THE INTERNAL DYNAMICS OF GLOBULAR PROTEINS

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I. INTRODUCTION

Although many globular proteins have a well-defined equilibrium geometry in the native state, their flexibility and structural fluctuations play an important role in their activity. Examination of the X-ray structures reveals that transient packing defects must occur in myoglobin and hemoglobin to allow oxygen penetration to the heme binding sites.[1] Functional interactions of flexible ligands with their binding sites often require conformational adjustments in both the ligands and the binding proteins; the ligands involved include drugs, hormones, and enzyme substrates.[2] The conformational adjustments in the binding proteins are known to be important in regulating the activity of many of these molecules through induced fit and allosteric effects.[3,4] The chemical transformations of substrates by enzymes typically involve significant atomic displacements in the enzyme-substrate complexes; the mechanisms and rates of such transformations are sensitive to the dynamical properties of these complexes since, for example, the differences in the vibrational properties of the initial and the transition states affect the activation free energies and catalytic rates.[4] Electron transfer processes may depend strongly on vibronic coupling and on fluctuations which alter the distance between the donor and acceptor.[5-7] The relative motion of distinct structural domains is important in the activities of myosin,[8-10] other enzymes,[11] and antibody molecules,[12,13] as well as in the assembly of supramolecular structures such as viruses.[14]

The examples provided above (as well as many others which could be cited) demonstrate that a full understanding of biochemical phenomena will require detailed information on the dynamical properties of molecules that play a role in living systems. In this paper recent experimental and theoretical studies of the internal dynamics of proteins are reviewed; particular emphasis is devoted to the theoretical work, which is of recent origin and has not been dealt with previously. Theoretical studies of protein structure and dynamics have both "pragmatic" and "fundamental" goals. Among the "pragmatic" goals, one may include (1) development of methods for predicting the possible conformations of a protein in a given environment, the relative populations of these conformations, and the rates of transitions among them; and (2) development of methods for predicting the rates of biomolecular transformations (e.g., allosteric transitions, enzyme catalysis) and the changes in these rates resulting from structural differences (e.g., different substrates, enzymes with sequence alterations) or environ-

mental changes. Such goals are termed "pragmatic" in consequence of their predictive character and potential for future use in the design of enzymes and drugs.[15] The "fundamental" goal of theoretical studies of protein structure and dynamics is the development of a body of physical principles which accounts for the observed properties of these molecules. Such physical principles would provide a language for the description of the properties of proteins and a quantitative framework within which the results of different experimental studies could be rationalized. Progress has been made toward identifying the specific factors responsible for the stability of the native conformations of proteins, although a complete description is not yet available. These factors include covalent and hydrogen-bonding, salt-bridges and other charge interactions, and hydrophobic and atom-packing effects.[16-21] The characteristics of dynamical processes in proteins are determined by the forces resulting from these interactions; in some cases effective frictional forces can be introduced to simplify the dynamical problem.[22-26]

Experimental and theoretical studies are described in the second and third parts of this review, respectively. Comparison of the experimental and theoretical results indicates that there is much room for progress. There is a need for experimental techniques which can provide more detailed information on dynamical processes in proteins, i.e., on the magnitudes of displacement of the atoms and the correlations of these displacements in space and time. On the other hand, further development of the theoretical techniques is necessary before studies of complex biological processes will be possible. As will be described in the following sections, there have been a variety of recent developments in both the experimental and theoretical areas. For a few "elementary" processes, there is now some overlap between experimental and theoretical results on protein dynamics. These processes include the rotation of the rings in certain aromatic side chains, the passage of small ligands through particular steric bottlenecks, and the motion of globular domains in antibody molecules.

II. EXPERIMENTAL RESULTS

X-ray diffraction results for globular proteins have revealed that they have a precisely defined equilibrium structure. Nevertheless, a considerable body of experimental evidence accumulated over the past 20 years reveals that the native structure has considerable flexibility, which permits sizable internal motions in response to random thermal forces or to specific mechanical forces associated, for example, with ligand binding or crystallization. Several general reviews of this experimental work are available. A useful discussion of the types and time scales of dynamical events in proteins and related model systems has been presented by Careri et al.[20] In their review, particular emphasis is given to electrical charge fluctuations. Citri has reviewed experimental studies which demonstrate the existence and importance of enzyme flexibility.[3] Also, Weber has discussed the internal dynamics of globular proteins, with particular regard to the energetic cost of local structural fluctuations and their relation to ligand-binding.[27] Two other reviews which emphasize the experimental results have recently been published.[28,29] In addition to these general discussions, a number of reviews which focus on particular experimental approaches are available and are cited in the appropriate sections. Because of the availability of these reviews, we concentrate on recent work and mention only selected earlier studies which have served as prototypes for subsequent investigations.

A. X-ray Evidence of Protein Flexibility and Mobility

1. Structural Changes due to Changes in Surroundings

It is generally accepted that the native structure of a globular protein determined by X-ray diffraction of a particular crystal form is preserved when the environment is

altered by dissolving the protein or by crystallizing it in a different form. This is a consequence of the facts that protein crystals contain a large fraction (30 to 80%) of solvent, that only a small part of the protein surface is usually involved in contacts with other protein molecules in the crystal, and that the stabilization energies of protein crystals are rather low.[30] A variety of studies have revealed the not unexpected result that there is some accommodation of the protein structure to its environment. This does not, per se, provide evidence for motion but gives some indication of the flexibility of certain parts of protein molecules. A consideration of environment effects on side-chain potentials in proteins is given in the theoretical section. Already in the earliest high resolution protein crystal structure, that of myoglobin, there was an indication of protein flexibility. The three N and C terminal residues were not resolved, indicating that disorder was present in the crystal; a number of the polar side chains appeared to be disordered, as well.[31]

Tulinsky et al. compared the structures at 2.8 Å resolution of the two crystallographically independent molecules which occur in crystals of α-chymotrypsin.[32] Although the overall tertiary fold was the same, they noted significant structural differences in about one sixth of each molecule, particularly in the dimer interface region and at the surface of the dimer. Side chains had more variation than the backbone. Detailed examination showed that some of the structural differences (e.g., different values of the dihedral angle χ^1 for the Phe 39 residues of the two monomers) are due to van der Waals repulsion in the dimer interface; other differences may be due to attractive interactions (e.g., H-bond formation).

Structural differences due to different crystal environments have also been observed in two independent X-ray studies of hen egg-white lysozyme; the triclinic and tetragonal crystal forms show a root-mean-square (rms) deviation of backbone atoms equal to 0.5 Å, with the largest changes at the surface of the protein.[33,34] In most cases involving backbone atoms, the observed differences were local in character and could be related to packing interactions in the crystals.

Comparison of the structures at 2.0 Å resolution of the two symmetry-independent molecules which occur in crystals of ferricytochrome c, and of these structures with that of ferrocytochrome c, showed that all of these structures are very similar.[35] Many small differences were observed, however; mean differences in atomic position of the backbone atoms were 0.8 — 1.0 Å, and somewhat larger differences were observed in the side chains. It was noted by the authors that some of these differences may be artifacts of the maps and the model-building process, but certain side-chain differences are clearly due to crystal packing effects.

Small angle, low resolution X-ray scattering studies of globular proteins in solution have indicated that the overall solution structure is the same as in the crystal. Attempts to extend the solution results to higher resolution suggest that there may be small differences in the crystal and solution structures; such differences have been detected in hen egg-white lysozyme,[36] phage T4 lysozyme,[37] and sperm whale myoglobin.[38,39] The solution scattering data are difficult to interpret uniquely, but are consistent with a small opening of the substrate binding cleft in hen egg-white lysozyme (reversed by inhibitor binding) and displacement of the G and H helices in myoglobin.

2. Structural Changes due to Ligand Binding

Other evidence of protein flexibility comes from X-ray studies of protein structural changes resulting from the binding of ligands. These studies are of particular interest since they reveal structural changes which are associated with protein function. Comparative X-ray studies of liganded and unliganded forms have yielded detailed descriptions of such structural changes in a number of proteins.[3] Classical cases are the allosteric transition in hemoglobin[40] and the displacement of a tyrosine side chain (Tyr

248) on substrate binding in carboxypeptidase A.[41] The investigations of trypsin and trypsinogen reported by Huber et al. provide a more recent example.[42,43] These enzymes contain an "activation domain" which is coupled to a "specificity pocket" adjacent to the active site. In trypsinogen, the activation domain and the specificity pocket are structurally disordered; the static or dynamic disorder of the three external loops which comprise most of the activation domain is such that these loops are not clearly visible in the X-ray maps. Upon activation, the N-terminal Ile 16 of trypsin interacts with and stabilizes the activation region. Then, as a consequence of the coupling of the activation region and specificity pocket (together with the direct interaction of Ile 16 with Asp 194 of the pocket), the specificity pocket appears to adopt a more ordered conformation. A corresponding effect is observed on binding certain inhibitors such as the bovine trypsin inhibitor (BPTI); both the specificity pocket and the loops of the activation domain are more rigidly ordered than in the absence of inhibitor.

In some cases, it has been possible to perform X-ray studies on stabilized intermediates. Studies of this kind can help to define the pathways of structural displacements which result from ligand binding. One such X-ray analysis has been reported by Anderson,[44] who was able to block quaternary structural changes in deoxyhemoglobin crystals (by polymerization of acrylamide in the solvent-filled cavities) and thereby observe the tertiary structural displacements which result from oxidation of the iron. They presumably represent the first steps of what would lead to quaternary structure change in an unconstrained system (see below). Studies of intermediates stabilized by low temperatures can provide similar structural information.[45]

3. X-Ray Temperature Factors

In the refinement of X-ray data, parameters are introduced to take account of the thermal motion of the atoms. In principle, these parameters can be related to the magnitude of mean square thermal fluctuations of the individual atoms and, with highly refined data, to the anisotropy and anharmonicity of these fluctuations;[46] no time-dependence is obtained, however. For relatively small molecules, detailed results have been obtained and related to the internal motions,[47] although difficulties in interpretation have been found even in such relatively simple systems.[48] For proteins, analyses up to the present have been limited to the determination of isotropic temperature factors and it is realized that in all but the best structures, any direct attempts to relate their values to positional fluctuations are not warranted. The primary difficulties are that the accuracy of the temperature factors is low because of the limited data available, that the constraints used in the refinement process may lead to unrealistic results and that even if these problems are overcome, the number obtained represents the combined effect of static disorder and thermal motion. Recently, an attempt has been made to overcome some of these difficulties for myoglobin by doing high-resolution measurements at several temperatures and using Mossbauer data for the iron to separate a static disorder contribution.[49] The resulting thermal parameters indicate that there is a wide variation in the fluctuations of residues close to each other in space. Further, there is significant correlation between the magnitude of a fluctuation and its temperature dependence; that is, the large fluctuations are less sensitive to temperature, suggesting a square-well potential and/or a static disorder contribution. Also, the temperature factors in the interior of the protein tend to be smaller than those near the surface. The individual atom values can of course be compared with the results of dynamic simulations; one for myoglobin is in progress. The more general features of the experimental conclusions are compared with the theoretical results for the bovine pancreatic trypsin inhibitor (see Section III).

In another recent study, the temperature factors of the atoms in tetragonal crystals

of hen egg-white lysozyme have been analyzed.[223] No attempt was made to eliminate static disorder contributions. Since limited diffraction data were available, only two temperature factors were used for each residue: one for the main-chain and C^β atoms, the other for any side chain atoms beyond C^β. It was found that, as in myoglobin, the temperature factors in the interior of the protein tend to be smaller than those near the surface. The apparent motion could be analyzed as an overall rigid-body translational vibration (with mean square amplitude ≈ 0.1 Å^2), plus a motion which could be described either as a rigid-body libration or as an intramolecular motion in which the amplitude increases with distance from the protein centroid. In addition to this general motion, backbone loops and side chains at the protein surface showed apparent motion which correlated with the exposure of these groups to solvent. Little evidence for relative motion of the two globular domains of the protein was found in the crystal.

A subsequent analysis[234] is concerned with the temperature factors of various parts of the lysozyme molecule. The active site is found to be located in a region for which the temperature factors are large, and it is suggested that this is due to protein mobility which may play a role in the enzymatic activity.

B. Experimental Evidence of Internal Mobility

In the preceding section evidence was cited which shows that in spite of their well defined structure, globular proteins have considerable flexibility in that they undergo spontaneous fluctuations and they exhibit structural displacements in response to applied stresses. This naturally raises questions about the time-dependence of the fluctuations and responses, e.g., how rapidly do the structural displacements occur, how are the motions of different atoms correlated in space and time, and to what damping effects are these motions subject? It is known that the thermal energy in a small flexible system can lead to sizable structural fluctuations even in the absence of systematically applied stresses; indeed, statistical mechanics yields definite relationships between the responses of a system to small stresses and the random thermal fluctuations of the system.[50,51] Further, for such small systems the magnitudes of fluctuations in energy and volume of individual molecules should be relatively large.[23,52]

Cooper[52] has used thermodynamic fluctuation formulas to estimate the magnitudes of the fluctuations in the internal energy and volume of proteins from their heat capacities and bulk compressibilities, respectively. For a globular protein of mol wt 25,000 daltons, the predicted rms fluctuation of the internal energy is 38 kcal/mol and that of the volume is 30 cm^3/mol. If the volume fluctuation consisted of a uniform expansion and contraction of the protein, the corresponding fluctuation in the radius of the protein would be about 0.01 Å, compared with an average radius of about 20 Å. The thermodynamic approach does not allow one to infer the microscopic character of the fluctuations; e.g., the volume changes could equally well represent the formation in the protein of a cavity with a typical radius of about 2 Å, or more likely, a number of smaller cavities.[16]

In the remainder of this section we review some of the experimental findings on the internal dynamics of proteins. As will be seen, the evidence for the existence of dynamical processes is compelling and the time scales of many such processes have been determined. Their review here should motivate the interpretation of these data in terms of the detailed structure of proteins; some preliminary attempts are given in the theoretical section.

1. Vibrations

The vibrational motions of protein molecules span the frequency range from 3×10^{11} to 10^{14} sec^{-1} (periods from 10^{-14} to 3×10^{-12} sec). They can be probed by a variety of different spectroscopic methods including infrared absorption, Raman scattering,

and inelastic neutron scattering. To date, only Raman spectroscopy has been extensively applied to proteins. Several recent reviews of this work are available.[53-55,224] In general, these studies have shown that high frequency vibrations (which typically correspond to localized bond length, bond angle, and stiff dihedral angle deformations) are comparable to those found in smaller molecules (amino acids and peptides) with similar local structure. Small shifts in the frequencies in some of these vibrations (e.g., amide and disulfide bonds) are observed on changing the environment of proteins, that is, between crystal and solution or between the native and denatured molecules.[56,57]

Of interest as an example of the possibility of looking at side chain oscillations by inelastic neutron scattering is the study of poly-L-alanine with α-helical and β-sheet secondary structure.[58] The principal observed peak is at 230 cm^{-1} (1.5×10^{-13} sec) and was assigned to the torsional motion of the methyl side chain of alanine.

Relatively recently, very low frequency (<50 cm^{-1}) vibrations have been detected by Raman and infrared absorption spectroscopy in several proteins.[59-61,169] The exact nature of these motions is not known, although the low frequencies involved suggest that the motions have a large-scale or collective character. Possible assignments include vibrations of structural elements such as α-helices,[61,62] vibrations which involve most or all of the atoms in a protein,[23] or vibrations involving different protein molecules in a crystal.[60] The corresponding Raman bands disappear upon denaturation of the proteins,[59] and it has been noted that the band observed at 25cm^{-1} in lysozyme crystals does not appear in lysozyme solutions.[60] Two explanations of the latter result are possible. If the lysozyme vibration is a collective motion of different protein molecules, the Raman band would vanish upon destruction of the crystal lattice.[60] On the other hand, if the vibration is largely intramolecular, solvent damping effects could account for the altered spectrum in solution.[24,25] A theoretical analysis of one such low frequency motion, the hinge bending mode, is given below.

