Nunes, A. C., and Schoenborn, B. P.,** Neutron diffraction analysis of myoglobin: structure of the carbon monoxide derivative, *Science*, 190, 568, 1975.

16. **Praissman, M. and Rupley, J. A.,** Comparison of protein structure in the crystal and in solution. III. Tritium-hydrogen exchange of lysozyme and a lysozyme-saccharide complex, *Biochemistry*, 7, 2446, 1968.
17. **Tüchsen, E., Hvidt, A., and Ottesen, M.,** Enzymes immobilized as crystals. Hydrogen isotope exchange of crystalline lysozyme, *Biochimie*, 62, 563, 1980.
18. **Karplus, M. and McCammon, J. A.,** The internal dynamics of globular proteins, *Crit. Rev. Biochem.*, 9, 293, 1981.
19. **Cooper, A.,** Conformational fluctuation and change in biological macromolecules, *Sci. Prog., Oxf.* 66, 473, 1980.
20. **Careri, G., Fasella, P., and Gratton, E.,** Enzyme dynamics: the statistical physics approach, *Ann. Rev. Biophys. Bioeng.*, 8, 69, 1979.
21. **Welch, G. R., Somogyi, B., and Damjanovich, S.,** The role of protein fluctuations in enzyme action, *Prog. Biophys. Molec. Biol.*, 39, 109, 1982.
22. **Citri, N.,** Conformational adaptability in enzymes, *Adv. Enzymol.*, 37, 397, 1973.
23. **Jardetzky, O. and Roberts, G. C. K.,** *NMR in Molecular Biology*, Academic Press, New York, 1981.
24. **Wüthrich, K. and Wagner, G.,** Internal motions in globular proteins, *Trends Biochem. Sci.*, 3, 227, 1978.
25. **Jardetzky, O.,** NMR Studies of Macromolecular Dynamics, *Acc. Chem. Res.*, 14, 291, 1981.
26. **Rosa, J. J. and Richards, F. M.,** Hydrogen exchange from identified regions of the S-protein component of ribonuclease as a function of temperature, pH, and the binding of S-peptide, *J. Mol. Biol.*, 145, 835, 1981.
27. **Woodward, C. K. and Hilton, B. D.,** Hydrogen exchange kinetics and internal motions in proteins and nucleic acids, *Ann. Rev. Biophys. Bioeng.*, 8, 99, 1979.
28. **Englander, S. W., Downer, N. W., Teitelbaum, H.,** Hydrogen exchange, *Ann. Rev. Biochem.*, 41, 903, 1972.
29. **Tsuboi, M. and Nakanishi, M.,** Overall and localized fluctuation in the structure of a protein molecule, *Adv. Biophys.*, 12, 101, 1979.
30. **Wagner, G. and Wüthrich, K.,** Structural interpretation of the amide proton exchange in the basic pancreatic trypsin inhibitor and related proteins, *J. Mol. Biol.*, 134, 75, 1979.
31. **Lumry, R. and Rosenberg, A.,** The mobile defect hypothesis of protein function, Coll. Int., C. N. R. S. l'Eau. Syst. Biol., 246, 53, 1975.
32. **Eftink, M. R. and Ghiron, C. A.,** Fluorescence quenching studies with proteins, *Anal. Biochem.*, 114, 199, 1981.
33. **McCammon, J. A., Gelin, B. R., and Karplus, M.,** Dynamics of folded proteins, *Nature (London)*, 267, 585, 1977.
34. **Karplus, M. and McCammon, J. A.,** Protein structural fluctuations during a period of 100 ps, *Nature (London)*, 277, 578, 1979.
35. **McCammon, J. A. and Karplus, M.,** Simulation of protein dynamics, *Ann. Rev. Phys. Chem.*, 31, 29, 1980.
36. **Levitt, M.,** Protein conformation, dynamics, and folding by computer simulation, *Ann. Rev. Biophys. Bioeng.*, 11, 251, 1982.
37. **Cantor, C. R. and Timasheff, S. N.,** Optical spectroscopy of proteins, in *The Proteins*, Vol. 5, Neurath, H. and Hill, R. L., Eds., Academic Press, New York, 1982, 145.
38. **Yu, Nai-Teng,** Raman spectroscopy: a conformational probe in biochemistry, *Crit. Rev. Biochem.*, 4, 229, 1977.
39. **Spiro, T. G. and Gaber, B. P.,** Laser raman scattering as a probe of protein structure, *Ann. Rev. Biochem.*, 46, 553, 1977.
40. **Peticolas, W. L.,** Low frequency vibrations and the dynamics of proteins and polypeptides, *Meth. Enzymol.*, 61, 425, 1979.
41. **Rigler, R. and Ehrenberg, M.,** Fluorescence relaxation spectroscopy in the analysis of macromolecular structure and motion, *Q. Rev. Biophys.*, 9, 1, 1976.
42. **Badea, M. G. and Brand, L.,** Time-resolved fluorescence measurements, *Meth. Enzymol.*, 61, 378, 1979.
43. **Eisinger, J.,** Energy transfer and dynamical structure, *Q. Rev. Biophys.*, 9, 21, 1976.
44. **Stryer, L.,** Fluorescence energy transfer as a spectroscopic ruler, *Ann. Rev. Biochem.*, 47, 819, 1978.
45. **Morrisett, J. D.,** The use of spin labels for studying the structure and function of enzymes, in *Spin Labeling*, Berliner, L. J., Ed., Academic Press, New York, 1976, 274.
46. **Hyde, J. S. and Dalton, L. R.,** Saturation transfer spectroscopy, in *Spin Labeling II*, Berliner, L. J., Ed., Academic Press, New York, 1979, 1.
47. **Christen, P. and Gehring, H.,** Detection of ligand-induced and syncatalytic conformational changes of enzymes by differential chemical modification, *Meth. Biochem. Anal.*, 28, 151, 1982.
48. **Gibb, T. C.,** *Principles of Mössbauer Spectroscopy*, Chapman and Hall, London, 1976, chap. 1.
49. **Parak, F. and Formanek, H.,** Untersuchungen des Schwingungsanteils und des Kristallgitterfehleranteils des Temperaturfaktors in Myoglobin durch Vergleich von Mössbauerabsorptionsmessungen mit Röntgenstrukturdaten, *Acta Cryst.*, A27, 573, 1971.

50. **Parak, F., Frolov, E. N., Mössbauer, R. L., and Goldanskii, V. I.,** Dynamics of metmyoglobin crystals investigated by nuclear gamma resonance absorption, *J. Mol. Biol.*, 145, 825, 1981.
51. **Richardson, J. S.,** The anatomy and taxonomy of protein structure, *Adv. Prot. Chem.*, 34, 167, 1981.
52. **Finch, J. T.,** Electron microscopy of proteins, in *The Proteins*, Vol. 1, Neurath, H. and Hill, R. L., Eds., Academic Press, New York, 1975, 413.
53. **Ottensmeyer, F. P.,** Molecular structure determination by high resolution electron microscopy, *Ann. Rev. Biophys. Bioeng.*, 8, 129, 1979.
54. **Kratky, O. and Pilz, I.,** A comparison of X-ray small-angle scattering results to crystal structure analysis and other physical techniques in the field of biological macromolecules, *Q. Rev. Biophys.*, 11, 39, 1978.
55. **Pilz, I., Glatter, O., and Kratky, O.,** Small-angle X-ray scattering, *Meth. Enzymol.*, 61, 148, 1979.
56. **Luzzati, V. and Tardieu, A.,** Recent developments in solution X-ray scattering, *Ann. Rev. Biophys. Bioeng.*, 9, 1, 1980.
57. **Kneale, G. G., Baldwin, J. P., and Bradbury, E. M.,** Neutron scattering studies of biological macromolecules in solutions, *Q. Rev. Biophys.*, 10, 485, 1977.
58. **Schurr, J. M.,** Relaxation of rotational and internal modes of macromolecules determined by dynamic scattering, *Q. Rev. Biophys.*, 9, 109, 1976.
59. **Bloomfield, V. A.,** Quasi-elastic light scattering applications in biochemstry and biology, *Ann. Rev. Biophys. Bioeng.*, 10, 421, 1981.
60. **Bloomfield, V. A.,** Hydrodynamics in biophysical chemistry, *Ann. Rev. Phys. Chem.*, 28, 233, 1977.
61. **Schoenborn, B. P. and Nunes, A. C.,** Neutron scattering, *Ann. Rev. Biophys. Bioeng.*, 1, 529, 1972.
62. **Blundell, T. L. and Johnson, L. N.,** *Protein Crystallography*, Academic Press, New York, 1976.
63. **Matthews, B. W.,** X-ray structure of proteins, in *The Proteins*, Vol. 3, Neurath, H. and Hill, R. L., Eds., Academic Press, New York, 1977, 403.
64. **Wyckoff, H. W.,** personal communication, 1982.
65. **Compton, A. H. and Allison, S. K.,** *X-rays in Theory and Experiment*, van Nostrand, Princeton, 1935, chap. 4.
66. **Willis, B. T. M. and Pryor, A. W.,** *Thermal Vibrations in Crystallography*, Cambridge University Press, London, 1975.
67. **Diamond, R.,** A real-space refinement procedure for proteins, *Acta Cryst.*, A27, 436, 1971.
68. **Lipscomb, W. N.,** Carboxypeptidase A mechanisms, *Proc. Natl. Acad. Sci. USA*, 77, 3875, 1980.
69. **Steitz, T. A. and Schulman, R. G.,** Crystallographic and NMR studies of the serine proteases, *Ann. Rev. Biophys. Bioeng.*, 11, 419, 1982.
70. **Huber, R. and Bode, W.,** Structural basis of the activation and action of trypsin, *Acc. Chem. Res.*, 11, 114, 1978.
71. **Amzel, L. M. and Poljak, R. J.,** Three-dimensional structure of immunoglobulins, *Ann. Rev. Biochem.*, 48, 961, 1979.
72. **Huber, R.,** Spatial structure of immunoglobulin molecules, *Klin. Wochenschrift*, 58, 1217, 1980.
73. **Marquart, M. and Deisenhofer, J.,** Three-dimensional structure of antibodies, *Immunol. Today*, 3, 160, 1982.
74. **Davies, D. R., Padlan, E. A., and Segal, D. M.,** Three-dimensional structure of immunoglobulins, *Ann. Rev. Biochem.*, 44, 639, 1975.
75. **Edelman, G. M. and Gall, W. E.,** The antibody problem, *Ann. Rev. Biochem.*, 38, 415, 1969.
76. **Putnam, F. W.,** Immunoglobulins I. Structure, in *The Plasma Proteins*, Vol. 3, Putnam, F. W., Ed., Academic Press, 1977, 1.
77. **Noelken, M. E., Nelson, C. A., Buckley, C. E., and Tanford, C.,** Gross conformation of rabbit 7 S γ-immunoglobulin and its papain-cleaved fragments, *J. Biol. Chem.*, 240, 218, 1965.
78. **Dorrington, K. J. and Tanford, C.,** Molecular size and conformation of immunoglobulins, *Adv. Immunol.*, 12, 333, 1970.
79. **Green, N. M.,** Electron microscopy of the immunoglobulins, *Adv. Immunol.*, 11, 1, 1969.
80. **Valentine, R. C. and Green, N. M.,** Electron microscopy of an antibody-hapten complex, *J. Mol. Biol.*, 27, 615, 1967.
81. **Yguerabide, J., Epstein, H. F., and Stryer, L.,** Segmental flexibility in an antibody molecule, *J. Mol. Biol.*, 51, 573, 1970.
82. **Terry, W. D., Matthews, B. W., and Davies, D. R.,** Crystallographic studies of a human immunoglobulin, *Nature (London)*, 220, 239, 1968.
83. **Edmundson, A. B., Wood, M. K., Schiffer, M., Hardman, K. D., Ainsworth, C. F., and Ely, K. R.,** A crystallographic investigation of a human IgG Immunoglobulin, *J. Biol. Chem.*, 245, 2763, 1970.
84. **Steiner, L. A. and Lopes, A. D.,** The crystallizable human myeloma protein Dob has a hinge-region deletion, *Biochemistry*, 18, 4054, 1979.
85. **Fett, J. W., Deutsch, H. F., and Smithies, O.,** Hinge region deletion localized in the IgG1-globulin Mcg, *Immunochemistry*, 10, 115, 1973.