2. Fluorescence Depolarization

The relative motion of distinct, rigid domains in protein molecules has been detected by fluorescence depolarization[63] and other methods. Antibody molecules, which contain a number of such domains linked by flexible polypeptide segments, have been the object of several fluorescence depolarization studies; the results show that the domains in these molecules exhibit relative rotational motions on time scales of 10 to 50 nsec.[64-66] These internal motions of antibody molecules have been studied with similar results by measuring the nuclear magnetic relaxation of solvent protons induced by lanthanide ions bound to intact antibody and antibody fragments.[67] The time scales of these motions are consistent with a simple diffusional model for the relative motion of the antibody domains[68] (see Section III.C.4). Fluorescence depolarization studies have also shown that the S1 domains of myosin molecules have considerable freedom of motion relative to the long tails or stems of these molecules.[8] Flexibility in the S2 region of myosin has also been detected by fluorescence depolarization[9] and electro-optic techniques.[10]

Synchrotron radiation has recently been used in fluorescence depolarization studies of the motion of tryptophan side chains in proteins.[69] Short high intensity polarized pulses of radiation (width at half height of 0.65 nsec) at 300 nm were employed for excitation and it was shown that rotational correlation times as short as 200 psec could be measured from the decay in the anisotropy in the fluorescence. A number of proteins, each containing only a single tryptophan, were investigated. In some cases (e.g., staphylococcus aureus nuclease B), the tryptophan behaved as if it were rigidly bound so that the observed correlation time (9.9 nsec) corresponded to that for reorientation of the protein as a whole. In others (e.g., serum albumin at 45°C, azurin) the trypto-

phan appeared to have large amplitude motions (semi-angle of 34°) with shorter rotational correlation times; values of 0.1 to 0.5 nsec fitted the depolarization data. For azurin, the crystal structure shows that the tryptophan is located in the hydrophobic interior of the protein. Such motional behavior for a tryptophan residue, which due to its large size and hydrogen bonding groups is expected to be one of the most rigidly bound amino-acid side chains, suggests that even shorter relaxation times may occur for other side chains. Tyrosines, for which calculated values exist (see below) would also be of interest for investigation. For this residue there are two important transitions, L_a parallel to the symmetry axis and L_b perpendicular to the symmetry axis of the benzene ring,[70] so that some care in the analysis will be required.

An alternative approach to measuring rapid reorientation of tryptophan side chains by fluorescence depolarization makes use of quenching by oxygen (see below) to shorten the excited state lifetime from its normal value.[71-73] In this way an upper limit is obtained for the time during which depolarization can have occurred. The results for a variety of proteins yield reorientation angles up to ~30° in times as short as 2 nsec, demonstrating that the rotational correlation times for certain tryptophans are significantly shorter than that for the protein as a whole.

3. Fluorescence and Phosphorescence Quenching

Fluorescence quenching studies of tryptophan residues by small molecules have been interpreted as providing evidence for widespread structural fluctuations in proteins. It is observed, as mentioned above, that molecules such as O_2 quench the optically excited state of tryptophan so as to reduce the fluorescence intensity relative to the unquenched system. Quenching effectively occurs instantaneously and with 100% efficiency upon collision of the quenching agent with the excited fluorophore. Since the fluorescence lifetimes of these groups are typically on the order of 1 to 10 nsec, a high collision rate is required. On the hypothesis that the tryptophans are located in the densely packed interior of the globular proteins studied, the apparent high mobility of the quenching molecules appears to reflect rapid structural fluctuations of the protein (but see below).

Lakowicz and Weber studied the quenching of tryptophan fluorescence by oxygen in 14 proteins.[74] No tryptophan residues were found to be inaccessible to oxygen. In particular, proteins with emission spectral maxima at relatively short wavelengths that correspond to tryptophan residues mainly buried in nonpolar regions of the protein interior did not show significantly less evidence of quenching than proteins which emit at longer wavelengths. Further, it was demonstrated that complexes between O_2 and tryptophan were not involved in the quenching, although the possibility that oxygen is concentrated in the protein has not been excluded. Interpretation of the dynamic quenching due to collision between the oxygen and the tryptophan yielded an effective diffusion constant for oxygen in the interior of proteins 20 to 50% of that for oxygen in water. Although no temperature dependence was measured, the observed relative rates make it unlikely that the effective activation energy for the diffusion process in the protein is much larger than that observed for tryptophan quenching in H_2O (3.1 ± 0.9 kcal/mol), a value corresponding to the activation energy for oxygen diffusion.[74]

Because the tryptophans in the excited state have a different (generally larger) dipole moment that in the ground state, the reorientation of the polar groups in the environment stabilizes the excited state and results in a redshift of the fluorescence if the polar group relaxation times are on the order of nanoseconds.[74] Such relaxation processes following tryptophan excitation have also been studied by Grinvald and Steinberg.[75]

Eftink and Ghiron have studied the quenching of tryptophan fluorescence by acrylamide, which is larger and more polar than oxygen.[76,77] Again, the quenching rates correspond to effective diffusion constants that range up to 50% of that for acrylamide

in water; as in the oxygen case, the effect on these rates of solvent accessible surface area of the tryptophans has to be considered. Unlike the case of oxygen quenching, however, proteins were found to contain tryptophan residues which are shielded from collisions with acrylamide. By studying the temperature dependence of the quenching of the buried tryptophan in RNase T_1, it was found that the Arrhenius activation energy for acrylamide quenching of this fluorophore was about 9 kcal/mole, or approximately twice as large as the activation energy for diffusion in water.[76] More detailed studies suggested that the penetration of acrylamide to different tryptophan fluorophores may be facilitated by protein fluctuations.[77] The rate constants for the quenching reactions in aldolase and RNase T_1 were found to represent balances between large activation energies (11.0 and 9.0 kcal/mol, respectively) and large activation entropies (14.5 and 10.0 e.u., respectively), while the rate constants for the quenching reactions in human serum albumin under low and high (0.2 *M* KCl) ionic strength conditions at pH 5.5 were found to represent balances between small activation energies (2.7 and 2.1 kcal/mol, respectively) and negative activation entropies (−9.5 and −11.0 e.u., respectively). The interpretation of this approximate energy/entropy compensation given by the authors is that access of acrylamide to the tryptophan in RNase T_1 and aldolase is facilitated by fluctuations in which favorable interactions within the protein (or protein plus solvent) are broken, with a resulting increase in structural disorder, softening of vibrations, etc. For human serum albumin (under the experimental conditions) quenching by acrylamide appears to be facilitated by fluctuations of a specific character, such as the formation of well-ordered channels. In another set of experiments, Eftink and Ghiron found that the binding to trypsin of inhibitors of widely varying size resulted in similar decreases of fluorescence quenching. These results were interpreted as evidence for a tightening of the protein structure upon ligand binding.[78] Saviotti and Galley have studied the quenching of tryptophan phosphorescence by oxygen.[79] No phosphorescence was observed in most of the proteins examined, a result which would be expected given the long lifetimes of tryptophan triplet states (>0.1 sec) and the oxygen quenching rates found by Lakowicz and Weber for the interior of proteins. Two proteins were observed to contain tryptophan residues which are sufficiently inaccessible to oxygen that phosphorescence is observed at room temperature. The triplet lifetime of the buried tryptophan in one of these proteins (liver alcohol dehydrogenase, LADH) decreased at high oxygen concentrations. This result was interpreted in terms of a slow opening-closing conformational fluctuation which periodically makes the tryptophan available for quenching. Of the two tryptophans in LADH, one is on the surface and the other is "buried" at the dimer interface. Transient exposure of the latter is probably observed in these experiments; this could be confirmed by studies of the dissociated species.

The relative uniformity and high rate of tryptophan quenching in proteins contrast sharply with the oxygen binding data for myoglobin and the hydrogen exchange results described below; the latter involve rates for a given protein that vary over 5 or more orders of magnitude and have activation energies that range from 10 to over 60 kcal/mole. Part of the difference in the two phenomena may be a consequence of the large size of the tryptophan residue and the corresponding high efficiency of the quenching process. However, it should also be noted that in many cases tryptophan side chains which are well buried by the usual criteria are actually very close to or in contact with the surface of the protein. In lysozyme, for example, four out of the five tryptophans are partly exposed to solvent by the Lee and Richards criterion,[80,235] and the fifth (Trp 28) is not far from the surface and is close to Trp 108. Thus, it is not clear, at least in some cases, whether the properties of the protein interior are being probed by the quenching studies.

4. Hydrogen Exchange

Historically, hydrogen exchange experiments provided some of the first results that were interpreted as evidence of substantial conformational fluctuations in proteins;[81] the subject has been reviewed recently by Englander et al.[82] and by Woodward and Hilton.[83] Exchange occurs when one isotope of hydrogen bound to an O, N or S atom of a protein is replaced by another isotope of hydrogen from the solvent water. Almost all of the experiments that have been analyzed in detail have been concerned with the exchange of hydrogens bonded to nitrogens and among those, mainly to the peptide nitrogens. The rate of exchange of a given protein hydrogen, relative to that of the hydrogen in the same amino acid incorporated into a small peptide, depends on a variety of factors, one of which is the accessibility to the solvent. For hydrogens which are normally well buried in the protein, particularly peptide hydrogens involved in backbone-backbone hydrogen bonds, a factor in the exchange must be structural fluctuations which make it possible for H_2O, OH^-, and/or H_3O^+ to penetrate to the exchange site. To attempt to obtain more specific information about the nature and frequency of these fluctuations, it is useful to summarize certain results that are emerging from the large number of experimental studies that have been made. Of most interest is the fact that at room temperature (25°C) while the rates for backbone NH groups in model "random-coil" peptides range over two orders of magnitude (1 to 10^{-2}min^{-1}) for the different amino acids, those for peptide hydrogens inside proteins range over eight orders of magnitude or more (1 to 10^{-8}min^{-1}). It is generally found that the exchange in proteins, as for free peptides, is both acid and base catalyzed, with a rate expression of the form

$$k_{exch} = k_H(H^+)^x + k_{OH}(OH^-)^y + k_{H_2O}$$

where k_H, k_{OH}, and k_{H2O} are effective rate constants, (H^+) and (OH^-) are the bulk solvent concentrations of these species, and x and y are constant exponents; x and y are equal to one for peptides but are found to range between 0.4 and 0.85 for native proteins.[83] It is becoming clear, in contrast to earlier analyses, that even for temperatures low enough that overall denaturation cannot contribute significantly to the exchange rate, the rates for proteins do have a significant temperature dependence beyond that found for peptides; the latter have hydrogen exchange activation energies equal to about 20 kcal/mol. It appears that the activation energies for exchange in proteins vary sufficiently that, to a first approximation, the wide range of observed exchange rates can be understood in terms of an Arrhenius expression with variable activation energy and an approximately constant pre-exponential factor; a factor of 10^{-8} in rate corresponds to an increase of only 11 kcal/mol in the activation energy, while a variation of over 20 kcal/mol has been observed.[227]

To use these general results and the many specific data available to infer something concerning mobility in proteins, it is necessary to have a detailed mechanism for the exchange phenomenon. Neither global unfolding nor large-scale structural changes can be responsible for the accessibility required for exchange, except at high temperatures or pH conditions near denaturation. Information concerning this point has been reviewed by Woodward and Hilton;[83] of particular importance in this regard are the myoglobin crystal and the trypsin-inhibitor complex exchange results,[84,85] as well as the large variation in the behavior of different protons in a given protein. Exclusion of total unfolding as the mechanism has reduced the published discussions to two primary alternatives: the first assumes that the protein unfolds locally so as to expose the exchanging hydrogen to solvent and the second assumes that the solvent penetrates into the protein to the site of the exchanging hydrogen. Both mechanisms require structural fluctuations but they are different in character. The local unfolding corresponds

essentially to a denaturation step, in which part of the polypeptide protrudes into the solvent, while a penetration model requires large enough fluctuations to allow solvent molecules to enter the protein.

Englander and co-workers have carried out extensive hydrogen exchange studies on hemoglobin and interpreted them by the local unfolding mechanism.[86] By incubating unlabeled hemoglobin in tritiated water for varying lengths of time, kinetically distinct classes of hydrogens are exchanged to different extents ("limited exchange-in"); these classes were then distinguished by following the exchange-out behavior of the tritium. The authors suggest that each exchanging class can be identified with a particular polypeptide segment which suffers occasional, cooperative local denaturation. Although this type of result is suggestive of local denaturation, the interpretation is not unequivocal. In particular, there is no evidence that the groups of protons with similar exchange characteristics come from a given region in the protein molecule. Furthermore, even if there were, the fluctuations that make it possible for solvent molecules to enter a given protein region might result in similar behavior without local folding. Also, the pH dependence ($x,y < 1$) suggests that the environment where hydrogen exchange is taking place deviates significantly from bulk solvent. As to the wide variation in rates and their correlation with activation energies, they both could be consequences of the local denaturation model; the observed excess energy would be that required to denature a given region. Within the context of the local denaturation model, the co-locality interpretation of the kinetic classes would appear tenable only if the exchange rate were limited by the frequency of local denaturation ("EX_1" mechanism). In this case, the pH dependence ($x,y < 1$) could result from the titration of groups involved in the local denaturation step. In the opposite case of rapid local denaturation ("EX_2"), which has customarily been assumed,[86] the observed exchange rates would be proportional to the intrinsic chemical exchange rates of the various residues; the latter rates, as mentioned before, vary over about two orders of magnitude. Within the context of the penetration exchange model, a co-locality interpretation could be tenable if the exchange rate were limited by a small activated diffusion rate of the exchanging species through the protein to the region under consideration.

The penetration exchange model has two limiting cases, both of which would yield a pH dependence different from the bulk solvent. The first considers that the solvent molecule or ion reaches the exchange site by jump diffusion in a manner analogous to that in a solid; that is, by following a complicated path through cavities that exist in the protein. The observed range in the rates would then result from the variation in the number of steps required to reach the internal site from the surface of the protein. With reasonable assumptions concerning the required cavity size, the number of such static cavities and the individual jump rate, it can be shown (S. Swaminathan and M. Karplus, unpublished; Reference 87) that the wide range of observed rates of hydrogen exchange cannot be explained. Further, in an Arrhenius or Kramers type interpretation of such jump diffusion, the rate variation appears mainly in the pre-exponential factor with an essentially constant activation energy (that associated with a single jump). The latter is inconsistent with the recent measurements on the variation in activation energies described above.

The second type of penetration model emphasizes the dynamics of the protein and assumes that the diffusion occurs due to fluctuations that appear and disappear on a fast time scale. This part of the process is analogous to diffusion in liquids and is expected to yield a rate that decreases slowly with increasing size of the protein. The rate-limiting step is assumed to involve a fluctuation in the structure of the protein; this could be a fluctuation that breaks a certain number of hydrogen bonds or creates a cavity of a certain size. The required cavity size could be determined by that needed for one or more water molecules in the neighborhood of the exchanging hydrogen or,

alternatively, for a chain of water molecules reaching from the external solvent to the site of the exchanging hydrogen. It is this step which could yield a wide range of activation energies because hydrogen bond breaking and/or cavity formation depends on the details of the local structure of the protein. An analogy is found in the tyrosine side-chain flips in the pancreatic trypsin inhibitor, where the activation energies range from 10 to 35 kcal/mol (see Section II.B.5).