86. **Klein, M., Haeffner-Cavaillon, N., Isenman, D. E., Rivat, C., Navia, M. A., Davies, D. R., and Dorrington, K. J.,** Expression of biological effector functions by immunoglobulin G molecules lacking the hinge region, *Proc. Natl. Acad. Sci. USA*, 78, 524, 1981.
87. **Palm, W. and Colman, P. M.,** Preliminary X-ray data from well-ordered crystals of a human immunoglobulin G molecule, *J. Mol. Biol.*, 82, 587, 1974.
88. **Ely, K. R., Colman, P. M., Abola, E. E., Hess, A. C., Peabody, D. S., Parr, D. M., Connell, G. E., Laschinger, C. A., and Edmundson, A. B.,** Mobile Fc region in the Zie IgG2 cryoglobulin: comparison of crystals of the $F(ab')_2$ fragment and the intact immunoglobulin, *Biochemistry*, 17, 820, 1978.
89. **Colman, P. M., Deisenhofer, J., Huber, R., and Palm, W.,** Structure of the human antibody molecule Kol (immunoglobulin G1): an electron density map at 5 Å resolution, *J. Mol. Biol.*, 100, 257, 1976.
90. **Sarma, V. R., Silverton, E. W., Davies, D. R., and Terry, W. D.,** The Three-dimensional structure at 6 Å resolution of a human γG1 immunoglobulin molecule, *J. Biol. Chem.*, 246, 3753, 1971.
91. **Silverton, E. W., Navia, M. A., and Davies, D. R.,** Three-dimensional structure of an intact human immunoglobulin, *Proc. Natl. Acad. Sci. USA*, 74, 5140, 1977.
92. **Edmundson, A. B., et al.,** unpublished results cited in ref. 88.
93. **Marquart, M., Deisenhofer, J., Huber, R., and Palm, W.,** Crystallographic refinement and atomic models of the intact immunoglobulin molecule Kol and its antigen-binding fragment at 3.0 and 1.9 Å resolution, *J. Mol. Biol.*, 141, 369, 1980.
94. **Edelman, G. M.,** The covalent structure of a human γ G-immunoglobulin. XI. Functional implications, *Biochemistry*, 9, 3197, 1970.
95. **Deisenhofer, J., Colman, P. M., Epp, O., and Huber, R.,** Crystallographic structural studies of a human Fc fragment. II. A complete model based on a Fourier map at 3.5 Å resolution, *Hoppe Seyler's Z. Physiol. Chem.*, 357, 1421, 1976.
96. **Deisenhofer, J., Jones, T. A., Huber, R., Sjödahl, J., and Sjöquist, J.,** Crystallization, crystal structure analysis and atomic model of the complex formed by a human Fc fragment and fragment B of protein A from *Staphylococcus aureus*, *Hoppe-Seyler's Z. Physiol. Chem.*, 359, 975, 1978.
97. **Deisenhofer, J.,** Crystallographic refinement and atomic models of a human Fc fragment and its complex with fragment B of protein A from *Staphylococcus aureus* at 2.9 and 2.8 Å resolution, *Biochemistry*, 20, 2361, 1981.
98. **Deisenhofer, J., Colman, P. M., Huber, R., Haupt, H., and Schwick, G.,** Crystallographic structural studies of a human Fc-fragment. I. An electron density map at 4 Å resolution and a partial model, *Hoppe-Seyler's Z. Physiol. Chem.*, 357, 435, 1976.
99. **Gally, J. A.,** Structure of immunoglobulins, in *The Antigens*, Vol. I, Sela, M., Ed., Academic Press, New York, 1973, 161.
100. **Crick, F. H. C. and Magdoff, B. S.,** The theory of the method of isomorphous replacement for protein crystals, *Acta Cryst.*, 9, 901, 1956.
101. **Dayhoff, M. O.,** *Atlas of Protein Sequence and Structure*, Vol. 5, Suppl. 2, National Biomedical Research Foundation, Washington, D.C., 1976.
102. **Steiner, L. A. and Porter, R. R.,** The interchain disulfide bonds of a human pathologcal immunoglobulin, *Biochemistry*, 6, 3957, 1967.
103. **Putnam, F. W., Easley, C. W., and Lynn, L. T.,** Site of cleavage of γ-globulins by papain, *Biochim. Biophys. Acta*, 58, 279, 1962.
104. **Palm, W.,** Über die physikalisch-chemische Charakterisierung und nachfolgende Kristallisation von Immunoglobulinen und deren Fragmenten: Ein Beitrag zur dreidimensionalen Strukturaufklärung der Antikoerper. IV, *Hoppe-Seyler's Z. Physiol. Chem.*, 357, 799, 1976.
105. **Matsushima, M., Marquart, M., Jones, T. A., Colman, P. M., Bartels, K., Huber, R., and Palm, W.,** Crystal structure of the human Fab fragment Kol and its comparison with the intact Kol molecule, *J. Mol. Biol.*, 121, 441, 1978.
106. **Celada, F. and Strom, R.,** Antibody-induced conformational changes in proteins, *Q. Rev. Biophys.*, 5, 395, 1972.
107. **Poljak, R. J., Amzel, L. M., Chen, B. L., Phizackerley, R. P., and Saul, F.,** The three-dimensional structure of the Fab fragment of a human myeloma immunoglobulin at 2.0 Å resolution, *Proc. Natl. Acad. Sci. USA*, 71, 3440, 1974.
108. **Segal, D. M., Padlan, E. A., Cohen, G. H., Rudikoff, S., Potter, M., and Davies, D. R.,** The three-dimensional structure of a phosphorylcholine-binding mouse immunoglobulin Fab and the nature of the antigen binding site, *Proc. Natl. Acad. Sci. USA*, 71, 4298, 1974.
109. **Navia, M. A., Segal, D. M., Padlan, E. A., Davies, D. R., Rao, N., Rudikoff, S., and Potter, M.,** Crystal structure of galactan-binding mouse immunoglobulin J539 Fab at 4.5 Å resolution, *Proc. Natl. Acad. Sci. USA*, 76, 4071, 1979.
110. **Abola, E. E., Ely, K. R., and Edmundson, A. B.,** Marked structural differences of the Mcg Bence-Jones dimer in two crystal sysems, *Biochemistry*, 19, 432, 1980.

111. **Panagiotopulos, N. C., Abola, E. E., Ely, K. R., Schiffer, M., and Edmundson, A. B.,** unpublished results cited in ref. 110.
112. **Firca, J. R., Ely, K. R., Kremser, P., Westholm, F. A., Dorrington, K. J., and Edmundson, A. B.,** Interconversion of conformational isomers of light chains in the Mcg immunoglobulins, *Biochemistry*, 17, 148, 1978.
113. **Schiffer, M., Girling, R. L., Ely, K. R., and Edmundson, A. B.,** Structure of a λ-type Bence-Jones protein at 3.5 Å resolution, *Biochemistry*, 12, 4620, 1973.
114. **Edmundson, A. B., Ely, K. R., Abola, E. E., Schiffer, M., and Panagiotopoulos, N.,** Rotational allomerism and divergent evolution of domains in immunogloblin light chains, *Biochemistry*, 14, 3953, 1975.
115. **Schiffer, M.,** Possible distortion of antibody binding site of the Mcg Bence-Jones protein by lattice forces, *Biophys. J.*, 32, 230, 1980.
116. **Ely, K. R., Firca, J. R., Williams, K. J., Abola, E. E., Fenton, J. M., Schiffer, M., Panagiotopoulos, N. C., and Edmundson, A. B.,** Crystal properties as indicators of conformational changes during ligand binding or interconversion of Mcg light chain isomers, *Biochemistry*, 17, 158, 1978.
117. **Saul, F. A., Amzel, L. M., and Poljak, R. J.,** Preliminary refinement and structural analysis of the Fab fragment from human immunoglobulin new at 2.0 Å resolution, *J. Biol. Chem.*, 253, 585, 1978.
118. **Sakano, H., Rogers, J. H., Hüppi, K., Brack, C., Traunecker, A., Maki, R., Wall, R., and Tonegawa, S.,** Domains and the hinge region of an immunoglobulin heavy chain are encoded in separate DNA segments, *Nature (London)*, 277, 627, 1979.
119. **Sarma, R. and Laudin, A. G.,** The three-dimensional structure of a human IgG1 immunoglobulin at 4 Å resolution: a computer fit of various structural domains on the electron density map, *J. Appl. Cryst.*, 15, 476, 1982.
120. **Cathou, R. E.,** Solution conformation and segmental flexibility of immunogloulins, *Compr. Immunol.*, 5, 37, 1978.
121. **Pecht, I.,** Dynamic aspects of antibody function, in *The Antigens*, Vol. VI, Sela, M., Ed., Academic Press, New York, 1982, 1.
122. **Schumaker, V. N., Seegan, G. W., Smith, C. A., Ma, S. K., Rodwell, J. D., and Schumaker, M. F.,** The free energy of angular position of the Fab arms of IgG antibody, *Mol. Immunol.*, 17, 413, 1980.
123. **Archer, B. G. and Krakauer, H.,** Thermodynamics of antibody-antigen reactions. 2. The binding of bivalent synthetic random coil antigens to antibodies having different antigen precipitating properties, *Biochemistry*, 16, 618, 1977.
124. **Reidler, J., Oi, V. T., Carlsen, W., Vuong, T. M., Pecht, I., Herzenberg, L. A., and Stryer, L.,** Rotational dynamics of monoclonal anti-dansyl immunoglobulins, *J. Mol. Biol.*, 158, 739, 1982.
125. **Wegener, W. A., Dowben, R. M., and Koester, V. J.,** Diffusion coefficients for segmentally flexible macromolecules: general formalism and application to rotational behavior of a body with two segments, *J. Chem. Phys.*, 73, 4086, 1980.
126. **Wegener, W. A.,** A swivel-jointed formalism for segmentally flexible macromolecules and its application to the rotational behavior of myosin, *Biopolymers*, 21, 1049, 1982.
127. **Hanson, D. C., Yguerabide, J., and Schumaker, V. N.,** Segmental flexibility of immunoglobulin G antibody molecules in solution: a new interpretation, *Biochemistry*, 20, 6842, 1981.
128. **Chan, L. M. and Cathou, R. E.,** The role of the inter-heavy chain disulfide bond in modulating the flexibility of the immunoglobulin G antibody, *J. Mol. Biol.*, 112, 653, 1977.
129. **Lovejoy, C., Holowka, D. A., and Cathou, R. E.,** Nanosecond fluorescence spectroscopy of pyrene butyrate-anti-pyrene antibody complexes, *Biochemistry*, 16, 3668, 1977.
130. **Pilz, I., Schwarz, E., and Palm, W.,** Small-angle X-ray studies of the Fab and Fc fragments from the human immunoglobulin molecule Kol, *Eur. J. Biochem.*, 71, 239, 1976.
131. **Pilz, I., Schwarz, E., and Palm, W.,** Small-angle X-ray studies of the human immunoglobulin molecule Kol, *Eur. J. Biochem.*, 75, 195, 1977.
132. **Cser, L., Franĉk, F., Gladkikh, I. A., Kunchenko, A. B., and Ostanevich, Y. M.,** General shape and hapten-induced conformational changes of pig antibody against dinitrophenyl, *Eur. J. Biochem.*, 116, 109, 1981.
133. **Schiffer, M., Stevens, F. J., Westholm, F. A., Kim, S. S., and Carlson, R. D.,** Small-Angle neutron Scattering Study of Bence-Jones Protein Mcg: Comparison of Structures in Solution and in Crystal, *Biochemistry*, 21, 2874, 1982.
134. **Dudich, E. I., Nezlin, R. S., and Franěk, F.,** Fluorescence polarization analysis of various immunoglobulins: dependence of rotational relaxation time on protein concentration and ability to precipitate with antigen, *FEBS Lett.*, 89, 89, 1978.
135. **Metzger, H.,** Effect of antigen binding on the properties of antibody, *Adv. Immunol.*, 18, 169, 1974.
136. **Sjöquist, J., Meloun, B., and Hjelm, H.,** Protein A isolated from *Staphylococcus aureus* after digestion with lysostaphin, *Eur. J. Biochem.*, 29, 572, 1972.
137. **Björk, I., Petersson, B.-A., and Sjöquist, J.,** Some physicochemical properties of protein A from *Staphylococcus aureus*, *Eur. J. Biochem.*, 29, 579, 1972.