From the above discussion, the penetration model with a rate-limiting fluctuation step is consistent with the general character of the hydrogen exchange kinetics. It has elements in common with the local denaturation model. In fact, the difference between the two mechanisms is a matter of degree; that is, it depends on the magnitude of the required fluctuation and on the extent to which it exposes the exchange site directly to the solvent environment. In certain cases, the denaturation description may be a valid approximation, but in the interior of some proteins it is likely that penetration of a limited number of solvent molecules is the essential element. More generally, it may be expected that the exchange process will involve a superposition of kinetic mechanisms, associated with the range of conformations that contribute to the native protein structure at ordinary temperatures.[88,89]

A complete analysis of the hydrogen exchange process will require identification of the individual protons and measurements of their kinetic parameters in a protein of known structure and fluctuation properties. Recently, the exchange rates of the slowest-exchanging peptide hydrogens in the bovine pancreatic trypsin inhibitor (BPTI) and related proteins have been determined by NMR over wide ranges of pH and temperature.[90,91,103,225-227] The resonances associated with at least 7 of the 11 identifiable slowest-exchanging hydrogens have been assigned to particular residues;[228] these are all in the buried β-sheet region of the protein, where the smallest motions are observed in dynamical simulations of BPTI.[88] The exchange rates of these slowest-exchanging peptide hydrogens exhibit different pH and temperature dependences, although the rates tend to converge to similar values at elevated temperatures.[225,227]

Analysis of these results has led to somewhat different mechanistic proposals by Wuthrich et al.[225] and Hilton and Woodward.[227] Wüthrich et al. propose that exchange occurs via a "local" EX_2 mechanism; i.e., the exchange rate is influenced by the frequency of local conformational fluctuations.[225] Moreover, they found that the exchange rates at pH 3 to 5 correlate with thermal stability in a series of proteins closely related to BPTI, so that the rate-limiting conformational fluctuations required for exchange in this pH region appear to be related to conformational transitions involved in protein denaturation.[226]

Hilton and Woodward note that the pH and temperature dependences of the exchange rates themselves depend on experimental conditions.[227] At sufficiently high temperature or sufficiently low pH, exchange occurs via a process with relatively high activation energy; this appears to be the process described by Wüthrich et al. At lower temperature or higher pH, however, the exchange rates are associated with a range of activation energies. This process is suggested to be of the penetration-exchange type. It is to be hoped that such specific data, when combined with detailed calculations of local structural fluctuations in BPTI (see below) will provide a satisfactory picture of hydrogen exchange, at least for this protein.

In addition to the intrinsic interest of hydrogen exchange, it can be used as a diagnostic tool for changes in the structure or other properties of a protein. One type of study is that of unliganded and liganded hemoglobin.[86] There are differences in the hydrogen exchange behavior that depend on the ligation of the protein used in the exchange experiments. It appears to be possible to identify particular classes of hydrogens which have different exchange properties in the quaternary structures corresponding to the liganded (oxy) or unliganded (deoxy) hemoglobin. In this way, several li-

gand-responsive classes, each containing 3 to 9 hydrogens per hemoglobin subunit, have been distinguished. The ligand-responsive classes are assumed to correspond to segments which have different equilibrium constants for local denaturation in the liganded and unliganded state, although other interpretation of this data may be possible.[83,92] These changes in equilibrium constants can be calculated from the kinetic data if the exchange rates are limited by the fraction of the time a segment is in the locally unfolded conformation; equivalently, the changes can be related to differences in the free energy of denaturation of the segments in the liganded and unliganded forms of hemoglobin. Differences ranging from 1.5 to about 4 kcal/mol have been found, with the deoxy form the more stable one in each case.[86] It has not been possible to identify the particular polypeptide segments which are presumed to correspond to the various kinetic classes observed in the hydrogen exchange experiments. Nor has it been possible to determine whether the ligand-induced destabilization occurs simultaneously or in a particular order for these segments. Hydrogen exchange studies of hemoglobins with mutations or chemically induced structural alterations should be helpful in clarifying these important questions. However, as in the pancreatic trypsin inhibitor, identification of specific hydrogens is required to draw unequivocal conclusions from the exchange data.

The effects of intersubunit contacts and of pH changes on the hydrogen exchange behavior of hemoglobin have been studied by Abaturov et al.[93] These authors find that the formation of native oxy-hemoglobin from separated oxy-α and oxy-β subunits results in a reduced hydrogen exchange rate relative to that of the isolated subunits, while the exchange behavior of oxy-hemoglobin appears to be similar to that of met-myoglobin. Moreover, they find that the hydrogen exchange of oxy-hemoglobin is sensitive to changes of pH in a range (pH 7 to 8) where physiologically significant pH effects on hemoglobin oxygen binding properties are observed; neither the hydrogen exchange nor ligand binding properties of isolated α chains show such effects. A substantial increase in exchange rate is observed upon removal of the heme group (i.e., in $\alpha\beta$ globin dimers). Another study is that of Wickett et al. of lysozyme;[94] they found that the binding of inhibitors induced a general reduction in the proton exchange rate, which was interpreted in terms of a widespread tightening of the structure of the protein. Similar results have been found in other systems, though great care is required to separate the effect of a large change in the rates of a few protons from a small change in the rates of many protons.

The hydrogen exchange properties of the soybean trypsin inhibitor-trypsin complex show that protons in the protein interface exchange with solvent with a pH and temperature dependence characteristic of the interior of a globular protein;[85] this complements the static structural similarities reported by Chothia and Janin.[95] It is suggested in that study, as pointed out above, that the dynamical processes that expose interfacial protons to solvent species cannot include localized reversible unfolding.[85]

5. Nuclear Magnetic Resonance

The most detailed experimental data on protein internal motions have been provided by NMR studies, particularly using ^{1}H and ^{13}C resonances, though other nuclei have been studied as well. The utility of the measurements is largely a consequence of recent advances in assignment techniques, which allow the association of particular resonances with particular atoms of a protein. Consequently, dynamical information obtained from NMR spectra can often be interpreted in specific structural terms. Several recent reviews of this work are available.[28,96-99,229]

For native proteins in solution, NMR studies indicate that side chains exhibit more sizable fluctuations than the polypeptide backbone, and surface groups often exhibit substantially more mobility than interior groups. In some cases, regions of proteins

which appear to undergo sizable fluctuations in NMR studies show evidence of disorder in the X-ray structure. In other cases, such disorder has not been seen in the X-ray structure; this could be due to the character of the motion (e.g., transitions between symmetrical states) or to crystal packing effects.[98] NMR has also been useful in helping to characterize the structural and dynamic properties of particularly flexible proteins that do not have a unique conformation in solution; two such proteins that have been studied are insulin[96] and glucagon.[100,231]

The measurements used include chemical shifts, coupling constants, longitudinal (T_1) and transverse (T_2) relaxation times, nuclear Overhauser enhancements (NOE), and lanthanide-induced shifts and relaxation. From data for a number of different identified protons and comparisons of the results calculated from the X-ray structure with the observed values (i.e., ring current shifts, lanthanide broadening and shifts), it has been concluded in a detailed study of lysozyme that the overall structure of the protein in solution is closely similar to that in the crystal.[101,102] Similar conclusions have been drawn for the bovine pancreatic inhibitor.[103] Some caution in evaluating these results is appropriate since the dominant effects on the spectrum require only local order, and it is possible that structural changes that shift one part of the protein relative to the other would be more difficult to determine from the NMR results. As to motions, the relaxation data for protons suggest a high degree of local internal mobility for main- and side-chain atoms on the nanosecond time scale,[98] but it is carbon 13 results which are likely to be most useful in this regard. ^{13}C NMR measurements, including T_1, T_2, and NOE studies, can be interpreted so as to provide detailed information concerning the motional properties of individual atoms. The essential element that makes possible such an analysis in the ^{13}C case is that the dominant interaction involved in most cases is the dipole-dipole coupling between the carbon nucleus of interest and the proton(s) bonded to it.[232] Thus it is the motion of the CH bonds that are being probed by the measurement. This contrasts with the result for protons, where the interactions (usually proton-proton) between nonbonded neighbors are most important. Because of the wider range of contributions in the latter plus the complication of cross-correlation effects,[104] the interpretation of the results is more difficult, though potentially also of great interest. As to the ^{13}C data, primary focus has been on α-carbons and on the methylene and methyl groups of amino acid side chains. The values of T_1, T_2, and NOE are affected by motions on the micro- to picosecond time scale, so that in addition to the overall protein tumbling (rotational correlation times of 10^{-8} to 10^{-9}sec for the proteins studied), local fluctuations can make an important contribution. The α carbon results indicate that the motional freedom that must exist (see Section III) does not lead to significant contributions to ^{13}C relaxation; i.e., the observed correlation times correspond to those of the protein as a whole,[105] although internal librational motion may influence the results somewhat.[106] By contrast, for the methylene groups in both aliphatic (e.g., isoleucine) and charged (e.g., lysine) side chains, the data clearly demonstrate the importance of additional motional freedom. Methyl groups have rotational correlation times on the order of 3 to 10 psec, suggesting that the surrounding protein medium has little effect on their motion.[107] Further, the results for methyl and methylene groups show that there is additional motional freedom in the side chains associated with oscillations around C–C bonds. Detailed results are available on side chains in myoglobin[110] and collagen.[233] Phenomenological models of the restricted diffusion and jump type[108,109] have been used to analyze the data. They suggest that large oscillations occur about the C–C bonds on the 10 psec time scale for residues in the interior of proteins. Because the analysis requires a number of assumptions to translate the NMR data into motional parameters, theoretical information on the detailed character of the side chain motions will be necessary to quantify the results.

The most fully characterized motions revealed by NMR studies are side chain conformational transitions, particularly the 180° rotations about $C^{\beta} - C^{\gamma}$ of tyrosine and phenylalanine rings. In a number of proteins tyrosine and phenylalanine rings have been shown to rotate with rates $> 10^4$ sec^{-1}, even when the residues are buried in the protein interior;[102] there are a few cases where evidence for slower reorientation rates has been found.[111,112] It has been possible to extract definite rate constants and estimates of the enthalpy and entropy of activation for aromatic ring rotation.[112,113] The enthalpies of activation measured to date range downward from 40 kcal/mol and the entropies of activation range downward from 70 e.u.; larger entropies of activation are associated with larger enthalpies of activation. The observed activation enthalpies are in reasonable agreement with theoretical calculations, as described in Section III A.1. Other side chains (e.g., valine, methionine) also appear to enjoy substantial rotational mobility,[98,107,109] while side-chain rotations which would involve greater disturbances of the surrounding protein matrix occur relatively infrequently. An example is the rotation of tyrosine side chains around $C^{\alpha} - C^{\beta}$.[114] It will be of interest to compare these results with the fluorescence depolarization measurements described above.

NMR studies, particularly proton NMR measurements, have also provided information on changes in the structure and dynamical fluctuations of proteins produced by changes in solution conditions (temperature, ionic strength) and ligand binding. Lysozyme has been the subject of extensive studies of this kind.[98] The binding of inhibitors to this enzyme appears to restrict the conformational mobility of residues not only at the active site, but also in other parts of the protein. The extent of structural displacements which accompany side-chain ionizations, inhibitor binding, and chemical modifications near the active site have also been determined by NMR. The displacements observed in NMR studies of lysozyme in solution sometimes appear to propagate further into the protein than do displacements observed in X-ray crystallographic studies. A displacement of Trp 108 induced by chemical modification results in displacements of groups up to 15 Å away, apparently as a result of successive contacts involving several intervening hydrophobic residues. Estimates or bounds on the rates of some structural transitions induced in lysozyme by ligand binding have also been obtained.

Evidence for long-range structural displacements resulting from ligand binding in myoglobin,[107] and for long-range alterations of residue mobility resulting from calcium ion binding in muscle calcium binding parvalbumin[115] have also been obtained by NMR methods.

6. Photochemical Probes

a. Dynamics of Heme Proteins

Rapid conformational changes and relaxation processes in proteins have been explored in a number of recent photoexcitation experiments. Perhaps the most detailed results to date are those which have been obtained in studies of the kinetics of carbon monoxide and oxygen rebinding in heme proteins following flash-photolysis;[116,117] a summary of this work has been presented by Eisenstein.[118] These studies indicate that a ligand (such as carbon monoxide) must cross several energy barriers in the course of moving from solvent to the binding site inside a protein. On the assumption of a simple Arrhenius type model with constant pre-exponential factors and activation energies, the data obtained over a very wide temperature range (40 to 340 K) have yielded the activation enthalpies and entropies for the individual barriers. The outermost barrier was originally associated with penetration through the solvent-protein interface;[116] more recently[230] it has been suggested that the interface offers no appreciable barrier and that the observed activation energy is simply that associated with diffusion through the solvent. The innermost barrier arises from properties of the heme group itself, as

modified by the globin surroundings. Both of these barriers are observed in rebinding to isolated heme groups in solution as well as in rebinding to heme proteins; for the proteins, however, at least one or two additional barriers due to the globin chain are found.

While the origin of these barriers is not yet known in detail, several important results have been reported. The effects of changing solvent viscosity upon the rate constants are markedly attenuated for the internal barriers. The internal barriers observed at room temperature appear to represent averages of rapidly interconverting ($\sim 10^5$ sec^{-1} at 300 K) locally different conformations. At low temperatures the rate of transition among these conformations becomes slow on the time scale of the rebinding reaction. A distribution of activation enthalpies for the innermost barrier is then obtained and interpreted as corresponding to the various conformations that are prevented from interconverting by the low temperatures. A similar freezing of conformational fluctuations is observed when the protein is embedded in a solid matrix. Finally, the sensitivity of the activation enthalpy spectrum of the innermost barrier and of the activation enthalpies and entropies of the other internal barriers to the details of the polypeptide chain conformation have been demonstrated by the study of different heme proteins and by chemical modification studies. Comparisons of the activation enthalpies and entropies of the innermost barrier in myoglobin and in modified and unmodified free subunits of hemoglobin has led to a tentative identification of the states separated by this barrier; it appears that in one state the ligand is bound to the heme group, while in the other the ligand is confined to the hydrophobic pocket formed by the heme group and the surrounding globin.[117] With this identification, the more negative ligand-binding activation entropy of α Hb chains compared to β Hb chains is related to the larger volume of the pocket in the αHb chains. Moreover, the substantially greater ligand-binding activation enthalpy of myoglobin compared to the Hb chains is consistent with a variety of experimental results, which suggest that myoglobin has a more rigid polypeptide cage surrounding the heme. Variations in the activation enthalpies of other barriers in the different proteins can also be rationalized qualitatively in terms of globin rigidity.[117] Comparative studies of some of the many mutant hemoglobins and of other modified heme proteins may help to provide a more detailed description of these barriers in terms of protein structure.

The time scales of the events which lead to quaternary structure changes in hemoglobin following ligand photodissociation have been examined by several different groups. Shank et al. found that CO is dissociated from the heme group within 2 psec after photoexcitation, but that O_2 appears to be dissociated more slowly.[119] More recent studies, however, indicate that CO dissociation takes place in about 11 psec.[120] The latter studies also indicate that CO recombination occurs with an apparent first order rate constant of $5 \times 10^3 s^{-1}$ in the incompletely dissociated species $Hb_4(CO)_3$ and $Hb_4(CO)_2$ (which may retain the oxy quaternary structure); recombination in more highly dissociated species occurs more slowly, probably reflecting conformational changes in the globin. After photodissociation of CO, time-resolved resonance Raman spectroscopy indicates that the heme group changes from its characteristic liganded conformation to its unliganded conformation in a time less than 7 nsec.[121,122] This time is substantially shorter than the 40 to 90 nsec required for substantial adjustment of the tertiary structure of the surrounding globin chain.[123] The overall quaternary structure change occurs on a much longer time scale still, requiring approximately 1 msec for the R (oxy) to T (deoxy) transition after CO photodissociation;[124] the reverse transition (T to R) requires a few tenths of a msec.[124]

The separation of the time scales for the heme and tertiary structural changes is consistent with the suggestion that the alteration in heme conformation on ligand binding triggers subsequent protein structure changes through local nonbonded atom inter-

actions.[125] In the present studies that involve the inverse process (ligand dissociation), it appears that removal of the ligand and the resulting doming of the heme leaves the globin in a strained, metastable conformation that exists for 40 to 90 nsec; during this time, the protein presumably must cross certain energy barriers and/or sample a variety of slightly different conformations (i.e., undergo processes whose rates are slowed by activation enthalpies and/or activation entropies) before the globin reaches the deoxy tertiary structure. The separation of the time scales for the tertiary and quaternary structure changes suggests that the rate of the quaternary structure change is also limited by activated processes.