138. **Sjödahl, J.,** Repetitive sequences in protein A from *Staphylococcus aureus*, *Eur. J. Biochem.*, 73, 343, 1977.
139. **Hjelm, H., Sjödahl, J., and Sjöquist, J.,** Immunologically active and structurally similar fragments of protein A from *Staphylococcus aureus*, *Eur. J. Biochem.*, 57, 395, 1975.
140. **Laskowski, M. and Kato, I.,** Protein inhibitors of proteinases, *Ann. Rev. Biochem.*, 49, 593, 1980.
141. **Weber, E., Papamokos, E., Bode, W., Huber, R., Kato, I., and Laskowski, M., Jr.,** Crystallization, crystal structure analysis and molecular model of the third domain of Japanese quail ovomucoid, a Kazal type inhibitor, *J. Mol. Biol.*, 149, 109, 1981.
142. **Papamokos, E., Weber, E., Bode, W., Huber, R., Empie, M. W., Kato, I., and Laskowski, M., Jr.,** Crystallographic refinement of Japanese quail ovomucoid, a Kazal-type inhibitor and model building studies of complexes with serine proteases, *J. Mol. Biol.*, 158, 515, 1982.
143. **Fujinago, M., Read, R. J., Sielecki, A., Ardelt, W., Laskowski, M., and James, M. N. G.,** Refined crystal structure of the molecular complex of *Streptomyces griseus* protease B, a serine protease, with the third domain of the ovomucoid inhibitor from turkey, *Proc. Natl. Acad. Sci. USA*, 79, 4868, 1982.
144. **Kretsinger, R. H. and Nockolds, C. E.,** Carp muscle calcium-binding protein. II. Structure determination and general description, *J. Biol. Chem.*, 248, 3313, 1973.
145. **Ploegman, J. H., Drent, G., Kalk, K. H., and Hol, W. G. J.,** Structure of bovine liver rhodanese. I. Structure determination at 2.5 Å resolution and a comparison of the conformation and sequence of its two domains, *J. Mol. Biol.*, 123, 557, 1978.
146. **Wright, C. S.,** The crystal structure of wheat germ agglutinin at 2.2 Å resolution, *J. Mol. Biol.*, 111, 439, 1977.
147. **Blundell, T., Lindley, P., Miller, L., Moss, D., Slingsby, C., Tickle, I., Turnell, B., and Wistow, G.,** The molecular structure and stability of the eye lens: X-ray analysis of γ-crystallin II, *Nature (London)*, 289, 771, 1981.
148. **Bolognesi, M., Gatti, G., Menegatti, E., Guarneri, M., Marquart, M., Papamokos, E., and Huber, R.,** Three dimensional structure of the complex between pancreatic secretory trypsin inhibitor (Kazal type) and trypsinogen at 1.8 Å resolution, *J. Mol. Biol.*, 162, 839, 1982.
149. **Porter, R. R. and Reid, K. B. M.,** Activation of the complement system by antibody-antigen complexes: the classical pathway, *Adv. Prot. Chem.*, 33, 1, 1979.
150. **Gilmour, S., Randall, J. T., Willan, K. J., Dwek, R. A., and Torbet, J.,** The conformation of subcomponent C1q of the first component of human complement, *Nature (London)*, 285, 512, 1980.
151. **Marchesi, V. T.,** Spectrin: present status of a putative cyto-skeletal protein of the red cell membrane, *J. Membrane Biol.*, 51, 101, 1979.
152. **Bennett, V., Davis, J., and Fowler, W. E.,** Brain spectrin, a membrane-associated protein related in structure and function to erythrocyte spectrin, *Nature (London)*, 299, 126, 1982.
153. **Cohen, C. M., Branton, D., and Tyler, J.,** Mapping functional sites on biological macromolecules, *Ultramicroscopy*, 8, 185, 1982.
154. **Speicher, D. W., Morrow, J. S., Knowles, W. J., and Marchesi, V. T.,** A structural model of human erythrocyte spectrin. Alignment of chemical and functional domains, *J. Biol. Chem.*, 257, 9093, 1982.
155. **Yurchenco, P. D., Speicher, D. W., Morrow, J. S., Knowles, W. J., and Marchesi, V. T.,** Monoclonal antibodies as probes of domain structure of the spectrin α subunit, *J. Biol. Chem.*, 257, 9102, 1982.
156. **Engel, J., Odermatt, E., Engel, A., Madri, J. A., Furthmayr, H., Rohde, H., and Timpl, R.,** Shapes, domain organizations and flexibility of laminin and fibronectin, two multifunctional proteins of the extracellular matrix, *J. Mol. Biol.*, 150, 97, 1981.
157. **Odermatt, E., Engel, J., Richter, H., and Hörmann, H.,** Shape conformation and stability of fibronectin fragments determined by electron microscopy, circular dichroism and ultracentrifugation, *J. Mol. Biol.*, 159, 109, 1982.
158. **Cooper, A.,** Thermodynamic fluctuations in protein molecules, *Proc. Natl. Acad. Sci. USA*, 73, 2740, 1976.
159. **Huber, R.,** Conformational flexibility and its functional significance in some protein molecules, *Trends Biochem. Sci.*, 4, 271, 1979.
160. **Huber, R. and Bennett, W. S.,** Functional significance of flexibility in proteins, *Biopolymers*, 22, 261, 1983.
161. **Biltonen, R. C. and Freire, E.,** Thermodynamic characterization of conformational states of biological macromolecules using differential scanning calorimetry, *Crit. Rev. Biochem.*, 5, 85, 1978.
162. **Privalov, P. L.,** Stability of proteins, *Adv. Prot. Chem.*, 33, 167, 1979.
163. **Metzger, H.,** The effect of antigen on antibodies. Recent studies, in *Contemporary Topics in Molecular Immunology*, Vol. 7, Reisfeld, R. A. and Inman, F. P., Eds., Plenum Press, New York, 1978, 119.
164. **Edmundson, A. B., Ely, K. R., and Abola, E. E.,** Conformational flexibility in immunoglobulins, in *Contemporary Topics in Molecular Immunology*, Vol. 7, Reisfeld, R. A. and Inman, F. P., Eds., Plenum Press, New York, 1978, 95.

165. **Kabat, E. A., Wu, T. T., and Bilofsky, H.,** Sequences of Immunoglobulin Chains, U.S. Department of Health, Education, and Welfare, NIH Publication No. 80-2008, 1979.
166. **Fehlhammer, H., Bode, W., and Huber, R.,** Crystal structure of bovine trypsinogen at 1.8 Å resolution. II. Crystallographic refinement, refined crystal structure and comparison with bovine trypsin, *J. Mol. Biol.*, 111, 415, 1977.
167. **Bode, W., Fehlhammer, H., and Huber, R.,** Crystal structure of bovine trypsinogen at 1.8 Å resolution. I. Data collection, application of Patterson search techniques and preliminary structural interpretation, *J. Mol. Biol.*, 106, 325, 1976.
168. **Kossiakoff, A. A., Chambers, J. L., Kay, L. M. and Stroud, R. M.,** Structure of bovine trypsinogen at 1.9 Å resolution, *Biochemistry*, 16, 654, 1977.
169. **Walsh, K. A., and Neurath, H.,** Trypsinogen and chymotrypsinogen as homologous proteins, *Proc. Natl. Acad. Sci. USA*, 52, 884, 1964.
170. **Mikěs, O., Tomášek, V., Holeyšovský, V., and Šorm, F.,** On proteins, CVI. Covalent structure of bovine trypsinogen, *Collect. Czech. Chem. Commun.*, 32, 655, 1967.
171. **Hartley, B. S.,** Amino-acid sequence of bovine chymotrypsinogen-A, *Nature (London)*, 201, 1284, 1964.
172. **Hartley, B. S. and Shotton, D. M.,** Pancreatic elastase, in *The Enzymes*, vol. 3., Boyer, P. D., Ed., Academic Press, New York, 1971, 323.
173. **Schroeder, D. D., and Shaw, E.,** Chromatography of trypsin and its derivatives. Characterization of a new active form of bovine trypsin, *J. Biol. Chem.*, 243, 2943, 1968.
174. **Birktoft, J. J., Blow, D. M., Henderson, R., and Steitz, T. A.,** The structure of α-chymotrypsin, *Phil. Trans. R. Soc. London*, B257, 67, 1970.
175. **Bode, W. and Schwager, P.,** The refined crystal structure of bovine β-Trypsin at 1.8 Å Resolution. II. Crystallographic refinement, calcium binding site, benzamidine binding site and active site at pH 7.0, *J. Mol. Biol.*, 98, 693, 1975.
176. **Chambers, J. L. and Stroud, R. M.,** Difference Fourier refinement of the structure of DIP-trypsin at 1.5 Å with a minicomputer technique, *Acta Cryst.*, B33, 1824, 1977.
177. **Huber, R., Kukla, D., Bode, W., Schwager, P., Bartels, K., Deisenhofer, J., and Steigemann, W.,** Structure of the complex formed by bovine trypsin and bovine pancreatic trypsin inhibitor. II. Crystallographic refinement at 1.9 Å resolution, *J. Mol. Biol.*, 89, 73, 1974.
178. **Fehlhammer, H. and Bode, W.,** The refined crystal structure of bovine β-trypsin at 1.8 Å resolution. I. Crystallization, data collection and application of Patterson search techniques, *J. Mol. Biol.*, 98, 683, 1975.
179. **Rühlmann, A., Kukla, D., Schwager, P., Bartels, K., and Huber, R.,** Structure of the complex formed by bovine trypsin and bovine pancreatic trypsin inhibitor. I. Crystal structure determination and stereochemistry of the contact region, *J. Mol. Biol.*, 77, 417, 1973.
180. **Sweet, R. M., Wright, H. T., Janin, J., Chothia, C. H., and Blow, D. M.,** Crystal structure of the complex of porcine trypsin with soybean trypsin inhibitor (Kunitz) at 2.6 Å resolution, *Biochemistry*, 13, 4212, 1974.
181. **Walter, J., Steigemann, W., Singh, T. P., Bartunik, H., Bode, W., and Huber, R.,** On the disordered activation domain in trypsinogen: chemical labelling and low-temperature crystallography, *Acta Cryst.*, B38, 1462, 1982.
182. **Stroud, R. M.,** personal communication, 1982.
183. **Bode, W. and Huber, R.,** Crystal structure analysis and refinement of two variants of trigonal trypsinogen, *FEBS Lett.*, 90, 265, 1978.
184. **Freer, S. T., Kraut, J., Robertus, J. D., Wright, H. T., and Xuong, Ng. H.,** Chymotrypsinogen: 2.5 Å crystal structure, comparison with α-chymotrypsin, and implications for zymogen activation, *Biochemistry*, 9, 1997, 1970.
185. **Wright, H. T.,** Comparison of the crystal structures of chymotrypsinogen-A and α-chymotrypsin, *J. Mol. Biol.*, 79, 1, 1973.
186. **Stroud, R. M. and Huber, R.,** personal communications, 1982.
187. **Neurath, H.,** Limited proteolysis and zymogen activation, in *Proteases and Biological Control*, Reich, E., Rifkin, D. B., and Shaw, E., Eds., Cold Spring Harbor Laboratory, 1975, 51.
188. **Vincent, J.-P. and Lazdunski, M.,** Trypsin-pancreatic trypsin inhibitor association, Dynamics of the interaction and role of disulfid bridges, *Biochemistry*, 11, 2967, 1972.
189. **Vincent, J.-P. and Lazdunski, M.,** Pre-existence of the active site in zymogens, the interaction of trypsinogen with the basic pancreatic trypsin inhibitor (Kunitz), *FEBS Lett.*, 63, 240, 1976.
190. **Bernstein, F. C., Koetzle, T. F., Williams, G. J. B., Meyer, E. F., Brice, M. D., Rodgers, J. R., Kennard, O., Shimanouchi, T., and Tasumi, M.,** The protein data bank: a computer-based archival file for macromolecular structures, *J. Mol. Biol.*, 112, 535, 1977.
191. **Bode, W., Schwager, P., and Huber, R.,** The transiton of bovine trypsinogen to a trypsin-like state upon strong ligand binding, *J. Mol. Biol.*, 118, 99, 1978.
192. **Perkins, S. J. and Wüthrich, K.,** Conformational transition from trypsinogen to trypsin. H nuclear magnetic resonance at 360 MHz and ring current calculations, *J. Mol. Biol.*, 138, 43, 1980.

193. **Antonini, E., Ascenzi, P., Bolognesi, M., Gatti, G., Guarneri, M., and Menegatti, E.,** The interaction between serine (pro)enzymes and Kazal and Kunitz inhibitors, *J. Mol. Biol.*, in press, 1983.
194. **Kumar, S., Dar, K., Ganno, S., and Hatano, H.,** Modification of Isoleucine-16 of acylated α-chymotrypsin, *J. Biol. Chem.*, 250, 5393, 1975.
195. **Kerr, M. A., Walsh, K. A., and Neurath, H.,** Catalysis by serine proteases and their zymogens. A study of acyl intermediates by circular dichroism, *Biochemistry*, 14, 5088, 1975.
196. **Bode, W. and Huber, R.,** Induction of the bovine trypsinogen-trypsin transition by peptides sequentially similar to the N-terminus of trypsin, *FEBS Lett.*, 68, 231, 1976.
197. **Bode, W.,** The transition of bovine trypsinogen to a trypsin-like state upon strong ligand binding, *J. Mol. Biol.*, 127, 357, 1979.
198. **Abita, J. P. and Lazdunski, M.,** On the structural and functional role of carboxylates in chymotrypsinogen A. A comparison with chymotrypsin, trypsinogen and trypsin, *Biochem. Biophys. Res. Commun.*, 35, 707 1969.
199. **Nolte, H. J. and Neuman, E.,** Kinetics and mechanism for the conformational transition in p-guanidinobenzoate bovine trypsinogen induced by the isoleucine-valine dipeptide, *Biophys. Chem.*, 10, 253, 1979.
200. **Butz, T., Lerf, A., and Huber, R.,** Intramolecular reorientational motion in trypsinogen studied by perturbed angular correlation of ^{199m}Hg labels, *Phys. Rev. Lett.*, 48, 890, 1982.
201. **Radhakrishnan, T. M., Walsh, K. A., and Neurath, H.,** The promotion of activation of bovine trypsinogen by specific modification of aspartyl residues, *Biochemistry*, 8, 4020, 1969.
202. **Abita, J. P., Delaage, M., and Lazdunski, M.,** The mechanism of activation of trypsinogen. The role of the four N-terminal aspartyl residues, *Eur. J. Biochem.*, 8, 314, 1969.
203. **Singh, T. P., Bode, W., and Huber, R.,** Low-temperature protein crystallography. Effect on flexibility, temperature factor, mosaic spread, extinction and diffuse scattering in two examples: bovine trypsinogen and Fc fragment, *Acta Cryst.*, B36, 621, 1980.
204. **de Haën, C., Neurath, H., and Teller, D. C.,** The phylogeny of trypsin-related serine proteases and their zymogens. New methods for the investigation of distant evolutionary relationships, *J. Mol. Biol.*, 92, 225, 1975.
205. **Ramachandran, G. N. and Sasisekharan, V.,** Conformation of polypeptides and proteins, *Adv. Prot. Chem.*, 23, 283, 1968.
206. **Birktoft, J. J. and Blow, D. M.,** Structure of crystalline α-chymotrypsin. V. The atomic structure of tosyl-α-chymotrypsin at 2 A resolution, *J. Mol. Biol.*, 68, 187, 1972.
207. **Beale, D. and Feinstein, A.,** Structure and function of the constant regions of immunoglobulins, *Q. Rev. Biophys.*, 9, 135, 1976.
208. **Caspar, D. L. D.,** Assembly and stability of the tobacco mosaic virus particle, *Adv. Prot. Chem.*, 18, 37, 1963.
209. **Champness, J. N., Bloomer, A. C., Bricogne, G., Butler, P. J. G., and Klug, A.,** The structure of the protein disk of tobacco mosaic virus to 5 Å resolution, *Nature (London)*, 259, 20, 1976.
210. **Stubbs, G., Warren, S., and Holmes, K. C.,** Structure of RNA and RNA binding site in tobacco mosaic virus from 4 Å map calculated from X-ray fibre diagrams, *Nature (London)*, 267, 216, 1977.
211. **Jardetzky, O., Akasaka, K., Vogel, D., Morris, S., and Holmes, K. C.,** Unusual segmental flexibility in a region of tobacco mosaic virus coat protein, *Nature (London)*, 273, 564, 1978.
212. **Perutz, M. F.,** Regulation of oxygen affinity of hemoglobin: influence of structure of the globin on the heme iron, *Ann. Rev. Biochem.*, 48, 327, 1979.
213. **Purich, D. L., Fromm, H. J., and Rudolph, F. B.,** The hexokinases: kinetic, physical, and regulatory properties, *Adv. Enzymol.*, 39, 249, 1973.
214. **Steitz, T. A., Fletterick, R. J., Anderson, W. F., and Anderson, C. M.,** High resolution X-ray structure of yeast hexokinase, an allosteric protein exhibiting a non-symmetric arrangement of subunits, *J. Mol. Biol.*, 104, 197, 1976.
215. **Anderson, C. M., McDonald, R. C., and Steitz, T. A.,** Sequencing a protein by X-ray crystallography. I. Interpretation of yeast hexokinase B at 2.5 Å resolution by model building, *J. Mol. Biol.*, 123, 1, 1978.
216. **Anderson, C. M., Stenkamp, R. E., and Steitz, T. A.,** Sequencing a protein by X-ray crystallography. II. Refinement of yeast hexokinase B co-ordinates and sequence at 2.1 Å resolution, *J. Mol. Biol.*, 123, 15, 1978.
217. **Steitz, T. A., Anderson, W. F., Fletterick, R. J., and Anderson, C. M.,** High resolution crystal structures of yeast hexokinase complexes with substrates, activators, and inhibitors. Evidence for an allosteric control site, *J. Biol. Chem.*, 252, 4494, 1977.
218. **Bennett, W. S. and Steitz, T. A.,** Glucose-induced conformational change in yeast hexokinase, *Proc. Natl. Acad. Sci. USA*, 75, 4848, 1978.
219. **Bennett, W. S. and Steitz, T. A.,** Structure of a complex between yeast hexokinase A and glucose. I. Structure determination and refinement at 3.5 Å resolution, *J. Mol. Biol.*, 140, 183, 1980.