Picosecond spectroscopic studies of heme proteins have been undertaken to study the nonradiative relaxation of the electronically excited heme group.[126] The results reported to date show that this relaxation is much faster when the metal atom (Fe^{2+} or Fe^{3+}) is bound to the heme; the possible influence of the protein itself on heme relaxation remains to be determined.

b. Dynamics of Rhodopsin

Another system which has been extensively studied in photoexcitation experiments is the visual pigment rhodopsin.[127,128] It is known that the chromophore retinal is bound to opsin as a Schiff base in the 11-cis form and ends up as all-trans after photobleaching. The geometric change in the chromophore is presumed to initiate alterations in the conformation of the opsin. Consequently, the conformational flexibility of this protein and its time scales are intimately involved in the visual process. To date, little is known about the structure of opsin and almost all the studies have concentrated on the chromophore. It has been shown[129] that a variety of modified forms of retinal (e.g., different number and positions of methyl groups) can bind to opsin and yield a photobleachable pigment. This may suggest that the binding pocket is rather open and flexible. Recent detailed studies have attempted to characterize the first metastable intermediate (bathorhodopsin or prelumirhodopsin) observed after excitation.[130,131] It has been shown to appear in less than 6 psec at room temperature after photoexcitation, but there is disagreement about whether the intermediate results from a cis-trans isomerization of the retinal chromophore in rhodopsin or from a less extensive chemical change of the chromophore, such as proton translocation.[130,131] Green et al.[130] have shown that bathorhodopsin is formed within a few psec after photoexcitation of either rhodopsin (containing 11-cis retinal) or isorhodopsin (containing 9-cis retinal) at room temperature and concluded that isomerization must occur. Also, it has been shown by resonance Raman spectroscopy that isorhodopsin forms from rhodopsin at 4 K.[132] This result must be interpreted with care since local heating may occur as a result of the photoexcitation; Green et al.[130] also suggest that the isomerized (bathorhodopsin) chromophore is essentially an all-trans retinal Schiff base. It appears that the protein-chromophore interactions must accelerate isomerization, since the analogous isomerization occurs on a nanosecond time scale in the absence of protein;[130,133-135] part of this difference may involve solvent damping of the free chromophore motion in solution. Peters et al.[131] have studied the kinetics of bathorhodopsin formation in low temperature glasses, where the reaction is slow enough to monitor in some detail. From the rapidity of the formation at low temperatures (36 psec at 4 K), the non-Arrhenius variation of the rate with temperature (interpreted as evidence for hydrogen tunneling), and a substantial isotope effect in deuterium-exchanged rhodopsin, these authors conclude that bathorhodopsin is formed by translocation of a proton to the Schiff base nitrogen of the retinal chromophore. Although proton translocation involving the protein may be part of the formation of the observed intermediate, it is not clear from these experiments that cis-trans isomerization is excluded.

The above discussion shows that the understanding of the changes that occur in

rhodopsin on photoexcitation is still in its infancy. The system has been discussed in the present review because protein-chromophore interactions and dynamical phenomena must play an essential role.

7. Other Methods

In some proteins, reactive groups may be transiently exposed to reagents in the solvent by structural fluctuations of the protein; it may then be possible to determine the rate constants characterizing the fluctuation by following the reaction kinetics. Vas and Boross have used this method to study the transient exposure of cys-153 in D-glyceraldehyde-3-phosphate dehydrogenase; this fluctuation occurs on a time scale of seconds.[136]

Chemical relaxation methods (e.g., measurement of spectral changes after a temperature jump) have been used to determine the kinetics of ligand binding and subsequent protein structural changes in a number of systems.[137,138] The flash and modulated photodissociation studies of CO binding to myoglobin and hemoglobin which were mentioned above are other examples of this general method. Temperature-jump studies of the binding of hapten to an immunoglobulin of the A class have yielded evidence for allosteric transitions on the millisecond time scale in this protein.[139]

III. THEORETICAL RESULTS

To develop a detailed theoretical description of the internal dynamics of a polypeptide or protein, it is necessary to have a model potential energy expression from which the forces acting on the atoms of the protein can be calculated as a function of the molecular conformation. In principle, the protein and its surroundings (solvent or crystal) should be treated on the same footing; in practice, detailed calculations on the protein alone are sufficiently difficult that the surroundings have been neglected or treated by continuum approximations in most of the theoretical studies reported thus far.

Several different approaches have been taken in the dynamical studies. In the harmonic vibration approach, it is assumed that the displacements of the atoms are of sufficiently small amplitude that the system remains in the quadratic part of a given potential energy well. This approach has the advantage that once the normal vibrational modes of the molecules are found, many structural and dynamical properties can be obtained in a straightforward manner by analytic calculations. In particular, the motion of the system is known for all time if the initial conditions are specified, and statistical characteristics of the system are given in terms of Gaussian distributions. The disadvantages of the harmonic vibration treatment are the difficulty of finding the normal modes of a large, unsymmetrical molecule and the implied assumption that anharmonic effects are not important in the motion.

In the molecular dynamics approach, one allows the protein to evolve from some initial state according to classical equations of motion. The full potential energy function is used to evaluate the forces acting on the atoms in the molecule, so that even highly anharmonic motions can be studied. Moreover, calculations can be performed on molecules which have too many atoms to be treated by conventional normal mode techniques. However, the usual molecular dynamics methods have the disadvantage that only limited time intervals (typically up to 10 to 100 psec) can be examined and for systems with long relaxation times, the calculation of thermal averages presents special difficulties. Also, processes that occur rarely due to high activation barriers or the intrinsic complexity of the event have to be studied by special methods, some of which are described in this section.

Finally, it is sometimes possible to obtain dynamical information concerning specific

motions by calculating effective potential surfaces which are then used in appropriate analytical models for the dynamics. This approach involves making certain assumptions concerning the separability of time scales of different processses and the magnitudes of frictional and entropic effects. The advantage of such methods is that they are simple and economical to apply and can often serve as useful starting points for more detailed dynamical investigations.

For all of the above approaches, the potential energy of the system as a function of the atomic coordinates is required. Although quantum mechanic calculations provide this for small molecules, empirical energy functions of the molecular mechanics type[140-142] are the only possible source of such information for proteins. Since most of the motions that occur at ordinary temperatures leave the bond lengths and bond angles of the polypeptide chains near their equilibrium values, which appear not to vary significantly throughout the protein (e.g., the standard dimensions of the peptide group first proposed by Pauling et al.),[143] the energy function representation of bonding can be hoped to have an accuracy on the order of that achieved in the vibrational analysis of small molecules. Where globular proteins differ from small molecules is that the contacts among nonbonded atoms play an essential role in the potential energy of the folded or native structure. From the success of the pioneering conformational studies of Ramachandran and co-workers[144] that made use of hard-sphere nonbonded radii, it is likely that relatively simple functions (Lennard-Jones nonbonded potentials supplemented by special hydrogen bonding terms and electrostatic interactions) can adequately describe the interactions involved. Detailed descriptions of the potential functions used in the various dynamical studies have been given recently;[26,145] they will not be reviewed here.

In energy refinement applications of potential functions, one is concerned only with the determination of a local minimum in the empirical energy surface of a protein. Application of these functions to the study of protein flexibility means that a knowledge of the shape of the energy surface in the neighborhood of the minimum is required. In studies of the small conformational fluctuations of proteins at sufficiently low temperatures, only the quadratic region near the equilibrium conformation is of interest. Studies of the behavior of proteins at higher temperatures or attempts to determine the response to sizable external forces require knowledge of the surface over correspondingly greater domains of configuration space; for such problems, anharmonic terms, as well as multiple minima, may play an important role.

In the following sections we review first effective potential surface studies and then proceed to harmonic vibration and molecular dynamics calculations. The final section introduces some possible extensions of dynamical models of proteins and suggests directions for future work. Some of these are treated in detail since much of this material has not been published elsewhere.

A. Effective Potential Surfaces

In this section we consider a number of dynamical problems that can be approached by the calculation of effective potential surfaces. The method is essentially static in character and usually involves the determination and analysis of a reaction path, in analogy to similar treatments of reactions in small molecules.[146]

In what follows we limit the examples to cases in which only empirical energy functions were used. There exist a number of studies of enzyme reaction paths which involve approximate quantum mechanical treatments of simplified models for the enzyme substrate complex[147-149] or attempts to combine quantum mechanical and empirical potential functions to take account of changes in the protein structure along the reaction path.[150,151]

1. Aromatic Ring Rotations

As discussed in the experimental section of this review, a variety of methods indicate that protein side chains have significant mobility even when they are buried in the protein interior. The torsional motions of aromatic side chains have been particularly well characterized since their protons are often resolved in proton NMR spectra. Gelin and Karplus were motivated by the measurements of the rates of ring "flipping" of the tyrosines and phenylalanines in the bovine pancreatic trypsin inhibitor (BPTI) to attempt to estimate the barriers by the reaction path approach.[142] The energy function used allowed both hard (bond lengths and bond angles) and soft (dihedral angles) internal coordinates to vary, but took only implicit account of hydrogen atoms by suitable adjustment of heavy atom parameters. With such a function,[145] it was found that rotation of the tyrosine and phenylalanine rings about the C^{β} — C^{γ} axes (i.e., rotation of the dihedral angle χ^2) yielded very high barriers ($\sim$100 kcal/mole) if the surrounding protein was fixed in the X-ray or energy-minimized structure. Analysis showed that the barrier for each ring arose from overlaps of the repulsive van der Waals cores of a few atoms in the ring with a few atoms in the surrounding matrix. Since the protein matrix would be unable to support the large stresses resulting from the rigid rotations, an attempt was made to include structural relaxation of the protein in the barrier calculations. To determine the effective barriers in the limit of complete relaxation (adiabatic approximation), the energy of the protein was minimized as a function of the ring orientation. A dramatic reduction of all rigid rotation barriers was observed. Analysis of the final coordinates showed that this relaxation resulted from small displacements (generally $\leq$ 0.5 Å) of atoms near the rings at the cost of modest amounts of residual strain (largely in bond angles and nonbonded interactions). Of the four tyrosines in BPTI, only Tyr 35 was found to have a final barrier high enough ($\Delta E \sim$23 kcal/mole) to make ring rotation slow on the NMR time scale. The barriers for the rings have subsequently been found to be in good agreement with the activation enthalpies determined in an NMR study.[112] Hetzel et al.[152] subsequently performed additional calculations on the aromatic ring rotations in BPTI. They made one set of calculations very similar to those of Gelin and Karplus and obtained corresponding results. In addition, they calculated the increases in protein potential energy produced by replacing each ring with a steeply repulsive sphere which had a volume similar to that which would be swept out by rotation of the ring. Even when protein relaxation is allowed, the energies of these structures relative to that of minimized, native BPTI are large; this suggests that ring rotation does not occur by a simple "free volume" mechanism.

2. Stereochemical Trigger of Hemoglobin

Potential surface calculations have been applied to the tertiary structural changes resulting from oxygenation of a hemoglobin subunit.[125] Preliminary energy refinement of the unliganded deoxy α subunit indicated that the heme group has a domed structure, with the top of the dome directed toward the proximal His F8. Although the existence of doming could not be confirmed in the 2.5-Å X-ray structure of deoxyhemoglobin,[153] it is observed in 2.0-Å X-ray structure of myoglobin[154] and in the model five-coordinate high-spin compound, $\alpha,\beta,\gamma,\delta$-tetraphenylporphinatoiron (II).[155,156] Calculation of the energy required for displacement of the domed heme perpendicular to its mean plane in the rigid deoxy globin (X-ray structure) showed that the heme is not subject to significant strain in this structure; this is in agreement with a number of experimental results.

From model compound studies, it is expected that the liganded heme has a planar rather than domed structure and that the length of the bond linking the heme iron to His F8 is somewhat reduced. Calculations for the heme in the α chain show that this

undoming would result in overlaps of the repulsive van der Waals cores of certain atoms in the heme with parts of His F8. These local nonbonded stresses could be removed by displacement of His F8 and/or tilting of the heme group. Several lines of experimental evidence (polarized UV absorption measurements of hemoglobin crystals[157] and X-ray studies of methemoglobin locked in the deoxy quaternary conformation[44]) indicate that tilting of the heme group does in fact occur on ligation. Examination of the X-ray structure and conformational energy calculations show that His F8 is fairly rigidly constrained by a surrounding cluster of hydrophobic residues and by the F helix itself. The structural and energetic consequences of the heme motion were investigated by tilting the heme in such a way as to remove the close contacts with His F8 (i.e., by rotating the heme through an angle of 20° about an axis through pyrrole nitrogens 2N and 4N) and allowing the globin to relax by energy minimization with the heme fixed in its new position. The largest response in the heme pocket occurs in Val FG5, which is pushed away from pyrrole 3 of the heme group by van der Waals repulsion; the average atom shift in Val FG5 is nearly 1 Å. The displacement of Val FG5 leads in turn to a more general movement of the FG corner and, again by nonbonded interactions, to movement of several residues in the C helix. Moreover, the large displacement of Val FG5 stretches and bends the hydrogen bond between its carbonyl oxygen and the hydroxyl group of Tyr HC2. These structural alterations in the α chain would be likely to affect the neighboring β subunit in the intact hemoglobin tetramer. The FG corner and C helix are components of the $\alpha_1\beta_2$ interface, which has been identified as an important element in the hemoglobin allosteric mechanism;[40,158] moreover, the C terminal residue next to Tyr HC2 is involved in α_1 - α_2 salt bridges, which may be weakened by deformation of the Val FG5 - Tyr HC2 hydrogen bond and result in destabilization of the deoxy quaternary structure.[44,159] Another interesting consequence of the displacements in the FG corner is the existence of a feedback loop whereby His F8, the residue which appears to induce heme tilting, is itself stabilized against displacement; i.e., the heme tilting displaces Val FG5, which in turn pushes Leu FG3 toward His F8. Some experimental support for the importance of Val FG5 displacement by pyrrole 3 and its vinyl group exists in the form of mutant or modified hemoglobins with alterations in these groups; such systems exhibit greatly reduced cooperativity. An important conclusion from these studies is that the reduced oxygen affinity of deoxy hemoglobin results from the strain on the *liganded* subunit in a tetramer with deoxy quaternary conformation, in contrast to earlier suggestions that there is strain in the unliganded subunit.

3. Ligand Motion in Myoglobin

Reaction path calculations[160] have been used to elucidate the experimental data on ligand motion in heme proteins (see Section II.B.6). Examination of the 2.0-Å resolution X-ray structure of myoglobin does not reveal any path by which even small ligands such as O_2 or CO can move between the heme binding site and the outside of the protein;[154] calculations suggest that the rigid protein would have barriers on the order of 100 kcal/mole. To locate pathways which might be available in the thermally fluctuating protein, ligand trajectories were calculated using the static myoglobin X-ray structure together with a test molecule of reduced effective diameter to compensate somewhat for the absence of fluctuations in the protein. The trajectory was determined by releasing the test molecule with substantial kinetic energy (15 kcal/mole) in the heme pocket and following its classical trajectory for a suitable length of time. A total of 80 such trajectories were computed; a given trajectory was terminated after 3.75 psec if the test molecule had not escaped from the protein. Slightly more than half the test molecules failed to escape from the protein in the allowed time; 25 molecules remained trapped near the heme binding site, while another 21 were trapped in two

cavities accessible from the heme pocket. Most of the molecules which escaped did so between the distal histidine (E7) and the sidechains of Thr E10 and Val E11. A secondary pathway was also found; this involved a more complicated motion along an extension of the heme pocket into a space between Leu B10, Phe B14, and Leu E4, followed by squeezing out between Leu E4 and Phe B14.