220. **Bennett, W. S. and Steitz, T. A.,** Structure of a complex between yeast hexokinase A and glucose. II. Detailed comparisons of conformation and active site configuration with the native hexokinase B monomer and dimer, *J. Mol. Biol.*, 140, 211, 1980.
221. **Lobo, Z. and Maitra, P. K.,** Genetics of yeast hexokinase, *Genetics*, 86, 727, 1977.
222. **Schmidt, J. J. and Colowick, S. P.,** Chemistry and subunit structure of yeast hexokinase isoenzymes, *Arch. Biochem. Biophys.*, 158, 458, 1973.
223. **Koshland, D. E.,** Mechanisms of transfer enzymes, in *The Enzymes*, Vol. 1, Boyer, P. D., Lardy, H., and Myrback, K., Eds., Academic Press, New York, 1959, 305.
224. **Kaji, A., and Colowick, S. P.,** Adenosine triphosphatase activity of yeast hexokinase and its relation to the mechanism of the hexokinase reaction, *J. Biol. Chem.*, 240, 4454, 1965.
225. **Dela Fuente, G., Lagunas, R., and Sols, A.,** Induced fit in yeast hexokinase, *Eur. J. Biochem.*, 16, 226, 1970.
226. **Dela Fuente, G. and Sols, A.,** The kinetics of yeast hexokinase in light of the induced fit involved in the binding of its sugar substrate, *Eur. J. Biochem.*, 16, 234, 1970.
227. **Wilkinson, K. D. and Rose, I. A.,** Glucose exchange and catalysis by two crystalline hexokinase-glucose complexes, *J. Biol. Chem.*, 255, 7569, 1980.
228. **Wilkinson, K. D. and Rose, I. A.,** Study of crystalline hexokinase-glucose complexes by isotope trapping, *J. Biol. Chem.*, 256, 9890, 1981.
229. **Steitz, T. A.,** unpublished observations cited in Ref. 218.
230. **Hoggett, J. G. and Kellet, G. L.,** Yeast Hexokinase. A fluorescence temperature-jump study of the kinetics of the binding of glucose to the monomer forms of hexokinases P-I and P-II, *Eur. J. Biochem.*, 68, 347, 1976.
231. **Feldman, I. and Kramp, D. C.,** Fluorescence-quenching study of glucose binding by yeast hexokinase isozymes, *Biochemistry*, 17, 1541, 1978.
232. **Mayes, E. L., Hoggett, J. G., and Kellet, G. L.,** The binding of glucose to yeast hexokinase monomers is independent of ionic strength, *Biochem. J.*, 203, 523, 1982.
233. **Rose, I. A., O'Connell, E. L., Litwin, S., and Bar Tana, J.,** Determination of the rate of hexokinase-glucose dissociation by the isotope-trapping method, *J. Biol. Chem.*, 249, 5163, 1974.
234. **Viola, R. E., Rauschel, F. M., Rendina, A. R., and Cleland, W. W.,** Substrate synergism and the kinetic mechanism of yeast hexokinase, *Biochemistry*, 21, 1295, 1982.
235. **Rose, I. A.,** The isotope trapping method: desorption rates of productive E·S. complexes, *Methods Enzymol.*, 64, Part B, 47, 1980.
236. **Eigen, M. and Hammes, G. G.,** Elementary steps in enzyme reactions, *Adv. Enzymol.*, 25, 1, 1963.
237. **McDonald, R. C., Steitz, T. A., and Engelman, D. M.,** Yeast hexokinase in solution exhibits a large conformational change upon binding glucose or glucose-6-phosphate, *Biochemistry*, 18, 338, 1979.
238. **Pickover, C. A. and Steitz, T. A.,** unpublished observations cited in Ref. 237.
239. **Pickover, C. A., McKay, D. B., Engelman, D. M., and Steitz, T. A.,** Substrate binding closes the cleft between the domains of yeast phosphoglycerate kinase, *J. Biol. Chem.*, 254, 11323, 1979.
240. **Pilz, I.,** Small-Angle X-ray Scattering, in *Physical Principles and Techniques of Protein Chemistry*, Part C, Leach, S. J., Ed., Academic Press, New York, 1973, 141.
241. **McDonald, R. C., Engelman, D. M., and Steitz, T. A.,** Small angle X-ray scattering of dimeric yeast hexokinase in solution, *J. Biol. Chem.*, 254, 2942, 1979.
242. **Newcomer, M. E., Lewis, B. A., and Quiocho, F. A.,** The radius of gyration of L-arabinose-binding protein decreases upon binding of ligand, *J. Biol. Chem.*, 256, 13218, 1981.
243. **Kramp, D. C. and Feldman, I.,** Tryptophan distribution in yeast hexokinase isoenzyme B, *Biochim. Biophys. Acta*, 537, 406, 1978.
244. **Feldman, I. and Norton, G. E.,** Effects of glucose and magnesium ion on the quenching of yeast hexokinase fluorescence by acrylamide, *Biochim. Biophys. Acta.*, 615, 132, 1980.
245. **Anderson, W. F., Fletterick, R. J., and Steitz, T. A.,** Structure of yeast hexokinase. III. Low resolution structure of a second crystal form showing a different quaternary structure, heterologous interaction of subunits and substrate binding, *J. Mol. Biol.*, 86, 261, 1974.
246. **Fletterick, R. J., Bates, D. J., and Steitz, T. A.,** The structure of a yeast hexokinase monomer and its complexes with substrates at 2.7 Å resolution, *Proc. Natl. Acad. Sci. USA*, 72, 38, 1975.
247. **Anderson, C. M., Stenkamp, R. E., McDonald, R. C., and Steitz, T. A.,** A refined model of the sugar binding site of yeast hexokinase B, *J. Mol. Biol.*, 123, 207, 1978.
248. **Fletterick, R. J. and Steitz, T. A.,** The combination of independent phase information obtained from separate protein structure determinations of yeast hexokinase, *Acta Cryst.*, A32, 125, 1976.
249. **Steitz, T. A., Fletterick, R. J., Anderson, W. F., and Anderson, C. M.,** High resolution X-ray structure of yeast hexokinase, an altosteric protein exhibiting a non-symmetric arrangement of subunits, *J. Mol. Biol.* 104, 197, 1976.
250. **Blake, C. C. F.,** The trouble with kinase crystals, in "News and Views", *Nature (London)*, 271, 707, 1978.

251. **Steitz, T.A., Shoham, M., and Bennett, W. S.,** Structural dynamics of yeast hexokinase during catalysis, *Phil. Trans. R. Soc. London*, B293, 43, 1981.
252. **Shoham, M. and Steitz, T. A.,** The 6-hydroxymethyl group of a hexose is essential for the substrate-induced closure of the cleft in hexokinase, *Biochim. Biophys. Acta*, 705, 380, 1982.
253. **Holmgren, A., Söderberg, B.-O., Eklund, H., and Brändén, C.-I.,** Three-dimensional structure of E. coli thioredoxin-S_2 at 2.8 Å resolution, *Proc. Natl. Acad. Sci. USA*, 72, 2305, 1975.
254. **Anderson, C. M., Zucker, F. H., and Steitz, T. A.,** Space-filling models of kinase clefts and conformational changes, *Science*, 204, 375, 1979.
255. **Takano, T.,** Structure of myoglobin refined at 2.0 Å resolution. I. Crystallographic refinement of met-myoglobin from sperm whale, *J. Mol. Biol.*, 110, 537, 1977.
256. **Phillips, S. E. V.,** Structure and refinement of oxymyoglobin at 1.6 Å resolution, *J. Mol. Biol.*, 142, 531, 1980.
257. **Perutz, M. F. and Mathews, F. S.,** An X-ray study of azide methaemoglobin, *J. Mol. Biol.*, 21, 199, 1966.
258. **Case, D. A. and Karplus, M.,** Dynamics of ligand binding to heme proteins, *J. Mol. Biol.*, 132, 343, 1979.
259. **Maley, F. and Lardy, H. A.,** Synthesis of N-substituted glucosamines and their effect on hexokinase, *J. Biol. Chem.*, 214, 765, 1955.
260. **Wilkinson, K. D. and Rose, I. A.,** Isotope trapping studies of yeast hexokinase during steady-state catalysis. A combined rapid quench and isotope trapping technique, *J. Biol. Chem.*, 254, 12567, 1979.
261. **Shoham, M. and Steitz, T. A.,** Crystallographic studies and model building of ATP at the active site of hexokinase, *J. Mol. Biol.*, 140, 1, 1980.
262. **Steitz, T. A., Harrison, R., Weber, I., and Leahy, M.,** Ligand induced conformational changes in proteins, in *Mobility and Function in Proteins and Nucleic acids, Ciba Foundation Symposium 93 (new series)*, Pitman, London, in press.
263. **Stachelek, C. and Konigsberg, W.,** unpublished results cited in references 261 and 262.
264. **Menezes, L. C. and Pudles, J.,** Effects of activators on chemically modified yeast hexokinase, *Biochimie*, 58, 51, 1976.
265. **Takahashi, K., Casey, J. L., and Sturtevant, J.,** Thermodynamics of the binding of D-glucose to yeast hexokinase, *Biochemistry*, 20, 4693, 1981.
266. **Tsong, T. Y., Hearn, R. P., Wrathall, D. P., and Sturtevant, J. M.,** A calorimetric study of thermally induced conformational transitions of ribonuclease A and certain of its derivatives, *Biochemistry*, 9, 2666, 1970.
267. **Sturtevant, J. M.,** Heat capacity and entropy changes in processes involving proteins, *Proc. Natl. Acad. Sci. USA*, 74, 2236, 1977.
268. **Jacrot, B., Cusack, S., Dianoux, A. J., and Engelman, D. M.,** Inelastic neutron scattering analysis of hexokinase dynamics and its modification on binding of glucose, *Nature (London)*, 300, 84, 1982.
269. **Colowick, S. P.,** The hexokinases, in *The Enzymes*, 3rd ed., Vol. 9, Boyer, P. D., Ed., Academic Press, New York, 1973, 1.
270. **Brändén, C.-I., Jörnvall, H., Eklund, H., and Furugren, B.,** Alcohol dehydrogenases, in *The Enzymes*, Vol. 11, Boyer, P. D., Ed., Academic Press, 1975, 103.
271. **Klinman, J. P.,** Probes of mechanism and transition-state structure in the alcohol dehydrogenase reaction, *Crit. Rev. Biochem.*, 10, 39, 1981.
272. **Eklund, H., Nordström, B., Zeppezauer, E., Söderlund, G., Ohlsson, I, Boiwe, T., Söderberg, B.-O., Tapia, O., and Brändén, C.-I.,** Three-dimensional structure of horse liver alcohol dehydrogenase at 2.4 Å resolution, *J. Mol. Biol.*, 102, 27, 1976.
273. **Eklund, H. and Bräendén, C.-I.,** Structural differences between apo- and holoenzyme of horse liver alcohol dehydrogenase, *J. Biol. Chem.*, 254, 3458, 1979.
274. **Eklund, H., Samama, J.-P., Wallén, L., Brändén, C.-I., Åkeson, Å. and Jones, T. A.,** Structure of a triclinic ternary complex of horse liver alcohol dehydrogenase at 2.9 Å resolution, *J. Mol. Biol.*, 146, 561, 1981.
275. **Eklund, H., Samama, J.-P., and Wallén, L.,** Pyrazole binding in crystalline binary and ternary complexes with liver alcohol dehydrogenase, *Biochemistry*, 21, 4858, 1982.
276. **Cedergren-Zeppezauer, E., Samama, J.-P., and Eklund, H.,** Crystal structure determinations of coenzyme analogue and substrate complexes of liver alcohol dehydrogenase: binding of 1,4,5,6-tetrahydronicotinamide adenine dinucleotide and trans-4-(N,N- dimethylamino) cinnamaldehyde to the enzyme, *Biochemistry*, 21, 4895, 1982.
277. **Eklund, H. and Jones, T. A.,** unpublished results cited in references 274 and 275.
278. **Zeppezauer, E., Söderberg, B.-O., Brändén, C.-I., Åkeson, Å., and Theorell, H.,** Crystallization of horse liver alcohol dehydrogenase complexes from alcohol solutions, *Acta Chem. Scand.*, 21, 1099, 1967.
279. **Plapp, B. V., Eklund, H., and Brändén, C.-I.,** Crystallography of liver alcohol dehydrogenase complexed with substrates, *J. Mol. Biol.*, 122, 23, 1978.

280. **Eklund, H., Plapp, B. V., Samama, J.-P., and Brändén, C.-I.,** Binding of substrate in a ternary complex of horse liver alcohol dehydrogenase, *J. Biol. Chem.*, 257, 14349, 1982.
281. **Brändén, C.-I.,** Structure of horse liver alcohol dehydrogenase. I. Structural symmetry and conformational changes, *Arch. Biochem. Biophys.*, 112, 215, 1965.
282. **Abdallah, M. A., Biellmann, J.-F., Nordström, B., and Brändén, C.-I.,** The conformation of adenosine diphosphoribose and 8-bromoadenosine diphosphoribose when bound to liver alcohol dehydrogenase, *Eur. J. Biochem.*, 50, 475, 1975.
283. **Samama, J.-P., Zeppezauer, E., Biellmann, J.-F., and Brändén, C.-I.,** The crystal structure of complexes between horse liver alcohol dehydrogenase and the coenzyme analogues 3-iodopyridine-adenine dinucleotide and pyridine-adenine dinucleotide, *Eur. J. Biochem.*, 81, 403, 1977.
284. **Samama, J.-P., Wrixon, A. D., and Biellmann, J.-F.,** 5-Methylnicotin amide-adenine dinucleotide. Kinetic investigation with major and minor isoenzymes of liver alcohol dehydrogenase and structural determination of its binary complex with alcohol dehydrogenase, *Eur. J. Biochem.*, 118, 479, 1981.
285. **Eklund, H. and Colonna, F.,** personal communication, 1982.
286. **Brändén, C.-I., and Eklund, H.,** Structure and mechanism of liver alcohol dehydrogenase, lactate dehydrogenase and glyceraldehyde-3-phosphate dehydrogenase, in *Dehydrogenases*, Jeffry, J., Ed., Birkhauser, Basel, 1980, 40.
287. **Bignetti, E., Rossi, G. L., and Zeppezauer, E.,** Microspectrophotometric measurements on single crystals of coenzyme containing complexes of horse liver alcohol dehydrogenase, *FEBS Lett.*, 100, 17, 1979.
288. **Yonetani, T. and Theorell, H.,** Crystallizaton of binary and ternary complexes of horse liver alcohol dehydrogenases with DPNH and with DPNH and isobutyramid, *Arch. Biochem. Biophys.*, 100, 554, 1963.
289. **Theorell, H. and Chance, B.,** Studies on liver alcohol dehydrogenase. II. The kinetics of the compound of horse liver alcohol dehydrogenase and reduced diphosphopyridine nucleotide, *Acta Chem Scand.*, 5, 1127, 1951.
290. **Danenberg, K. D. and Cleland, W. W.,** Use of chromium-adenosine triphosphate and lyxose to elucidate to the kinetic mechanism and coordination state of the nucleotide substrate for yeast hexokinase, *Biochemistry*, 14, 28, 1975.
291. **Czeisler, J. L. and Hollis, D. P.,** Nuclear magnetic resonance studies of alcohol dehydrogenases. Binding of inhibitors to the yeast and liver enzymes, *Biochemistry*, 12, 1683, 1973.
292. **Wolfe, J. K., Weidig, C. F., Halvorson, H. R., Shore, J. D., Parker, D. M., and Holbrook, J. J.,** pH-Dependent conformational states of horse liver alcohol dehydrogenase, *J. Biol. Chem.*, 252, 433, 1977.
293. **Coates, J. H., Hardman, M. J., Shore, J. D., and Gutfreund, H.,** Pressure relaxation studies of isomerisations of horse liver alcohol dehydrogenase linked to NAD^+ binding, *FEBS Lett.*, 84, 25, 1977.
294. **Dalziel, K.,** Kinetic studies of liver alcohol dehydrogenase and pH effects with coenzyme preparations of high purity, *J. Biol. Chem.*, 238, 2850, 1963.
295. **White, J. L., Hackert, M. L., Buehner, M., Adams, M. J., Ford, G. C., Lentz, P. J., Smiley, I. E., Steindel, S. J., and Rosmann, M. G.,** A comparison of the structures of apo dogfish M_4 lactate dehydrogenase and its ternary complexes, *J. Mol. Biol.*, 102, 759, 1976.
296. **Dalziel, K.,** Dynamic aspects of enzyme specificity, *Phil. Trans. R. Soc. Lond.*, B272, 109, 1975.
297. **Maret, W., Andersson, I., Dietrich, H., Schneider-Bernlöhr, H., Einarsson, R., and Zeppezauer, M.,** Site-specific substituted Co(II) horse liver alcohol dehydrogenases, *Eur. J. Biochem.*, 98, 501, 1979.
298. **Dietrich, H., Maret, W., Wallén, L. and Zeppezauer, M.,** Active-site-specific reconstituted cobalt(II) horse-liver alcohol dehydrogenase, *Eur. J. Biochem.*, 100, 267, 1979.
299. **Subramanian, S. and Ross, P. D.,** Thermodynamics of binding of oxidized and reduced nicotinamide adenine dinucleotides, adenosine-5′-diphosphoribose, and 5′-Iodosalicylate to dehydrogenase, *Biochemistry*, 17, 2193, 1978.
300. **Jörnvall, H.,** Differences between alcohol dehydrogenases structural properties and evolutionary aspects, *Eur. J. Biochem.*, 72, 443, 1977.
301. **Jörnvall, H., Eklund, H., and Brändén, C.-I.,** Subunit conformation of yeast alcohol dehydrogenase, *J. Biol. Chem.*, 253, 8414, 1978.
302. **Spector, L. B.,** Citrate cleavage and related enzymes, in *The Enzymes*, Vol. 7, Boyer, P. D., Ed., Academic Press, New York, 1972, 357.
303. **Srere, P. A.,** The enzymology of the formation and breakdown of citrate, *Adv. Enzymol.*, 43, 57, 1975.
304. **Remington, S., Wiegand, G., and Huber, R.,** Crystallographic refinement and atomic models of two different forms of citrate synthase at 2.7 and 1.7 Å resolution, *J. Mol. Biol.*, 158, 111, 1982.
305. **Johansson, C.-J. and Petterson, G.,** Evidence that citrate synthase operates by an ordered ternary-complex mechanism, *Eur. J. Biochem.*, 42, 383, 1974.
306. **Johansson, C.-J. and Petterson, G.,** Substrate-inhibition by acetyl-CoA in the condensation reaction between oxaloacetate and acetyl-CoA catalyzed by citrate synthase from pig heart, *Biochim. Biophys. Acta*, 484, 208, 1977.
307. **Eggerer, H.,** Zum Mechanismus der Biologischen Umwandlung von Citronensäure. VI. Citrat-synthtase ist eine Acetyl-CoA-Enolase, *Biochim. Z.*, 343, 111, 1965.

308. **Wiegand, G., Remington, S. J., and Huber, R.,** unpublished observations, 1982.
309. **Harris, J. F. and Waters, M.,** Glyceraldehyde-3-phosphate dehydrogenase, in *The Enzymes*, Vol. 13, Boyer, P. D., Ed., Academic Press, New York, 1976, 1.
310. **Biesecker, G., Harris, J. I., Thierry, J. C., Walker, J. E., and Wonacott, A. J.,** Sequence and structure of D-glyceraldehyde 3-phosphate dehydrogenase from *Bacillus stearothermophilus*, *Nature (London)*, 266, 328, 1977.
311. **Moras, D., Olsen, K. W., Sabesan, M. N., Buehner, M., Ford, G. C., and Rossmann, M. G.,** Studies of asymmetry in the three-dimensional structure of lobster D-glyceraldehyde-3-phosphate dehydrogenase, *J. Biol. Chem.*, 250, 9137, 1975.
312. **Rossmann, M. G., Moras, D., and Olsen, K. W.,** Chemical and biological evolution of a nucleotide-binding protein, *Nature (London)*, 250, 194, 1974.
313. **Biesecker, G. and Wonacott, A. J.,** Coenzyme binding and cooperativity in D-glyceraldehyde 3-phosphate dehydrogenase, *Biochem. Soc. Trans.*, 5, 647, 1977.
314. **Wonacott, A. J. and Biesecker, G.,** Symmetry and NAD^+-dependent structural changes, in D-Glyceraldehyde-3-phosphate Dehydrogenase, in *Pyridine Nucleotide-Dependent Dehydrogenases*, Sund, H., Ed., de Gruyter, Berlin, 1977, 140.
315. **Dalziel, K., McFerran, N. V., and Wonacott, A. J.,** Glyceraldehyde-3-phosphate dehydrogenase, *Phil. Trans. R. Soc. London*, B293, 105, 1981.
316. **Wonacott, A. J.,** personal communication, 1982.
317. **DeVijlder, J. J. M., Boers, W., and Slater, E. C.,** Binding and properties of NAD^+ in glyceraldehydephosphate dehydrogenase from lobster-tail muscle, *Biochim. Biophys. Acta*, 191, 214, 1969.
318. **Berni, R., Mozzarelli, A., Rossi, G. L., Bolognesi, M., and Oberti, R.,** Crystallographic symmetry and coenzyme binding properties of D-glyceraldehyde-3-phosphate dehydrogenase from the tail muscle of *Palinurus vulgaris*, *J. Biol. Chem.*, 254, 8004, 1979.
319. **Murthy, M. R. N., Garavito, R. M., Johnson, J. E., and Rossmann, M. G.,** Structure of lobster Apo-D-glyceraldehyde-3-phosphate dehydrogenase at 3.0 Å resolution, *J. Mol. Biol.*, 138, 859, 1980.
320. **Berni, R., Mozzarelli, A., Pellacani, L., and Rossi, G. L.,** Catalytic and regulatory properties of D-glyceraldehyde-3-phosphate dehydrogenase in the crystal. Spectral properties and chemical reactivity of a chromophoric acyl-enzyme intermdiate, *J. Mol. Biol.*, 110, 405, 1977.
321. **Fuller-Noel, J. K. and Schumaker, V. N.,** Sequential conformational changes with the binding of NAD^+ to rabbit muscle glyceraldehyde-3-phosphate dehydrogenase, *J. Mol. Biol.*, 68, 523, 1972.
322. **Smith, G. D. and Schachman, H. K.,** Effect of D_2O and nicotinamide adenine dinucleotide on the sedmentation properties and structure of glyceraldehyde-3-phosphate dehydrogenase, *Biochemistry*, 12, 3789, 1973.
323. **Jaenicke, R. and Gratzer, W. B.,** Cooperative and non-cooperative conformational effects of the coenzyme on yeast glyceraldehyde-3-phosphate dehydrogenase, *Eur. J. Biochem.*, 10, 158, 1969.
324. **Durchschlag, H., Puchwein, G., Kratky, O., Schuster, I., and Kirschner, K.,** X-ray small-angle scattering of yeast glyceraldehyde-3-phosphate dehydrogenase as a function of saturation with nicotinamide-adenine dinucleotide, *Eur. J. Biochem.*, 19, 9, 1971.
325. **Simon, I.,** Study of the position of NAD and its effect on the conformation of D-glyceraldehyde-3-phosphate dehydrogenase by small-angle X-ray scattering, *Eur. J. Biochem.*, 30, 184, 1972.
326. **Park, J. H., Agnello, C. F., and Mathew, E.,** S-N transfer and dual acetylation in the S-acetylation and N-acetylation of 3-phosphoglyceraldehyde dehydrogenase by substrates, *J. Biol. Chem.*, 241, 769, 1966.
327. **Mathew, E., Meriwether, B. P., and Park, J. H.,** The enzymatic significance of S-acetylation and N-acetylation of 3-phosphoglyceraldehyde dehydrogenase, *J. Biol. Chem.*, 242, 5024, 1967.
328. **Braunstein, A. E.,** Amino group transfer, in *The Enzymes*, Vol. 9, Boyer, P. D., Ed., Academic Press, New York, 1973, 379.
329. **Borisov, V. V., Borisova, S. N., Kachalova, G. S., Sosfenov, N. I., Vainshtein, B. K., Torchinsky, Yu. M., and Braunstein, A. E.,** Three-dimensional structure at 5 Å resolution of cytosolic aspartate transaminase from chicken heart, *J. Mol. Biol*, 125, 275, 1978.
330. **Ford, G. C., Eichele, G., and Jansonius, J. N.,** Three-dimensional structure of a pyridoxal-phosphate-dependent enzyme, mitochondrial aspartate aminotransferase, *Proc. Natl. Acad. Sci. USA*, 77, 2559, 1980.
331. **Arnone, A., Briley, P. D., Rogers, P. H., Hyde, C. C., Metzler, C. M., and Metzler, D. M.,** Changes in the tertiary structure of cytosolic aspartate aminotransferase induced by the binding of inhibitors and substrates, in *Molecular Structure and Biological Activity*, Griffin, J. F. and Duax, W. L., Eds., Elsevier, New York, 1982, 57.
332. **Metzler, D. E., Jansonius, J. N., Arnone, A., Martinez-Carrion, M., and Manning, J. M.,** Mini-symposium summary-transaminases, *Fed. Proc., Fed. Am. Soc. Exp. Biol.*, 41, 2432, 1982.
333. **Eichele, G., Ford, G. C., Glor, M., Jansonius, J. N., Mavrides, C., and Christen, P.,** The three-dimensional structure of mitochondrial aspartate aminotransferase at 4.5 Å resolution, *J. Mol. Biol.*, 133, 161, 1979.