In the rigid X-ray structure, both of these pathways present insurmountable barriers to a thermalized ligand of normal size. Thus, it was necessary to study the energetics of barrier relaxation to see whether either of these pathways could have acceptable activation enthalpies. Local dihedral rotations of key side chains in the otherwise rigid myoglobin were investigated and it was found that the bottleneck on the primary pathway could be relieved at the expense of modest strain in the protein (5 to 8 kcal/mole) by rigid rotations of the side chains of His E7, Val E11, and Thr E10. On the secondary path, however, no simple torsional motions reduced the barrier due to Leu E4 and Phe B14 since the necessary rotations lead to larger strain energies. A test sphere with van der Waals radius of 3.2 Å was then fixed in the energy-refined structure at either of two positions in the bottleneck on the primary path (between His E7 and Val E11, or between His E7 and Thr E10) or in the bottleneck on the secondary path (between Phe B14 and Leu E4). The protein was allowed to relax by energy minimization in the presence of the ligand and the resulting displacements in the polypeptide chains were monitored. The displacements involved local changes in side-chain dihedral angles and bond angles. In addition, neighboring sidechains and the backbones of helices D and E participated in the globin response, mostly by small dihedral angle changes. Approximate values or the relaxed barrier heights were found to be 13 kcal/mol and 6 kcal/mol for the two primary path positions and 18 kcal/mol for the secondary path position. These barriers are on the order of those estimated in the photolysis, rebinding studies described in Section II.B.6.[116-118]

4. Large-Scale Structural Changes

Honig et al. have applied static deformation techniques to study the packing of rigid structural elements in subtilisin BPN.[161] They examined the changes in van der Waals and electrostatic energy associated with conformational changes in the reverse turns of this protein and found that deformations of some turns required substantial rearrangements of the rigid fragments, while other turns did not. Both relatively short-range interactions (i.e., between residues near the turn in the polypeptide sequence) and longer range interactions between pairs of rigid fragments appeared to be involved in stabilizing portions of the native structure.

Large-scale structural fluctuations in the lysozyme molecule have also been studied by a static deformation method. This work is reviewed in Section III.B.3.

5. Limitations of Effective Potential Calculations

While static deformation studies have the virtue of simplicity and appear to yield reasonable values for the potential energy costs of structural deformations, they do suffer from two limitations. The first limitation is that such studies yield only the minimum potential energy and a corresponding single set of atomic coordinates for a given point on the reaction coordinate of interest. In a thermally fluctuating protein, a given point on a reaction coordinate may correspond to a large number of possible atomic configurations, and the thermodynamic cost of the deformation should be measured in terms of a potential of mean force or free energy.[26] As a specific example, consider the rotation of an aromatic ring in the interior of a protein. Many different microscopic configurations of the protein will be compatible with a given torsional angle of the ring. Thus the probability that the ring will be found at a given torsional angle will be determined by entropic as well as energetic factors. Indeed, the NMR

results[112] indicate that entropies of activation play an important role in determining the observed rates of ring rotations in BPTI. The second limitation of static deformation studies is that they provide no direct information on the dynamics of the atomic interactions which drive or oppose structural fluctuations, or on the frictional effects which may act to damp these fluctuations (see Section III.C.).

B. Vibrational Calculations

Early attempts to calculate dynamical properties of proteins or their fragments were motivated by vibrational spectroscopic studies.[162] The calculation of normal mode frequencies by use of empirical potential energy functions has long been a standard step in the assignment of infrared spectral bands.[163] In calculating the normal vibrational modes of a molecule, one assumes that the vibrational displacements of the atoms from their equilibrium positions are small enough that the potential energy can be approximated as a sum of terms which are quadratic in the displacements. The coefficients of these quadratic terms form a matrix of force constants which, together with the atomic masses, can be used to set up a matrix equation for the vibrational modes of the molecule.[163] Solution of this matrix equation generally requires the diagonalization of a 3N dimensional matrix, where N is the number of atoms in the molecule. The result is obtained as a set of 3N eigenvalues (vibrational frequencies) and 3N eigenvectors (normal modes). Six of these eigenvectors are associated with eigenvalues of zero, corresponding to overall translation and rotation of the molecule. The remaining 3N-6 eigenvalues are the internal vibrational frequencies of the molecule; the associated eigenvectors give the directions and relative amplitudes of the atomic displacements in each normal mode.

Although the harmonic model may not provide an adequate description for the motional properties of a protein, it is nevertheless of considerable importance because it does serve as a first approximation for which the theory is highly developed. Further, the harmonic model is essential for some quantum mechanical treatments of vibrational contributions to the heat capacity and free energy[164] and for certain treatments of unimolecular reactions.[165]

1. Secondary Structural Elements

In general, it is not feasible to carry out a detailed normal mode calculation for a molecule containing more than about 200 atoms, since the matrix diagonalization becomes very difficult for large N. This fact has prohibited direct calculations of the normal modes of protein molecules to date, though analyses in which only the dihedral angles are included should be possible. Some of the important structural elements of protein such as α-helices and β-sheets have intrinsic symmetries (in the case of "regular" structures) which allow factorization of the dynamical matrices and a substantial simplification of the normal mode calculations.[162] A number of theoretical calculations of the vibrational properties of such structures have been carried out and applied to the interpretation of IR absorption, Raman, and neutron scattering studies of polypeptides with regular secondary structures.[62,166,167] Calculations of the lowest frequency modes can be used to interpret the low-temperature heat capacities of such polypeptides.[168]

In general, the normal mode calculations on regular helices show that higher frequency vibrations ($\geq$400 cm^{-1}) are associated with the displacements within single residues, while lower frequency vibrations may be associated with collective displacements of atoms from many residues.[169] These extensive, low frequency vibrations can be characterized in terms of the overall elastic properties of the helices and used to calculate macroscopic elasticity parameters such as Young's modulus.[62,170,171]

The vibrations of α-helices in the quadratic potential approximation can be analyzed

by the methods of statistical mechanics to obtain the magnitudes of and correlation between fluctuations of the internal coordinates of the helices, the time dependence of these fluctuations, the elastic constants associated with stretching, bending, and twisting of helices, and the contributions of various intramolecular interactions to these properties. Gō and Gō studied the equilibrium fluctuations of an infinitely long α-helix; in their model helix, only the backbone dihedral angles ϕ and ψ were allowed to vary.[172] They found that the rms fluctuations of ϕ and ψ about their equilibrium values were about 8° and 7°, respectively, for the polyalanine helix at 300 K; slightly larger fluctuations were found for the polyglycine helix. They also found that the fluctuations of these dihedral angles were significantly correlated over a range of about eight residues for polyalanine or six residues for polyglycine and noted that the pattern of these correlations was such as to minimize disturbances of the overall structure of the helix. In a subsequent paper based on the same model, Suezaki and Gō calculated the stretching, bending, and twisting fluctuations for sections of various lengths in an infinite helix and calculated a number of elastic constants for the helix.[170] The fluctuations were found to be relatively large for short sections of helix (i.e., for sections shorter than about five residues for polyglycine or ten residues for polyalanine); within these short sections the helix was sufficiently rigid that the correlation of dihedral angle fluctuations could only partly suppress the effects of local fluctuations on neighboring residues. The mechanical rigidities for stretching, bending, and twisting of the polyalanine helix were found to be about twice those of the polyglycine helix, but this difference was attributed to the difference in cross-sectional areas of the helices; the Young's moduli and shear moduli of the two helices were estimated to have similar values of approximately 10^{11} dyn/cm^2. It was noted that the helices bend more easily in some directions than others.

The fluctuations of a finite α-helix (hexadecaglycine) have been studied in the quadratic potential approximation by Levy and Karplus.[171] Most of their calculations were based on an unconstrained model of the helix (i.e., bond lengths, bond angles, and all dihedral angles were allowed to vary), although additional calculations were performed in which the peptide dihedral angles ω_i were kept rigid; also, comparison studies were made in which the hydrogen-bond interactions were omitted. In the completely flexible model at 300 K, rms fluctuations of ϕ and ψ about their equilibrium values were about 12 to 13° in the middle of the helix and somewhat larger near the ends; the dihedral angle fluctuations were significantly correlated over a range of about two residues. The difference between these results and those of Gō and Gō[172] mentioned above were attributed to the more flexible character of the model; that is, bond lengths, bond angles, and the dihedral angles ω_i were available to absorb local stresses in the helix as was shown, e.g., by the substantial correlation of fluctuations between neighboring ϕ and ω angles ϕ_i, ω_{i-1}. Fluctuations in the lengths between adjacent residues (defined as the projection onto the helix axis of the vector connecting the centers of mass of adjacent residues) ranged from about 0.15 Å in the middle of the helix to about 0.25 Å at the ends. These length fluctuations were negatively correlated for residue pairs (i−1,i) and (i,i + 1), preserving the overall length of the helix; positive correlations were observed for the pairs (4,5), (8,9) and for (8,9), (12,13), suggesting that the motion of residue 8 is coupled to the motions of residues 4 and 12 so as to preserve optimal hydrogen bonding. When the above calculations were repeated with rigid peptide dihedral angles ω_i, the rms fluctuations of ϕ were reduced by about 2° and the pattern (but not the range) of dihedral angle fluctuation correlations was changed. Elimination of hydrogen bond interactions had little effect on the magnitude or correlations of dihedral angle fluctuations, but substantially increased the fluctuations in the lengths between adjacent residues (which ranged from 0.20 to about 0.5 Å) and greatly diminished the correlation of these fluctuations between formerly hydrogen bonded sections of the helix.

The time evolution of internal coordinate fluctuations in the harmonic α-helix were also analyzed[171] by use of time correlation functions.[26,51] If A(s) represents the fluctuation of some dynamical variable away from its mean value at a time s, the (unnormalized) time correlation function $C_A(t) = \langle A(t)A(0)\rangle$ gives the mean square fluctuation of the variable if $t = 0$ (for an equilibrated system) and gives the average decay behavior of the fluctuation for $t>0$. For the dihedral angle fluctuations of the helix, these time correlation functions exhibit an apparent damped behavior with a decay time on the order of 0.1 psec. In the harmonic model, which permits no energy transfer between normal modes, this apparent damping of a given internal coordinate fluctuation is a consequence of the destructive interference of the normal mode components.

In addition to α-helices, β-sheets and various turns are considered as protein secondary structural elements. There are few theoretical studies of their dynamical properties, even in the harmonic approximation. For parallel β-sheets, there is a recent study[173] of the origin of the twisting observed in protein structures. An extension of the model to the dynamics of such a twisting mode would be of interest, particularly in view of the β-sheet results obtained in the simulation of the basic pancreatic trypsin inhibitor (see Section III.C.2). Normal mode calculations have been made for tetrapeptides in β-turn conformations (types I and II) with special emphasis on the effects of the geometry, including internal hydrogen bonds, on the well-known amide frequencies.[174]

2. Globular Proteins

As noted above, some of the low-frequency vibrations of polypeptides correspond to collective oscillations of atoms from many different residues. When the wave lengths of such vibrations are much longer than the sizes of individual residues, these motions can be approximated by the methods of continuum elasticity theory. This kind of approach has been used to estimate the frequencies of large-scale vibrations of whole proteins. In the simplest model, a globular protein is represented as a sphere of homogeneous elastic material. The lowest frequencies of vibration of such a sphere were analyzed by deGennes and Papoular.[22] The fundamental vibration is a radial pulsation in which the displacement $\underset{\sim}{s}$ of a material element is given by

$$\underset{\sim}{s}(r) = \hat{r}\,\xi\, j_1(k_1 r)\, e^{-i\omega_1 t}$$

where r is the equilibrium distance of the element from the center of the sphere, $\hat{r}$ is the radial unit vector, ξ is an amplitude factor, j_1 is the spherical Bessel function of orer one, k_1 is a wavenumber, and ω_1 is the angular frequency of the vibration. The wavenumber depends on the radius of the sphere and on two elastic parameters, Young's modulus and Poisson's ratio. The frequency and amplitude factor also depend on these three quantities; the frequency contains an additional dependence on the density of the sphere and the amplitude factor contains an additional dependence on the temperature of the sphere. Other vibrations involving angular deformations (shearing, twisting) of the protein were also considered. Suezaki and Gō[23] have calculated the properties of the fundamental vibration of a spherical, homogeneous, elastic protein using this model. They assumed a radius of 20 Å, a density of 1 g cm^{-3}, and a Young's modulus of 10^{11} dyne cm^{-2}; the value used for Poisson's ratio was not quoted but from the frequency obtained, it was assumed to be about 0.3. For this elastic model, the frequency of the fundamental vibration is 26 cm^{-1}. The amplitude of this vibration was given as 0.35 Å at room temperature, but this result appears to conflict with the rms displacement of elements at the surface of the sphere as obtained by methods described by deGennes and Papoular;[22] using a Poisson's ratio of 0.3, this rms displacement is only 0.032 Å at 300 K. It should be noted that the calculated frequencies and amplitudes of the fundamental vibration are sensitive to the value

chosen for Poisson's ratio. For the protein model described above, one finds frequencies of 76 cm^{-1}, 26 cm^{-1}, 18 cm^{-1} and surface element rms displacements (at 300 K) of 0.053, 0.032, 0.015 Å for Poisson's ratios of 0.0, 0.3, 0.45, respectively. Of course, the rms displacements of the surface will be somewhat larger when superposition of higher frequency modes is taken into account; comparison can be made with fluctuations in the protein radius of gyration obtained in a molecular dynamics simulation (see Section III.C.3). For a rod of homogeneous elastic material, Poisson's ratio is simply the ratio of the transverse contraction to the longitudinal extension when the rod is stretched by forces applied at its ends; in principle, this ratio may take any value in the range −1 to + ½, although only values greater than zero are found in practice.[175] The use of values near 0.5 (similar to what is found for rubber) for biomolecules is advocated by deGennes and Papoular.[22]

For a protein which is immersed in solvent surroundings, it is necessary to take account of viscous damping in treating the low-frequency vibrations which involve significant displacements of the surface.[22,23] Suezaki and Gō estimated the magnitude of the damping for the fundamental vibration of their model protein and found that the vibration of the sphere would be underdamped in water, i.e., in the absence of driving forces the amplitude of the fundamental vibration would decay with time in an oscillatory manner.[23] This damping would result in a decrease in the effective frequency (to about 20 cm^{-1}) and was predicted to result in a vibrational spectral linewidth of 8 cm^{-1}. Suezaki and Go noted that these spectral characteristics correspond roughly to the low-frequency Raman results of Brown et al.,[59] though it seems possible that higher frequency modes of the sphere and anharmonicity of the fundamental mode could also contribute to the shape of the Raman band. For a discussion of this point, see the recent review by Peticolas.[169]

It should be noted that the global elastic mode discussed here need not be the lowest vibrational frequency of a protein. As mentioned previously, bending or twisting oscillations of secondary structural elements of proteins can also have very low frequencies. These elements could retain some of their independent vibrational characteristics; in such cases, the protein should be described as an inhomogeneous elastic material.

Gō has recently considered the thermodynamic role played by the collective vibrations of an homogeneous, elastic protein.[176,177] The number of such modes was estimated by modeling the protein as a cube with periodic boundary conditions and counting only vibrations with wavelengths longer than characteristic lengths of single amino acid residues; the resulting number was found to be roughly three times the number of amino acid residues in the protein. By setting Young's modulus equal to 10^{11} dyn cm^{-2}, Poisson's ratio equal to zero, and the diameter of the protein equal to 40 Å, the frequency range of the collective vibrations was obtained; usingslightly different approximations, ranges of 18 to 210 cm^{-1} and 26 to 120 cm^{-1} were found[176,177]. From these results it was shown that the energies of typical configurations of this model protein at 300 K correspond to energies of at least 150 kcal mol^{-1} above the minimum (zero-point) conformational energy; this conclusion requires the assumption that the protein behaves harmonically over the range of typical atomic displacements observed at 300 K.[176] Under this same assumption, it was shown that these modes contribute about 0.11 cal $deg^{-1}g^{-1}$ to the entropy and about 0.06 cal $deg^{-1}g^{-1}$ to the heat capacity of the protein.[177] The entropy contribution is sensitive to the details of the vibrational spectrum, and it was suggested that the elastic modes may therefore serve as an entropy resevoir in processes which affect the elasticity of the protein. Possible contributions of low frequency vibrations, as well as other effects, to the heat capacity and entropy of proteins have been discussed by Sturtevant, who quotes a variety of relevant data as well.[178] It is clear that for a more satisfactory analysis of such possibilities a detailed

dynamic approach to the low-frequency motions of a protein is required (see Section III.C).

3. Interdomain Vibrations

A large number of enzymes and other protein molecules (e.g., immunoglobulins) consists of two or more distinct domains connected by a few strands of polypeptide chain which may be viewed as "hinges". In solution, the relative motion of two such domains may range from underdamped oscillation (if the connecting hinge is stiff and damping due to solvent drag on the domains and internal frictional effects in the protein is small) to Brownian motion (if the hinge is highly flexible or damping effects are large). To assess the magnitudes of some of these factors, a model study was made of lysozyme,[24] which has two globular domains separated by a cleft including the active site. In this calculation the stiffness of the hinge was evaluated by the use of an empirical potential energy function. An angle bending potential was obtained by rigidly rotating one of the globular domains relative to a bending axis which passes through the hinge and calculating the changes in the protein conformational energy. This procedure is expected to overestimate the bending potential, since no allowance is made for the relaxation of unfavorable contacts between atoms whch may have been generated during rotation.[142] To take account of the relaxation, an adiabatic bending potential was calculated by holding the bending angle fixed at various values and permitting the positions of atoms in the hinge and adjacent regions of the two globular domains to adjust themselves so as to minimize the total potential energy. As in the adiabatic ring rotation study, only small (<0.3 Å) atomic displacements occurred in the relaxation process. The rigid and adiabatic bending potentials were approximately parabolic, with the restoring force constant for the adiabatic potential about an order of magnitude smaller that that for the rigid potential. However, even in the adiabatic case, the effective force constant was about 20 times the bond-angle bending force constant of an α carbon (i.e., N−C$^{\alpha}$−C) the dominant contributions to the force constant come from repulsive nonbonded interactions involving on the order of 50 contacts. Solvent damping was treated by modeling the two globular domains as spheres and calculating the viscous frictional drag accompanying the relative motion of these spheres by use of a modified Stokes law. The internal friction of the protein was considered to be negligible compared to the hydrodynamic frictions. From these estimates of the hinge stiffness and solvent damping, it was found that the relative motion of the two globular domains in lysozyme was overdamped, i.e., in the absence of driving forces the domains would relax to their equilibrium separation without oscillating. The decay time for this relaxation was estimated to be about 2×10^{-11} sec. Actually, the lysozyme molecule will experience a randomly fluctuating driving force due to collisions with the solvent molecules, so that the distance between the globular domains will fluctuate in a Brownian manner over a range limited by the bending potential; the rms fluctuation of the cleft width was estimated to be about 0.5 Å.[24]

The assumptions which were used in the calculation of the stiffness and hydrodynamic damping in the lysozyme study warrant some comments. The hinge bending potential was calculated in the spirit of elasticity theory; in principle, it would be possible to calculate this potential and the vibrational frequency of the bending mode from the bending Young's modulus and the shear modulus of the protein if these quantities were known.[179] The equations for the bending and vibration of a curved rod exist, but the available solutions do not apply for the case of a thick rod with substantial curvature.[180] Nevertheless, it is possible to estimate the values of the mechanical moduli of lysozyme by assuming that they are of similar orders of magnitude and comparing the calculated stress-strain ratio with the equations for the deformation of a thick, linear beam.[181] One finds that the mechanical moduli corresponding to the

adiabatic bending potential are on the order of 10^{-11} dyn cm^{-2}, in the range expected for proteins,[23] and that bending dominates shear by about an order of magnitude in determining the stiffness of the protein.[236] From a more detailed molecular point of view, it is important to note that the difference between the rigid and adiabatic bending potentials is largely due to small shifts in the relative positions of a few atoms which have been forced too close together by the rigid rotation model. The relief of these contacts can be effected by localized motions (e.g., bond angle and local dihedral angle deformations).[24] The frequencies associated with these local deformations (>100 cm^{-1}) are expected to be much greater than the hinge bending frequency (≈4 cm^{-1}), so that the use of the adiabatic bending potential appears to be appropriate in this case.

It was noted in the lysozyme study that the use of a Stokes law to describe the solvent damping could only be justified as a first approximation. This is because the hinge-bending motion is sufficiently rapid that the hydrodynamic assumptions implicit in Stokes law break down to some extent. This question has been examined in detail by McCammon and Wolynes,[25] who showed that corrections due to the nonsteadiness of the solvent flow could produce special qualitative features in the Raman spectra of molecules with hinge-bending motions. It should also be noted that in estimating the relative diffusion coefficient of globular domains which are close together, special consideration must be given to the hydrodynamic boundary conditions at the domain surfaces. For impermeable domains with the usual stick boundary conditions, the relative diffusion coefficient vanishes for domains which are approaching contact; such limiting hydrodynamic interaction results from the fact that a moving domain has a strong tendency to drag the surrounding solvent with stick boundary conditions.[182] However, the roughness of protein surfaces allows some solvent penetration into the moving domains;[183,184] the appropriate boundary condition is then one of partial slip, resulting in diminished hydrodynamic coupling of the domains.[185] As a numerical example, consider the relative diffusion coefficient D_{12} of two spherical domains of radius 25 Å which are immersed in water at room temperature and which are separated by a 5-Å gap. For stick boundary conditions, one finds $D_{12} = 1.7 \times 10^{-6}$ $cm^2 sec^{-1}$ when the hydrodynamic interaction is neglected and $D_{12} = 0.34 \times 10^{-6}$ cm^2 sec^{-1} when this interaction is taken into account. If, however, the spheres are conidered to have rough surfaces that approximate those of proteins (e.g., a 10 Å thick outer layer with an inverse hydrodynamic shielding length of 0.4 $Å^{-1}$,[184] one finds $D_{12} = 1.9 \times 10^{-6}$ cm^2 sec^{-1} when the hydrodynamic interaction is neglected and $D_{12} = 1.2 \times 10^{-6}$ cm^2 sec^{-1} when this interaction is taken into account. Thus, in the latter case the effect of hydrodynamic interaction is much reduced. Finally, it should be mentioned that direct evidence of relative domain motions in lysozyme has been provided by comparison of X-ray structures with and without ligands bound in the cleft.[33,186]

An important class of proteins with hinged domain structure is provided by the immunoglobulins. X-ray[187] and fluorescence depolarization studies[65,66] suggest that immunoglobulin hinges may be highly flexible. Relative motion of the globular domains in antibody molecules has been suggested to play a role in their function.[12,13,187] Even if there is no direct allosteric effect, as originally suggested, the inherent flexibility may make it possible for the antibody molecule to bind a greater range of antigens.[13] The results of the lysozyme study described above suggest that the relative motion of immunoglobulin domains is of a diffusional nature. McCammon and Karplus have provided theoretical estimates of the characteristic times of these motions for antibodies of the IgG class;[68] these times are predicted to be on the order to 10^{-9} to 5×10^{-9} sec.

Since the hinge bending motion in lysozyme and in other enzymes involves the active site cleft, it may play a role in enzymatic activity of these systems. In addition to the possible difference in the binding equilibrium of the open and closed state, the motion itself could result in a coupling between the entrance and exit of the substrate and the

opening and closing of the cleft. More detailed calculations, including the effect of substrates on the interdomain motion, are required to substantiate this suggestion. Ptitsyn has recently called attention to the possibility of interdomain flexibility of the lysozyme type in other enzymes and suggested that they may play a role in substrate binding or release and in catalysis.[188] The kinases represent a particularly interesting case for dynamic study since large structural changes (much greater than in lysozyme) involving closing of the active site cleft occur on substrate binding.[11,189] In some sense, these interdomain motions represent a special case of the induced fit model of Koshland, who has often stressed the possible role of enzyme flexibility in catalysis and specificity.[190]

C. Molecular Dynamics Calculations

The internal motions of proteins have been studied recently by the molecular dynamics method.[26,88,89,204,210] In a molecular dynamics simulation, one assigns initial positions and velocities to all the atoms in the system of interest and then solves the classical simultaneous equations of motion for the atoms with forces determined from a known potential over some period of time. The resulting atomic trajectories are analyzed to determine the magnitudes of structural fluctuations, the correlations of these fluctuations in space and time, and other structural and dynamical properties of the system. In practice, the system is usually allowed to equilibrate for a period of time before the trajectories are subjected to analysis; this allows for the decay of transient dynamical effects associated with the initial conditions.

The molecular dynamics method has been extensively applied in past years to characterize the microscopic structural and dynamical properties of homogeneous gases, liquids, and solids, and of heterogeneous systems such as droplets and interfaces.[191] Related trajectory calculations have long been employed for the study of collisions and reactions of small, isolated molecules in the gas phase.[192] The application of such methods to the internal motions of proteins is a natural extension of this earlier work; the large size and high density of proteins, which endows them with bulk character, suggest that the simulation of a single isolated protein molecule is of considerable interest.

1. Technical Comments

The initial molecular dynamics simulation of BPTI[88,210] made use of the empirical potential function employed previously in a study of aromatic ring rotations[142] (see Section III.A.1). A fixed list of nonbonded interactions (all pairs which were closer than 8 Å in the X-ray structure) was used throughout the simulation. The classical equations of motion for the heavy atoms (including four internal water molecules) of which the protein is composed were solved by means of the fifth-order Gear algorithm with a time step of about 10^{-15} sec. The X-ray coordinates were used for the initial atomic positions and the atomic velocities were allowed to evolve from zero initial values by relaxation of stresses in the X-ray structure. During the first 40 steps, the "kinetic" temperature of the protein (determined from the atomic velocities by application of the equipartition theorem) rapidly rose to about 140 K as the stresses due to nonbonded interactions, bond-length strain, and bond-angle strain were largely relieved. The temperature fluctuated around 140 K for the next 60 steps, after which all velocities were multiplied by 1.5 and an additional 250 equilibration steps were taken; the temperature at the end of this period was 285 K.

Statistical data on the motions of the protein were collected during a subsequent 9000-step (8.8-psec) calculation. A slow phase of relaxation was apparent in a moderate decrease of electrostatic energy and smaller rises in the bond angle and bond length energies during the first 3 psec of this calculation; much of the change in electrostatic energy was associated with rearrangements of the charged sidechains on the surface

of the protein. This slow phase of relaxation resulted in a small rise of the temperature of the protein; the average temperature over the 8.8-psec simulation was 295 K. It was also noted that the distribution of atomic mean kinetic energies was not uniform in the protein at the start of the 8.8-psec simulation. This was a consequence of the lack of uniformity of stresses in the X-ray structure. Most notably, the C terminal residues 56 to 58 were about 60% warmer than the rest of the molecule at the outset due to the relaxation of structural strains induced by crystal interactions;[89] all but 10 to 20% of this excess kinetic energy was spread through neighboring residues within about 1 psec (see below). The total energy of the protein was well conserved during the 8.8-psec simulation; its change by only 0.7 kcal/mol is an indication of the stability of the numerical integration.

In subsequent molecular dynamics calculations on BPTI, a number of improvements in the molecular model and in system equilibration have been introduced. A simulation[26] was initiated by assigning all heavy atoms (in the X-ray structure) equal thermal velocities with randomized directions. For the first 2.0 psec of the equilibration period (i.e., as some of the initial kinetic energy was converted to potential energy) the system was gradually brought to 300 K with systematic damping of "hot" atoms, so that the mean atomic kinetic energies were more uniformly distributed throughout the molecule. (This special treatment of certain atoms introduced a small net linear and angular momentum into the protein; this overall motion was eliminated before the actual simulation to simplify statistical analysis of coordinate displacements.) The equilibration period continued without external intervention for an additional 2.9 psec to allow further relaxation of the atomic positions and velocities. The average temperature thus obtained was 308 K, which remained fairly constant through the following 9.8-psec simulation. In a new, long simulation of BPTI dynamics, provision was made for regeneration of the nonbonded list to reflect structural changes during the calculation.[89] The nonbonded interaction potentials (van der Waals and electrostatic) had the usual functional form up to a separation of 6.0 Å, beyond which they were smoothly reduced to zero at 6.5 Å by multiplication with a sigmoid damping function. All appropriate pairs with a distance of less than 7.0 Å were included in the nonbonded list, which was regenerated every 25 steps. This procedure eliminated fluctuations in the nonbonded energy and forces which would result from abrupt inclusion (or deletion) of a nonbonded pair subject to a sharply truncated potential. Introduction of this improved method of handling nonbonded pairs appeared to significantly increase the configurational relaxation time of the system, possibly because the protein configuration space was not restricted by use of a fixed set of nonbonded interactions; in particular, it was found that a longer equilibration period was required than in the initial simulation. Equilibration was started in the same manner as described above, but the thermalization with damping of "hot" atoms was applied for 3.9 psec, followed by a 35-psec period of unperturbed dynamics during which slow relaxation processes (lasting about 20 psec) raised the kinetic temperature from 300 to 327 K. After this period, the molecule was subjected to gentle cooling with damping of "hot" atoms for 4.9 psec, then allowed to continue unperturbed for 28 psec of equilibration, during which the average temperature of the protein was 306 K. The final phase of the simulation (96 psec) provided the trajectory used for statistical analysis; the average temperature during this period was again 306 K.

In the dynamic simulations under discussion, all heavy atoms were allowed to move freely in the presence of the forces due to the empirical potential function. A method for simplifying the calculation by use of fixed bond lengths has been formulated and tested on BPTI with the same potential function. The method appears to work well and may make it possible to take time steps that are about three times longer than in the unconstrained simulation.[193]

2. Results of the Initial Simulation

With minor exceptions (described in the following section) the general qualitative features of the BPTI internal dynamics observed in all of the simulations are very similar. In the initial study,[88,210] the rms deviation of the dynamical average atom positions from their positions in the X-ray structure was 1.7 Å. Part of this deviation was certainly due to the absence of crystal packing and solvent effects,[145] since the six residues with the largest deviations (Ala 58 and the side chains of Lys 15, Arg 17, Lys 26, Lys 46, Arg 53) had contacts with neighboring protein molecules in the crystal (residues 17, 26, 58) or would have substantial solvent exposure if the protein in its X-ray conformation were rigidly immersed in water (all would be more than 60% exposed to solvent.[235] Other residues which were involved in protein-protein contacts in the crystal (e.g., Arg 39, Glu 49, Met 52) or were substantially exposed to solvent (e.g., Asp 3, Arg 39, Arg 42) exhibited somewhat smaller deviations, despite substantial mobility in some cases (see below).

The mean rms fluctuation of the atom positions about their dynamical average positions was 0.9 Å. The most mobile groups were Ala 58 and the sidechains of Pro 13, Lys 26, Arg 39, Arg 42, Lys 46, and Met 52. All of these groups except Met 52 would be more than 50% exposed to solvent upon rigid immersion of the protein in water. Most of them had large temperature factors (or could not be located) in the X-ray diffraction work (Ala 58 and the side chains of Lys 26, Arg 42, Lys 46;[237]) or were groups which had been involved in protein-protein contacts in the crystal (Arg 39, Met 52). Overall, the most mobile groups were the side chains on the surface of the molecule. The buried side chains exhibited somewhat more mobility than the backbone atoms, and the backbone showed more mobility in the loop regions (residues 25 to 28 and, to a lesser extent, 12 to 17 and 36 to 42) than in the β sheet (residues 18 to 24 and 29 to 35).

The fluctuations in the internal coordinates range widely in magnitude. The average rms dynamical fluctuations of the backbone bond lengths, bond angles, and peptide dihedral angles ω were about 0.02 to 0.03 Å, 5° and 8° to 9°, respectively; the fluctuations did not vary substantially from residue to residue. Similar values were found in the vibrational analysis of an α-helix by Levy and Karplus.[171] Quantum corrections to these classical results are expected to be significant for these "hard" coordinates (See Section III.C.6). Rms fluctuations of the softer dihedral angles ϕ and ψ exhibited a much larger variation along the backbone, ranging typically from 10 to 40°; ϕ,ψ fluctuations tended to be smaller in the β sheet and α-helix than in the loops. The atom position fluctuations were smaller than these large dihedral angle fluctuations might suggest, principally because of a substantial correlation of fluctuations in ψ_{i-1} with those in ϕ_i; a similar phenomenon has been observed in the α-helix vibrational studies.[171,172]

The magnitudes of fluctuations depend only on the shape of the protein potential surface and could also be obtained by Monte Carlo simulation methods.[191] The important advantage of the molecular dynamics method is that it also provides information on the time dependence of the protein fluctuations. The decay of structural fluctuations in proteins is best characterized in terms of time correlation functions.[51] Moreover, Fourier transformation of the time correlation functions yields power spectra which characterize the dominant frequencies contributing to the time course of the fluctuations; these correspond to the frequencies of external forces that can effectively couple to the fluctuations.[25,194]

Time correlation functions of the atom displacements in BPTI generally exhibit a decay that is essentially monotonic with superimposed small high-frequency oscillations. In addition, there is the suggestion of components with (low <100 cm^{-1}) but finite frequencies; analyses of longer simulations are needed to establish the character-

istics of these low-frequency motions. The decay times for theatom displacement fluctuations are roughly on the order of 1 psec. The irregular character of the atomic motions reflected in the decay of the time correlation functions makes it possible to model them in terms of a largely diffusional (Brownian) picture on a picosecond time scale, although the physical processes responsible for this behavior can only be determined by considering other data; this is discussed more fully in Section III.C.4. Velocity correlation functions of the backbone α-carbons typically exhibit oscillatory behavior (apparent frequencies $\sim$1200 cm^{-1}) with a substantial loss of amplitude during the first oscillation and much slower decay thereafter. The initial rapid decay is qualitatively similar to behavior observed in condensed media,[195] but the persistent oscillations are due to the covalent bonds connecting the atoms (see below).