334. **Metzler, C. M., Metzler, D. E., Martin, D. S., Newman, R., Arnone, A., and Rogers, P.,** Crystalline enzyme substrate complexes of aspartate aminotransferase, *J. Biol. Chem.*, 253, 5251, 1978.
335. **Jansonius, J. N.,** personal communication, 1982.
336. **Eichele, G., Karabelnik, D., Halonbrenner, R., Jansonius, J. N., and Christen, P.,** Catalytic activity in crystals of mitochondrial aspartate aminotransferase as detected by microspectrophotometry, *J. Biol., Chem.*, 253, 5239, 1978.
337. **Blake, C. C. F. and Evans, P. R.,** Structure of horse muscle phosphoglycerate kinase. Some results on the chain conformation, substrate binding and evolution of the molecule from a 3 Å Fourier map, *J. Mol. Biol.*, 84, 585, 1974.
338. **Schulz, G. E., Elzinga, M., Marx, F., and Schirmer, R. H.,** Three-dimensional structure of adenylate kinase, *Nature (London)*, 250, 120, 1974.
339. **Levine, M., Muirhead, H., Stammers, D. K., and Stuart, D. I.,** Structure of pyruvate kinase and similarities with other enzymes: possible implications for protein taxonomy and evolution, *Nature (London)*, 271, 626, 1978.
340. **Evans, P. R. and Hudson, P. J.,** Structure and control of phosphofructokinase from *Bacillus stearothermophilus*, *Nature (London)*, 279, 500, 1979.
341. **Banks, R. D., Blake, C. C. F., Evans, P. R., Haser, R., Rice, D. W., Hardy, G. W., Merrett, M., and Phillips, A. W.,** Sequence, structure and activity of phosphoglycerate kinase: a possible hinge-bending enzyme, *Nature (London)*, 279, 773, 1979.
342. **Blake, C. C. F. and Rice, D. W.,** Phosphoglycerate kinase, *Phil. Trans. R. Soc. London*, B293, 93, 1981.
343. **Sachsenheimer, W. and Schulz, G. E.,** Two conformations of crystalline adenylate kinase, *J. Mol. Biol.*, 114, 23, 1977.
344. **Pai, E. F., Sachsenheimer, W., Schirmer, R. H., and Schulz, G. E.,** Substrate positions and induced-fit in crystalline adenylate kinase, *J. Mol. Biol.*, 114, 37, 1977.
345. **Stammers, D. K. and Muirhead, H.,** Three-dimensional structure of cat muscle pyruvate kinase at 6 Å resolution, *J. Mol. Biol.*, 95, 213, 1975.
346. **Evans, P. R., Farrants, G. W., and Hudson, P. J.,** Phosphofructokinase: structure and control, *Phil. Trans. R. Soc. London*, B293, 53, 1981.
347. **Gilliland, G. L. and Quiocho, F. A.,** Structure of the L-arabinose-binding protein from *Escherichia coli* at 2.4 Å resolution, *J. Mol. Biol.*, 146, 341, 1981.
348. **Newcomer, M. E., Gilliland, G. L., and Quiocho, F. A.,** L-arabinose-binding protein-sugar complex at 2.4 Å resolution, *J. Biol. Chem.*, 256, 13213, 1981.
349. **Kumosinski, T. F., Pessen, H., and Farrell, H. M., Jr.,** Structure and mechanism of action of riboflavin-binding protein: small-angle X-ray scattering, sedimentation, and circular dichroism studies on the holo- and apoproteins, *Arch. Biochem. Biophys.*, 214, 714, 1982.
350. **Huxley, H. E.,** The mechanism of muscular contraction, *Science*, 164, 1356, 1969.
351. **Eisenberg, E. and Hill, T. L.,** A cross-bridge model of muscle contraction, *Prog. Biophys. Mol. Biol.*, 33, 55, 1978.
352. **Tregear, R. T. and Marston, S. B.,** The Crossbridge theory, *Ann. Rev. Physiol.*, 41, 723, 1979.
353. **Taylor, E. W.,** Mechanism of actomyosin ATP and the problem of muscle contraction, *Crit. Rev. Biochem.*, 6, 103, 1979.
354. **Harvey, S. C. and Cheung, H. C.,** Myosin flexibility, in *Cell and Muscle Motility*, Vol. 2, Dowben, R. M. and Shay, J. W., Eds., Plenum Press, New York, 1982, 279.
355. **Mendelson, R. A.,** Fluorescent-probe studies of contractile proteins, in *Cell and Muscle Motility*, Vol. 2, Dowben, R. M. and Shay, J. W., Eds., Plenum Press, New York, 1982, 257.
356. **Huxley, H. E., Faruqi, A. R., Bordas, J., Koch, M. H. J., and Milch, J. R.,** The use of synchrotron radiation in time-resolved X-ray diffraction studies of myosin layer-line reflections during muscle contraction, *Nature (London)*, 284, 140, 1980.
357. **Huxley, H. E., Simmons, R. M., Faruqi, A. R., Kress, M., Bordas, J., and Koch, M. H. J.,** Millisecond time-resolved changes in X-ray reflections from contracting muscle during rapid mechanical transients, recorded using synchrotron radiation, *Proc. Natl. Acad. Sci. USA*, 78, 2297, 1981.
358. **Phillips, G. N., Jr., Lattman, E. E., Cummins, P., Lee, K. Y., and Cohen, C.,** Crystal structure and molecular interactions of tropomyosin, *Nature (London)*, 278, 413, 1979.
359. **Harrison, S. C., Olson, A. J., Schutt, C. E., Winkler, F. K., and Bricogne, G.,** Tomato bushy stunt virus at 2.9 Å resolution, *Nature (London)*, 276, 368, 1978.
360. **Harrison, S. C.,** Protein interfaces and intersubunit bonding. The case of tomato bushy stunt virus, *Biophys. J.*, 32, 139, 1980.
361. **Robinson, I. K. and Harrison, S. C.,** Structure of the expanded state of tomato bushy stunt virus, *Nature (London)*, 297, 563, 1982.
362. **Schimmel, P. R. and Söll, D.,** Aminoacyl-tRNA synthetases: general features and recognition of transfer RNAs, *Ann. Rev. Biochem.*, 48, 601, 1979.

363. **Irwin, M. J., Nyborg, J., Reid, B. R., and Blow, D. M.,** The crystal structure of tyrosyl-tRNA synthetase at 2.7 Å resolution, *J. Mol. Biol.*, 105, 577, 1976.
364. **Risler, J. L., Zelwer, C., and Brunie, S.,** Methioyl-tRNA synthetase show the nucleotide binding fold observed in dehydrogenases, *Nature (London)*, 292, 384, 1981.
365. **Bhat, T. N., Blow, D. M., Brick, P., and Nyborg, J.,** Tyrosyl-tRNA synthetase forms a mononucleotide-binding fold, *J. Mol. Biol.*, 158, 699, 1982.
366. **Bhat, T. N. and Blow, D. M.,** A density-modification method for the improvement of poorly resolved protein electron-density maps, *Acta Cryst.*, A38, 21, 1982.
367. **Zaccaï, G., Morin, P., Jacrot, B., Moras, D., Thierry, J.-C., and Giegé, R.,** Interaction of yeast valyl-tRNA synthetase with RNAs and conformational changes of the enzyme, *J. Mol. Biol.*, 129, 483, 1979.
368. **Koch, G. L. E. and Bruton, C. J.,** The subunit structure of methionyl-tRNA synthetase from *Escherichia coli*, *FEBS Lett.*, 40, 180, 1974.
369. **Waller, J.-P., Risler, J. L., Monteilhet, C., and Zelwer, C.,** Crystallization of trypsin-modified methionyl-tRNA synthetase from *E. coli*, *FEBS Lett.*, 16, 186, 1971.
370. **Dessen, P., Blanquet, S., Zaccaï, G., and Jacrot, B.,** Anticooperative binding of initiator transfer RNA^{MET} to methionyl-transfer RNA synhetase from *E. coli*: neutron scattering studies, *J. Mol. Biol.*, 126, 293, 1978.
371. **Dessen, P., Fayat, G., Zaccaï, G., and Blanquet, S.,** Neutron-scattering studies of the binding of initiator $tRNA^{MET}$ to *E. coli* trypsin-modified methionyl-tRNA synthetase, *J. Mol. Biol.*, 154, 603, 1982.
372. **McKay, D. B. and Steitz, T. A.,** Structure of catabolite gene activator protein at 2.9 Å resolution suggests binding to left-handed B-DNA, *Nature (London)*, 290, 744, 1981.
373. **McKay, D. B., Weber, I. T., and Steitz, T. A.,** Structure of catabolite gene activator protein at 2.9 Å resolution. Incorporation of amino acid sequence and interactions with cyclic AMP, *J. Biol. Chem.*, 257, 9518, 1982.
374. **Anderson, W. F., Ohlendorf, D. H., Takeda, Y., and Matthews, B. W.,** Structure of the cro repressor from bacteriophage λ and its interaction with DNA, *Nature (London)*, 290, 754, 1981.
375. **Pabo, C. O. and Lewis, M.,** The operator-binding domain of λ repressor: structure and DNA recognition, *Nature (London)*, 298, 443, 1982.
376. **Wade-Jardetzky, N., Bray, R. P., Conover, W. W., Jardetzky, O., Geisler, N., and Weber, K.,** Differential mobility of the N-terminal headpiece in the *lac*-repressor protein, *J. Mol. Biol.*, 128, 259, 1979.
377. **Krebs, E. G. and Beavo, J. A.,** Phosphorylation-dephosphorylation of enzymes, *Ann. Rev. Biochem.*, 48, 923, 1979.
378. **Graves, D. J. and Wang, J. H.,** α-Glucan phosphorylases — chemical and physical basis of catalysis and regulation, in *The Enzymes*, Vol. 7, Boyer, P. D., Ed., Academic Press, New York, 1972, 435.
379. **Fletterick, R. J. and Madsen, N. B.,** The structure and related functions of phosphorylase *a*, *Ann. Rev. Biochem.*, 49, 31, 1980.
380. **Fletterick, R. J., Sygusch, J., Murray, N., Madsen, N. B., and Johnson, L. N.,** Low-resolution structure of the glycogen phosphorylase *a* monomer and comparison with phosphorylase *b*, *J. Mol. Biol.*, 103, 1, 1976.
381. **Sprang, S. and Fletterick, R. J.,** The structure of glycogen phosphorylase *a* at 2.5 Å resolution, *J. Mol. Biol.*, 131, 523, 1979.
382. **Madsen, N. B., Kasvinsky, P. J., and Fletterick, R. J.,** Allosteric transitions of phosphorylase *a* and the regulation of glycogen metabolism, *J. Biol. Chem.*, 253, 9097, 1978.
383. **Fletterick, R. J., Sprang, S., and Madsen, N. B.,** Analysis of the surface topography of glycogen phosphorylase *a*: implications for metabolic interconversion and regulatory mechanisms, *Can. J. Biochem.*, 57, 789, 1979.
384. **Sprang, S. and Fletterick, R. J.,** Subunit interactions and the allosteric response in phosphorylase, *Biophys. J.*, 32, 175, 1980.
385. **Kasvinsky, P. J., Madsen, N. B., Sygusch, J., and Fletterick, R. J.,** The regulation of glycogen phosphorylase *a* by nucleotide derivatives, *J. Biol. Chem.*, 253, 3343, 1978.
386. **Johnson, L. N., Stura, E. A., Wilson, K. S., Sansom, M. S. P., and Weber, I. T.,** Nucleotide binding to glycogen phosphorylase *b* in the crystal, *J. Mol. Biol.*, 134, 639, 1979.
387. **Kasvinsky, P. J., Madsen, N. B., Fletterick, R. J., and Sygusch, J.,** X-ray crystallographic and kinetic studies of oligosaccharide binding to phosphorylase, *J. Biol. Chem.*, 253, 1290, 1978.
388. **Kasvinsky, P. J. and Madsen, N. B.,** Activity of glycogen phosphorylase in the crystalline state, *J. Biol. Chem.*, 251, 6852, 1976.
389. **Eagles, P. A. M., Iqbal, M., Johnson, L. N., Mosley, J., and Wilson, K. S.,** A tetragonal crystal form of phosphorylase *b*, *J. Mol. Biol.*, 71, 803, 1972.
390. **Johnson, L. N., Madsen, N. B., Mosley, J., and Wilson, K. S.,** The crystal sructure of phosphorylase *b* at 6 Å resolution, *J. Mol. Biol.*, 90, 703, 1974.
391. **Weber, I. T., Johnson, L. N., Wilson K. S., Yeates, D. G. R., Wild, D. L., and Jenkins, J. A.,** Crystallographic studies on the activity of glycogen phosphorylase *b*, *Nature, (London)*, 274, 433, 1978.