Time correlation functions for the bond lengths are oscillatory and show a pattern of "beats", indicating that the oscillations result from the superposition of a few high frequency components. Some decay of amplitude is apparent during the first 0.1 psec. Fourier analysis of the time correlation function of a given bond length demonstrates that the frequency components contributing to the fluctuations are just those of the localized normal modes which involve substantial displacements of the given bond length. For the bond angles and the peptide dihedral angles ω, Fourier analysis of the time correlation functions also indicates that the localized normal modes contribute substantially to the fluctuations of these moderately hard internal coordinates; however, frequencies below 300 cm^{-1} are significant, suggesting greater involvement of the protein matrix in these fluctuations. A similar analysis of the fluctuations of the softer dihedral angles ϕ and ψ shows that these fluctuations are dominated by low frequency (<100 cm^{-1}) processes.

The displacements of the atoms within the protein are dominated by torsional motions around single bonds, since the components due to the deforation of hard internal coordinates have small amplitude. Vibrations involving the hard internal coordinates make a significant contribution to the instantaneous velocity of a given atom, but this contribution largely reflects the simple relative oscillation within local bonded groups and is not effective in producing net displacements. This can be seen directly by calculating time correlation functions for the total linear or angular velocity of groups of atoms; the high-frequency components associated with harder internal coordinates are largely suppressed in these correlation functions.[26] Moreover, the effective structural fluctuations exhibit a diffusional character; that is, the motions are substantially damped. The degree of damping is related to the size of the locally rigid group, in analogy with diffusional processes in condensed media.

Several structural fluctuations observed during the dynamical simulation are of particular interest. A low frequency ($\sim$15 cm^{-1}) elastic motion involving much of the β sheet was found in the correlation of ϕ and ψ dihedral angles in different strands of the β sheet. The corresponding atom position fluctuations are small because of the equal-time ϕ_i,ψ_j correlations mentioned previously. This may result in sufficiently small frictional effects that underdamped oscillation is possible. Analysis of longer simulations will be required to obtain adequate statistics on such low-frequency processes. In a few instances at the surface of the molecule, dihedral angles ψ_{i-1} and ϕ_i were observed to undergo anticorrelated changes by 180°, resulting in complete rotation of the intervening amide group but overall preservation of the backbone direction and side chain orientations. While these particular structural transitions are likely to be artifacts (due to the neglect of solvent), they illustrate some of the special features which characterize structural transitions in proteins. There is a high degree of correlation in the internal coordinate changes to minimize steric requirements of the transitions and substantial dynamical involvement of the surrounding protein matrix. This results in a low barrier and diffusion-like transition for the peptide group.

Another feature of the BPTI dynamics noted in this initial study relates to the question of energy transfer in proteins. The carboxy-terminal end of the molecule was not well localized in the X-ray structure and interacted strongly with a neighboring protein in the crystal. Thus, in the isolated molecule the atoms in the C-terminal region are subject to sizable stresses. In the molecular dynamics calculation the system was started in the X-ray structure with its internal stresses, so that the C-terminal residues attained a higher kinetic temperature than the rest of the molecule during the equilibration period which preceded the actual 8.8-psec simulation. At the start of the simulation, residues 56 to 58 had a temperature which was about 60% higher than the molecule as a whole; this corresponds to an excess kinetic energy of about 12 kcal/mole in the three residues. Within about 1 psec, a rapid phase of relaxation distributed this excess kinetic energy through the C-terminal α-helix (residues 47 to 56) and the spatially adjacent N-terminal residues. During the remainder of the simulation, this group of residues remained 10 to 20% higher in kinetic energy than the rest of the protein. The somewhat larger (10 to 20%) bond length and bond angle fluctuations of these residues suggests that much of this slowly relaxing excess energy resided in localized high-frequency vibrational modes.

In principle, knowledge of the classical heat capacity of the isolated protein provides valuable information on the shape of the portion of the potential surface which is sampled at a given temperature; for a discussion of quantum corrections, see Section III.C.6. At sufficiently low temperatures the amplitudes of atomic motions in a protein are small, the protein samples only the quadratic region of a conformational energy well, and the heat capacity is that of a classical harmonic solid (C_v = 3.0 k per atom, where k is the Boltzmann constant). At higher temperatures, the local atomic motions are limited in amplitude by collisions with the steeply repulsive van der Waals cores of neighboring atoms. Consequently, the effective potentials sensed by the atoms are anharmonic in character and correspond to steep-walled wells, the bottoms of which are relatively flat on the scale of kT. The heat capacity of the protein is then reduced toward the value expected for a gas of hard spheres (C_v = 1.5 k per atom). The temperature at which these hard-core effects become significant depends on the density of the protein and on the character of the interatomic potentials of the system. The interior of a protein molecule is governed by both hard (bond lengths, bond angles, hydrogen bonds) and soft (torsional, van der Waals, electrostatic) interactions. The hard interactions tend to enhance the harmonic character of interatomic motions, while the soft interactions tend to introduce anharmonic effects of the kind just described. At elevated temperatures, the heat capacity may include additional contributions arising from structural transitions (i.e., the displacement of the protein from the region of one local potential energy minimum to another) as well as from the fluctuations described above. Evidence for such structural transition contributions at 300 K has, in fact, been found in the 96-psec dynamical simulation, as described in the following section.

An attempt was made to the estimate the classical heat capacity of BPTI from the magnitude of the kinetic energy fluctuations in the initial molecular dynamics study.[88] Because of the slow relaxation of stresses present in the initial (X-ray) structure, the kinetic energy fluctuations were averaged over only the final 2 psec of the simulation. The mean square fluctuation of kinetic energy was found to be 87 $kcal^2/mol^2$, which corresponds to a heat capacity C_v = 2.3 k per atom or 0.33 cal g^{-1} deg^{-1}. Calorimetric measurements yield similar values of C_v for proteins in solution,[52] although the apparent agreement is in part accidental because of the absence of quantum corrections in the calculation (see Section III.C.6); the neglect of hydrogen has a small effect due to their high-frequency modes. Also, more recent calculations have shown that the mean decay time for kinetic energy fluctuations is on the order of 0.1 psec or longer; applying

the theory of Zwanzig and Ailawadi,[196] the realtive error limits for the quoted values of the mean square kinetic energy fluctuation and of the heat capacity may exceed 30 and 20%, respectively. It appears that analysis of a long, well pre-equilibrated dynamical simulation would be required to establish C_v more accurately before conclusions about the anharmonicity of protein motion could be safely drawn from this approach. From the discussion given in the preceding paragraph, it may be appreciated that one of the difficulties in the estimation of anharmonic effects from the heat capacity is intrinsic to the molecular properties of a protein. Unlike a monatonic liquid, there are a large number of harmonic modes associated with hard internal coordinates, which tend to mask the anharmonic effects that appear in some of the softer modes. This suggests that parallel simulations with suitably modified potential functions (e.g., Lennard-Jones or hard sphere atoms connected by links which permit only torsional motions) may be helpful in detecting and characterizing anharmonicity in protein motions.

In this discussion of anharmonicity, it is important to remember that the *structure* of the protein is certainly governed to a large extent by the hard-sphere-like interatomic repulsions. Within the constraints imposed by the requirement that internal hydrogen and ion pair bonds be formed, excluded volume effects largely determine the packing of locally bonded segments to produce the observed tertiary structure[16] so that the atom density in the protein interior is near that expected for close packing. Direct evidence for the role of atomic cores in determining structure was described earlier in connection with the static effective potential energy determination of various motional paths (cf. Section III.A). Here the question is the role played by the atomic cores in determining the *dynamics* of interesting structural fluctuations. The nature of interatomic potential effects on the structure can be qualitively assessed by considering the variation of the potential for displacements on the order of an atomic diameter (~2 Å), while the effects on dynamics must be assessed by considering the variation of the potential over characteristic atomic thermal path lengths (~0.2 Å).

Although the analysis of the various contributions to the heat capacity suggests that its measurement as a function of temperature would be of great interest, the complication of quantum corrections has to be recognized. These are considered in Section III.C.6.

3. Results of the 96-Psec Simulation

Preliminary analysis of the 96-psec dynamical simulation of BPTI has confirmed most of the results of the initial study, but has also revealed significant features which can be observed only over the longer time period.[89] The time-average structure of the protein exhibits a somewhat greater deviation from the X-ray structure than in the previous calculation; the rms deviation for the α carbons is 2.1 Å (previously 1.2 Å) and that for all the atoms is 2.9 Å (previously 1.7 Å). As before, the largest backbone deviations are in the two ends of the molecule and, to a lesser extent, in the external loop (residues 25 to 28) and the two loops connected by the 14 to 38 disulphide bridge. The largest side-chain deviations are mostly in the charged groups which are exposed to solvent or involved in contacts with neighboring protein molecules in the BPTI crystal structure. As a result of these surface rearrangements, the radius of gyration of the protein decreased somewhat during the equilibration and then remained fairly constant during the 96 psec of actual simulation. The radius of gyration for the X-ray structure is 10.96 Å and that for the time-average structure is 10.22 Å; the fluctuations in the radius of gyration are on the order of 0.1 Å. The buried "core" of the protein has a density close to that of the X-ray structure. Within a sphere of radius 8 Å centered at the protein center of mass, the heavy atom number density for the time-average structure is 0.063 atoms $Å^{-3}$ while that for the X-ray structure is 0.060 atoms $Å^{-3}$.

The fluctuations of the protein about its time-average structure are slightly smaller than in the initial calculation. The average rms fluctuation for the α carbon positions is 0.60 Å (previously 0.74 Å) while that for all atoms is 0.75 Å (previously 0.90 Å). These values are comparable to those obtained from the experimental temperature factors. Omitting several atoms which were difficult to locate in the electron density map, the average temperature factor for α carbons is 12.0 Å^2 and that for all atoms is 14.4 Å^2; the corresponding rms fluctuations are 0.68 and 0.74 Å, respectively. However, it is likely that the other contributions to the thermal parameters (e.g., local and global disorder, rigid body motion) make the actual fluctuations smaller than those given here (see also Section II.A.3 above). Also, it should be pointed out that the calculated atomic fluctuations are highly anisotropic, i.e., for a given atom the largest component of mean square fluctuation can be ten times the value of the smallest. The variation of mobility along the backbone is similar to that seen in the initial calculation, with large α carbon rms fluctuations in the two ends of the molecule, in the external loop (residues 25 to 28) and in the two loops connectd by the 14 to 38 disulphide bridge. The fluctuations in the ends of the chain are significantly smaller than in the previous calculation (e.g., α carbon rms fluctuations of 0.63 vs. 1.20 Å for residue 1 and 1.12 vs. 2.22 Å for residue 58); as noted earlier, the fluctuations in the ends of the chain in the initial simulation are somewhat too large due to an excess kinetic energy in this part of the molecule.

The regions in which the largest fluctuations occur are the same as those which differ most on the average from the X-ray structure (see above). Both are a consequence of the fact that the calculated effective potentials for the isolated molecule are very permissive in these regions. For the molecule in the crystal, constraining forces due to protein neighbors are certainly present. Since many of the regions are highly exposed, the effect of solvent on the average position and magnitude of the fluctuations is also likely to be more important than in the protein interior.

The rms dynamical fluctuations of the backbone dihedral angles are somewhat more uniform along the chain than in the previous calculation; these fluctuations typically fall in the ranges 10 to 20° for ϕ and ψ and 7 to 9° for ω. During the equilibration period, two backbone amide groups (ψ_3, ϕ_4; ψ_{41}, ϕ_{42}) underwent 180° flips; no transitions of this kind occurred during the final 96 psec of simulation, although a number of side-chain torsional transitions did occur (including transitions in several buried side chains).

The length of the new simulation allows observation of a variety of structural and energetic phenomena which were not seen previously. Particularly striking in this regard are sizable (5 to 15 kcal/mol), long-lived (2 to 15 psec) fluctuations of 1 psec averages of the individual components of the potential energy about their 96-psec average values; the components referred to here are the total bond-stretching energy, total bond-angle-bending energy, etc., for the whole protein. These "coarse-grained" fluctuations tend to compensate one another (e.g., fluctuations of the bond and bond angle energies tend to balance fluctuations of the van der Waals energy), so that the total potential energy (and total kinetic energy) exhibits more modest "coarse-grained" fluctuations (1 to 4 kcal/mol, 2 to 5 psec). The normalized time correlation function for fluctuations in total kinetic energy falls from 1.0 to about 0.05 within 0.1 psec, after which it decays very slowly (on the order of 10 psec); the long tail of this function is apparently due to the long-lived fluctuations described above.

One consequence of the long-lived fluctuations in the total kinetic energy is that long periods of averaging are required to establish the rms fluctuation in this energy. Computation of rms fluctuations over different segments of the whole 96-psec simulation showed that values obtained upon averaging over 20 psec agreed within about 1% with the value obtained for the whole run, but that values obtained upon averaging

over shorter times tend to be too low (e.g., about 3 to 4% low for 10 psec and about 4 to 6% low for 5 psec. Using the rms fluctuation in kinetic energy over the whole 96 psec (11.5 ± 0.1 kcal/mol), the classical heat capacity of the protein is calculated to be 3.14 ± 0.1 k per atom (k is the Boltzmann constant); for an estimate of the quantum correction to this value, see Section III.C.6. The initial calculation yielded a smaller value for the rms kinetic energy fluctuation (9.3 kcal/mol) and a correspondingly smaller classical heat capacity (2.3 k per atom); these low values are evidently due to the short time interval used for averaging (2 psec).

In addition to the long-lived fluctuations in the energy components, long-lived fluctuations in the atom positions appear to occur during the simulation. Computation of average rms fluctuations in the α carbon positions over different segments of the whole 96-psec simulation showed that values obtained upon averaging over periods of much less than 20 psec tend to be too low.

The long-lived fluctuations described above could conceivably be due in part to very low-frequency elastic distortions within specific structural elements of BPTI (e.g., the β sheet) or between such structural elements and their surroundings. They are unlikely to represent simple distortions of BPTI considered as a uniform elastic body, since the longest period of such oscillations would be on the order of 0.6 psec.[23] It seems most likely, however, that these fluctuations largely represent transitions of the protein from one well in the protein potential energy surface to another. Direct evidence for this interpretation arises from the correlation of several of the large fluctuations in components of the total potential energy with unusually large excursions or complete transitions (from one rotameric state to another) of side-chain dihedral angles. Thus, long periods of averaging are necessary to sample the relatively infrequent structural transitions, and the calculated classical heat capacity quoted above contains a contribution arising from isomerization transitions of the protein among different thermally accessible states in the general neighborhood of the time-averaged structure. Because these larger-scale fluctuations can involve significant rearrangements of the protein surface, it will be important to assess the effect of the presence of solvent on their properties.