392. **Jenkins, J. A., Johnson, L. N., Stuart, D. I., Stura, E. A. Wilson, K. S., and Zanotti, G.,** Phosphorylase: control and activity, *Phil. Trans. R. Soc. Lond.*, B293, 23, 1981.
393. **Johnson, L. N.,** personal communication, 1982.
394. **Fletterick, R. J., Sygusch, J., Semple, H., and Madsen, N. B.,** Structure of glycogen phosphorylase *a* at 3.0 Å resolution and its ligand binding sites at 6 Å, *J. Biol. Chem.*, 251, 6142, 1976.
395. **Dijkstra, B. W., Kalk, K. H., Hol, W. G. J., and Drenth, J.,** Structure of bovine pancreatic phospholipase A2 at 1.7 Å resolution, *J. Mol. Biol.*, 147, 97, 1981.
396. **Dijkstra, B. W., van Nes, G. J. H., Kalk, K. H., Brandenburg, N. P., Hol, W. G. J., and Drenth J.,** The structure of bovine pancreatic prophospholipase A_2 at 3.0 Å resolution, *Acta Cryst.*, B38, 793, 1982.
397. **Dessen, P., Zaccaï, G., and Blanquet, S.,** Neutron scattering studies of *Escherichia Coli* tyrosyl-tRNA synthetase and of its interaction with $tRNA^{tyr}$, *J. Mol. Biol.*, 159, 651, 1982.
398. **Holbrook, J. J., Liljas, A., Steindel, S. J., and Rossmann, M. J.,** Lactate dehydrogenase, in *The Enzymes*, Vol. 11, Boyer, P. D., Ed., Academic Press, 1975, 191.
399. **Parker, D. M., Holbrook, J. J., Birdsall, B., and Roberts, G. K. C.,** H NMR studies of Tyr237 of lactate dehydrogenase, *FEBS Lett.*, 129, 33, 1981.
400. **Parker, D. M., Jeckel, D., and Holbrook, J. J.,** Slow structural changes shown by the 3-nitrotyrosine-237 residue in pig heart [$Tyr(3NO_2)^{237}$]lactate dehydrogenase, *Biochem. J.*, 201, 465, 1982.
401. **Trommer, W. E. and Gloegler, K.,** Solution conformation of lactate dehydrogenase as studied by saturation transfer ESR spectroscopy, *Biochim. Biophys. Acta*, 571, 186, 1979.
402. **Musick, W. D. L. and Rossmann, M. G.,** The structure of mouse testicular lactate dehydrogenase isozyme C_4 at 2.9 Å resolution, *J. Biol. Chem.*, 254, 7611, 1979.
403. **Grau, U. M., Trommer, W. E., and Rossmann, M. G.,** Structure of the active ternary complex of pig heart lactate dehydrogenase with S-lac-NAD at 2.7 Å resolution, *J. Mol. Biol.*, 151, 289, 1981.
404. **James, M. N. G. and Sielecki, A. R.,** Structure and refinement of penicillopepsin at 1.8 Å resolution, *J. Mol. Biol.*, 163, 299, 1983.
405. **James, M. N. G., Sielecki, A., Salituro, F., Rich, D. H., and Hofmann, T.,** Conformational flexibility in the active sites of aspartyl proteinases revealed by a pepstatin fragment binding to penicillopepsin, *Proc. Natl. Acad. Sci. USA*, 79, 6137, 1982.
406. **James, M. N. G., Hsu, I., and Delbaere, L. T. J.,** Mechanism of acid protease catalysis based on the crystal structure of penicillopepsin, *Nature (London)*, 267, 808, 1977.
407. **Alber, T., Banner, D. W., Bloomer, A. C., Petsko, G. A., Phillips, D., Rivers, P. S., and Wilson, I. A.,** On the three-dimensional structure and catalytic mechanism of triose phosphate isomerase, *Phil. Trans. R. Soc. Lond.*, B293, 159, 1981.
408. **Winn, S. I., Watson, H. C., Harkins, R. N., and Fothergill, L. A.,** Structure and activity of phosphoglycerate mutase, *Phil. Trans. R. Soc. Lond.*, B293, 121, 1981.
409. **Monod, J., Wyman, J., and Changeux, J.-P.,** On the nature of allosteric transitions: a plausible model, *J. Mol. Biol.*, 12, 88, 1965.
410. **Rossmann, M. G. and Argos, P.,** Protein folding, *Ann. Rev. Biochem.*, 50, 497, 1981.
411. **Jackson, C. M. and Nemerson, Y.,** Blood coagulation, *Ann. Rev. Biochem.*, 49, 765, 1980.
412. **Müller-Eberhard, H. J.,** Complement, *Ann. Rev. Biochem.*, 44, 697, 1975.
413. **Chance, B. and Ravilly, A.,** Reactivity of crystalline ferrihemoglobin towards azide, *J. Mol. Biol.*, 21, 195, 1966.
414. **Chien, J. C. W. and Dickerson, L. C.,** EPR crystallography of metalloproteins and spin-labelled enzymes, in *Biological Magnetic Resonance*, Vol. 3, Berliner, L. C. and Reuben, J., Eds., Academic Press, New York, 1981, 155.
415. **Rothgeb, T. M. and Oldfield, E.,** Nuclear magnetic resonance of heme protein crystals. General aspects, *J. Biol. Chem.*, 256, 1432, 1981.
416. **Rice, D. M., Wittebort, R. J., Griffin, R. G., Meirovitch, E., Stimson, E. R., Meinwald, Y. C., Freed, J. H., and Scheraga, H. A.,** Rotational jumps of the tyrosine side chain in crystalline enkephalin. ^2H NMR line shapes for aromatic ring motion in solids, *J. Am. Chem. Soc.*, 103, 7707, 1981.
417. **Meares, C. F. and Westmoreland, D. G.,** The study of biological macromolecular using perturbed angular correlation of gamma radiation, *Cold Spring Harbor Symp. Quant. Biol.*, 36, 511, 1972.
418. **Abramov, V. M., Arkhangelskaya, Z. A., and Zav'yalov, V. P.,** Conformational properties of human immunoglobulin G subclasses. Analysis by temperature-perturbation and solvent-perturbation spectroscopy, *Biochim. Biophys. Acta*, 742, 295, 1983.
419. **Pabo, C. O., Sauer, R. T., Sturtevant, J. M., and Ptashne, M.,** The λ repressor contains two domains, *Proc. Natl. Acad. Sci. USA*, 76, 1608, 1979.
420. **Zavodszky, P., Jaton, J. C., and Medgyesi, G. A.,** Domain-domain interactions in homogeneous rabbit IgG, *Hoppe Seyler's Z. Physiol. Chem.*, 362, 20, 1981.
421. **Roustan, C., Fattoum, A., Jeanneau, R., and Pradel, L.-A.,** Yeast 3-phosphoglycerate kinase: sulfate and substrate binding, their effect on the conformational state of the enzyme, *Biochemistry*, 19, 5168, 1980.

422. **Bryant, T. N., Watson, H. C., and Wendell, P. L.,** Structure of yeast phosphoglycerate kinase, *Nature (London)*, 247, 14, 1974.
423. **Blake, C. C. F. and Evans, P. R.,** Structure of horse muscle phosphoglycerate kinase, *J. Mol. Biol.*, 84, 585, 1974.
424. **Watson, H. C., Walker, N. P. C., Shaw, P. J., Bryant, T. N., Wendell, P. L., Fothergill, L. A., Perkins, R. E., Conroy, S. C., Dobson, M. J., Tuite, M. F., Kingsman, A. J., and Kingsman, S. M.,** Sequence and structure of yeast phosphoglycerate kinase, *EMBO J.*, 1, 1635, 1982.
425. **Ladner, J. E., Kitchell, J. P., Honzatko, R. B., Ke, H. M., Volz, K. W., Kalb (Gilboa), A. J., Ladner, R. C., and Lipscomb, W. N.,** Gross quaternary changes in aspartate carbamoyltransferase are induced by the binding of N-(phosphonacetyl)-L-aspartate: a 3.5 Å resolution study, *Proc. Natl. Acad. Sci., USA*, 79, 3125, 1982.
426. **Austin, R. H., Beeson, K. W., Eisenstein, L., Frauenfelder, H., and Gunsalus, I. C.,** Dynamics of Ligand Binding to Myoglobin, *Biochemistry*, 14, 5355, 1975.
427. **Frauenfelder, H., Petsko, G. A., and Tsernoglou, D.,** Temperature-dependent X-ray diffraction as a probe of protein structural dynamics, *Nature (London)*, 280, 558, 1979.
428. **Hartmann, H., Parak, F., Steigemann, W., Petsko, G. A., Ponzi, D. R., and Frauenfelder, H.,** Conformational substrates in a protein: structure and dynamics of metmyoglobin at 80 K, *Proc. Nat. Acad. Sci., USA*, 79, 4967, 1982.
429. **Harutyunyan, E. G., Malashkevich, V. N., Tersyan, S. S., Kochkina, V. M., Torchinsky, Yu. M., and Braunstein, A. E.,** Three-dimensional structure at 3.2 Å resolution of the complex of cytosolic aspartate aminotransferase from chicken heart with 2-oxoglutarate, *FEBS LeH.*, 138, 113, 1982.
430. **Janin, J. and Wodak, S. J.,** Structural domains in proteins and their role in the dynamics of protein function, *Prog. Bioph. Molec. Biol.*, 42, 21, 1983.
431. **Wrigley, N. G., Brown, E. B., and Skehel, J. J.,** Electron microscopic evidence for the axial rotation and inter-domain flexibility of the Fab regions of immunoglobulin G., *J. Mol. Biol.*, 169, 771, 1983.
432. **Rajan S. S., Ely, K. R., Abola, E. E., Wood, M. K., Colman, P. M., Athay, R. J., and Edmundson, A. B.,** Three-dimensional structure of the Mcg IgG1 immunoglobulim, *Molec. Immunol.*, 20, 787, 1983.
433. **Schneider, G., Eklund, H., Cedergren-Zeppezauer, E., and Zeppezauer, M.,** Structure of the complex of active site metal-depleted horse liver alcohol dehydrogenase and NADH, *EMBO J.*, 2, 685, 1983.
434. **Saper, M. A. and Quiocho, F. A.,** Leucine, isoleucine, valine-binding protein from *Escherichia coli*, *J. Biol. Chem.*, 258, 11057, 1983.
435. **Ottonello, S., Mozzarelli, A., Rossi, G. L., Carotti, D., and Riva, F.,** Interaction of a coenzyme analog with aspartate aminotransferase isoenzymes in the crystal, *Eur. J. Biochem.*, 133, 47, 1983.
436. **Vergé, D., Tardieu, A., and Arrio-Dupont, M.,** Solution X-ray scattering study of pig heart cytosolic aspartate aminotransferase, *FEBS Lett.*, 154, 277, 1983.