4. Protein Matrix and Frictional Effects

The initial molecular dynamics study of BPTI revealed that there are sizable frictional effects in the displacements of atoms and groups of atoms;[88] that is, fluctuations from the average structure were found to be subject to rapid damping. These effects have been analyzed in greater detail in a subsequent study that was focused on the relaxation of torsional fluctuations of two buried tyrosine rings in BPTI.[26] They were chosen because they represent well-defined internal probes that are convenient for analysis. The role of the protein matrix surrounding the rings in producing the observed frictional damping was demonstrated by comparison of the ring dynamics in three independent molecular dynamics calculations. Two of these calculations were dynamical simulations of BPTI with different torsional potential functions for the dihedral angle χ^2 of aromatic residues; the third calculation was a dynamical simulation of an isolated tyrosine "dipeptide", in which nonbonded interactions of the ring with the surrounding protein matrix were omitted except for local ring-backbone interactions. In the dipeptide fragment, the ring plane was observed to undergo nearly harmonic oscillations with relatively rare perturbations due to ring-backbone interactions. By contrast, the tyrosine in the presence of the rest of the protein showed frequent ring-atom, matrix-atom collisions which strongly altered the motion.

An important result of the protein simulation is that the magnitude of the torsional fluctuations is larger than would have been allowed if the protein matrix around the tyrosines were rigidly fixed in the X-ray conformation. This was demonstrated by comparing the energy required to rotate the Tyr 21 ring in the rigid X-ray structure with

the potential of mean force for Tyr 21 torsional fluctuations in the dynamical simulation; the latter potential corresponds to the free energy required for Tyr 21 ring torsional motion in the fluctuating protein. The potential of mean force is signficantly softer than the rigid rotation potential; for example, a ring with the typical thermal torsional energy of 0.6 kcal/mol (RT at 300 K) would experience torsional displacements of ± 10° in the rigid X-ray structure compared to the observed displacements of ± 15° in the fluctuating protein. The rigid rotation potential arises almost entirely from repulsive van der Waals contacts between a small number of ring and matrix atoms; the dihedral-angle torsional potential makes a negligible contribution. The softening of the torsional potential in the fluctuating protein, relative to the rigid protein, results from the correlated displacements of ring and matrix atoms. This can be seen by examining rigid rotation curves for coordinate sets selected from different times in the dynamical simulation; the minima of these curves vary over a range of about ± 10° from the dynamic average torsional angle. The typical displacements of matrix atoms which contribute to the softening of the effective torsional potential were estimated to be about 0.2 Å. These results tend to validate the use of adiabatic potentials in the static calculations of effective side-chain torsional potentials;[142,160] see Section III.A.

The frictional damping effects acting on the tyrosine rings are evident in the time correlation functions for the torsional fluctuations. For the BPTI simulations, these functions exhibited nearly monotonic decay with relaxation times of about 0.1 and 0.2 psec for the calculations with stronger and weaker dihedral angle (χ^2) restoring potentials, respectively. By contrast, the time correlation function for torsional fluctuations of the isolated tyrosine fragment exhibited pronounced, weakly damped oscillations. Time correlation functions for the squared torsional fluctuations and for the torsional angular velocity were also calculated to further characterize the ring dynamics.

For the time interval over which the BPTI correlation functions are statistically reliable, it was found that the dynamical behavior of the buried rings corresponds to that described by the Langevin equation for an harmonic oscillator. This equation is

$$I \frac{d^2\phi}{dt^2} + f \frac{d\phi}{dt} + k\phi = N_r(t)$$

where $\phi(t)$ is the torsional displacement, $I = 7.5 \times 10^{-15}$ g cm^2 mol^{-1} is the moment of inertia of the ring about the torsional (C^γ - C^ζ) axis, f is a friction constant, k is a harmonic restoring force constant and $N_r(t)$ represents the random torques acting on the ring due to fluctuations in its environment. The Langevin equation is appropriate if $N_r(t)$ varies more rapidly than $\phi(t)$; in this limit, $N_r(t)$ may be regarded as a Gaussian random process and it is not necessary to specify the mechanism by which the torque fluctuations arise. Moreover, if the fluctuations giving rise to $N_r(t)$ are more rapid than variations in ϕ, it is appropriate to choose a value of k which includes the effects of the relaxation of the protein matrix. From the potentials of mean force, values of k were estimated to be 1.3×10^{12} erg rd^{-2} mol^{-1} and 5.5×10^{11} erg rd^{-2} mol^{-1} for the simulations with the stronger and weaker dihedral angle restoring poentials, respectively. The only unknown coefficient in the Langevin equation is the friction constant f; this can be estimated from the observed decay times τ_* of the torsional fluctuation correlation function by the relation $\tau_* = f/k$. The friction constant turns out to be relatively insensitive to the dihedral angle restoring potential; for Tyr 21, one obtains f = 0.13 g cm^2 sec^{-1} mol^{-1} in the simulation with the stronger restoring potential and f = 0.11 g cm^2 sec^{-1} mol^{-1} in the simulation with the weaker restoring potential. For the isolated tyrosine fragment, the frictional effects and random torques were sufficiently weak that the torsional oscillations of the ring reflected its initial conditions through most

of the simulation. Taken together, these results support the idea that the frictional effects in the dynamics of a buried tyrosine ring derive largely from the nonbonded interactions of the ring with the surrounding protein matrix.

The friction constants quoted above may be related to angular diffusion constants by use of the Einstein formula $D = k_BT/f$. The values obtained from the two dynamical simulations of BPTI, $D = 1.9 \times 10^{11}$ sec^{-1} and $D = 2.3 \times 10^{11}$ sec^{-1}, are several times larger than experimental diffusion constants for the corresponding rotational motions of small aromatic molecules in organic solvents (e.g., $D = 5.7 \times 10^{10}$ sec^{-1} for neat benzene and $D = 7.9 \times 10^{10}$ sec^{-1} for benzene in the low viscosity solvent isopentane).[26] Since the average density of matrix atoms in the neighborhood of the tyrosine ring is close to that expected for a hydrocarbon solvent, the difference in diffusion constants may result from the covalent connectivity of the protein atoms; that is, that there are fewer matrix atoms in positions which allow for effective perturbation of the ring angular velocity than would be found in ordinary solvent surroundings. Similar effects have recently been detected in the rotational motion of iodine molecules in liquid paraffins.[197]

The described ring behavior found in the BPTI simulations is consistent with a collisional model based on the assumption that the ring suffers independent, relatively impulsive encounters with the protein matrix atoms; in this model, the rapid fluctuations in the random torque appear as a random succession of sharp peaks with varying amplitude.[26] Models of this kind have provided a starting point for theories of molecular reorientation in liquids.[198] The collisional model (with theoretical estimates for the torsional restoring force of the ring and the mean time between ring-matrix collisions) produces good agreement with the time correlation functions from the dynamic simulation.

A direct examination has been made of the individual torques exerted on the ring due to ring-matrix nonbonded interactions.[207] It was found that many of the torques act for sufficiently short intervals ($\leqslant$0.1 psec) that, during these intervals, changes in torsional angle are similar to what would be found if the torques had not acted. Thus, many of the torques have an impulsive character in accord with impulsive collisional models. However, most of the torques are not sufficiently strong to randomize the angular velocity of the ring. Also, several impulses in rapid succession are sometimes observed for particular ring-atom, matrix-atom pairs (most commonly when the matrix atom is part of the local backbone). An adequate description of such phenomena would require a somewhat more sophisticated model than the independent, binary collision model presented earlier.[26]

It should be pointed out that the Langevin-like behavior of the torsional coordinate could be observed in a purely harmonic system.[26,171] Since the torsional coordinate would then correspond to the superposition of a large number of normal modes, their dephasing could result in the rapid decay observed for the angular correlation function. In the harmonic model, the rapid fluctuations in the random torque can be represented by a smoothly varying function of time. The torque varies as the normal mode displacements of the matrix atoms near the ring intermittently come into phase; if many modes are involved, the net torque variations can be described stochastically and the ring motion will have a diffusional character. Although the two decay mechanisms (collision energy transfer and harmonic dephasing) give similar behavior for the correlation function on a short time scale, it appears from the detailed analysis of the interactions that the collisional model is the best zero-order description for the tyrosine rings. However, other parts of the protein (e.g., H-bonded groups) may behave differently and additional studies will be required to completely resolve this aspect of the dynamics.

5. Activated Processes

Activated processes play an important role in many protein functions. In an activated process, the system of interest must surmount an energy barrier to move from some initial state to some final state. Examples of such processes in proteins include the rotation of side chains, the motion of ligand and substrate molecules through steric bottlenecks in a protein matrix as the ligand approaches or leaves a binding site, and the chemical events associated with enzyme catalysis.

Experimental kinetic studies have yielded quantitative data concerning the free energy barriers, as well as activation energies and entropies associated with several activated processes in proteins. Examples of detailed studies include the rotations of the rings of certain aromatic side chains[112,113] (see section II.B.5), the motion of diatomic ligands through the globin of several heme proteins[116-118] (see Section II.B.6), and enzymatic reactions, such as the transformation of dihydroxyacetone phosphate to gylceraldehyde 3-phosphate by the enzyme triosephosphate isomerase.[199] Theoretical estimates of the potential energy barriers for ring rotations[142,152] (see Section III.A.1) and ligand motions through globin bottlenecks[160] (see Section III.A.3) have been made by reaction path calculations.

These experimental and theoretical studies have provided valuable information on the magnitude of energy barriers and on the role of the barriers in the biological activity of proteins. At the same time, these studies have raised new questions concerning the microscopic character of activated processes in proteins. Many of these questions pertain even to the simplest activated processes, those which do not involve rearrangements of the covalent structure of a protein or a protein-ligand complex. Among the essential questions are the following:

1. What are the structural elements of a protein which are responsible for the barriers in a given process? For example, can one identify particular groups of atoms which must be displaced to allow a small ligand to move from one site to another in a protein? Due to fluctuations in the protein interior, the atoms which are responsible for the barrier will generally differ somewhat at different instants. Can one calculate the instantaneous potential energy barriers and do the appropriate averaging to obtain the experimental barriers? What does this imply concerning the temperature dependence of the effective barriers, since the relative rates of barrier crossing and of protein fluctuations and the contributions of different fluctuations to the barrier are expected to vary with temperature?
2. What are the mechanisms by which the barrier traversal is effected? On the one hand, transient relaxation of the barrier may play a role. In the case of ligand motions, such relaxation may take the form of accommodating displacements of the barrier atoms; one can imagine that such displacements could precede or be concerted with the transition of interest. On the other hand, specific driving forces must be present to effect the transition over whatever residual barrier exists at a given instant. The nature, strength, and duration of the interactions which produce these forces need to be determined.
3. What is the dynamical character of the barrier crossing? In the absence of significant frictional effects, the group undergoing the transition would be expected to cross the barrier in a simple inertial manner upon experiencing a suitable driving force. The density of atoms in the protein interior is such that it is possible that significant frictional effects may occur during the transition as a result of interactions between the group undergoing the transition and the surrounding protein matrix.[26] Such collisional damping can affect the barrier-crossing dynamics, leading (in the high-friction limit) to Brownian rather than inertial crossings and increased transition-state lifetimes.[200]

To provide answers to the questions discussed above, it is necessary to carry out detailed studies of the atomic dynamics inside proteins. While the conventional molecular dynamics simulations described in the preceding sections provide much useful information on the equilibrium structural fluctuations of a protein, they do not allow one to study activated processes directly. Activated processes are by their nature rare events. Many of them have rate constants of 10^9 sec^{-1} or less so that they cannot be expected to occur in a conventional molecular dynamics simulation that is 10^{-11} to 10^{-10} sec in length. Fortunately, special simulation techniques have been developed which make possible the efficient determination of trajectories for activated processes. These techniques, which correspond to a synthesis of molecular dynamics and transition state theory concepts, have been employed in calculations of small-molecule collision dynamics[201,202] and of vacancy diffusion dynamics in regular solids.[203] The first step in such a calculation is to generate a variety of "transition state" configurations; these configurations correspond to thermally accessible points near the ridge which separates the initial and final-state valleys in the potential energy surface of the system. The second step consists of assigning velocities (randomly chosen from appropriate distributions) to atoms in these transition state configurations and of computing the corresponding trajectories which pass through these configurations. Analysis of the transition state configurations generated in the first step yields information on the kinds of molecular strain which produce the activation energy for the process, while analysis of the trajectories yields information on its dynamical character.

McCammon and Karplus have recently reported a preliminary calculation which demonstrates that such methods can be useful in the study of activated processes in proteins.[204,207] The particular process which was studied was the rotation of the well-buried aromatic ring of tyrosine 35 in BPTI. The transition state coordinate sets of BPTI (i.e., thermodynamically possible configurations, subject to the constraint that the ring be near the top of its rotational potential energy barrier) were prepared in the following manner: a coordinate set was selected from an equilabrium dynamical simulation of BPTI (see above), the ring was rigidly rotated to a high energy orientation, and the surrounding protein matrix was allowed to relax by Metropolis Monte Carlo methods[191] while the ring orientation was held fixed by appropriate constraints. Adjustments of the ring orientation were made by rotation through small angles followed by continued Monte Carlo sampling until trial trajectory calculations (see below) resulted in successful barrier crossings. At this point, a longer Monte Carlo calculation was performed to generate transition state configurations for detailed study. To obtain more adequate coverage of configuration space and to test the adequacy of the Monte Carlo method for generating a representative set of configurations, the whole procedure was repeated starting with a different coordinate set from the equilibrium molecular dynamics simulation.

For computing a trajectory which passes through a given transition state configureation, it is necessary to assign appropriate velocities to the atoms in the given configuration. With these positions and velocities as initial conditions, one half of the trajectory (e.g., the ring falling from the transition state into the final state valley of the potential surface) was calculated by molecular dynamics methods. The other half of the trajectory (e.g., the ring rising from the initial state valley to the transition state) was obtained by using velocities with opposite signs in the initial conditions, calculating the corresponding trajectory and then reversing it in time.

In these preliminary calculations, the velocity assignments were carried out in the following manner. A reaction coordinate for ring rotation was defined as the torsional angle of the best plane through the ring atoms about the axis which passes through C^{γ}_{35} and C^{ζ}_{35}.[26] In principle, to assign a specified velocity to one such internal coordinate requires assigning velocities to all the atoms of the protein so that there is no

resulting motion in the remaining internal coordinates or of the protein as a whole.[205] This procedure was simplified by assuming that the protein has effectively infinite mass and moment of inertia, and that the aromatic ring is effectively rigid. Thus, reaction coordinate velocities (i.e., torsional angular velocities for the ring) were specified by assigning velocities perpendicular to the plane of the ring for the atoms $C_{35}^{\delta 1}$, $C_{35}^{\delta 2}$ $C_{35}^{\epsilon 1}$ $C_{35}^{\epsilon 2}$; these velocities had equal magnitudes and the signs were consistent with overall rotation of the ring. The magnitudes of these velocitis were chosen by sampling an effusion velocity distribution[206] for the torsional motion of the ring, since the initial conditions represent states which are passing through a surface in phase space.[201] Velocities for all other atoms of the protein and for the in-plane motions of the above four atoms were chosen by sampling a Maxwellian distribution.[206] Both distributions corresponded to 300 K. Using this procedure, trajectories were calculated for five transition states from each of the two Monte Carlo series, which can be thought of as representing crossings through the two local regions of the transition state domain. The equations of motion were integrated by the same method used in the equilibrium dynamics simulations;[26] the total length of each trajectory was 1.17 psec.

Analysis of the transition state configurations and the associated trajectories yielded a number of conclusions.

1. In all trajectories, the Tyr 35 ring crossed the rotational potential energy barrier successfully; the times required to complete these ring rotations were 0.5 to 1.0 psec. None of the trajectories exhibited path reversals (torsional angular velocity sign changes) during the barrier crossings. However, several trajectories did exhibit appreciable slowing down (angular velocities approaching zero). Thus, damping or frictional effects are evident but do not dominate inertial effects in the barrier-crossing dynamics. This qualitative result is in accord with predictions based on the properties of equilibrium torsional fluctuations of tyrosine rings in BPTI,[26] and indicate that transition state theory with the appropriate choice of reaction coordinate is approximately valid for this rotational isomerization problem.[207]
2. The torsional displacements and angular velocity changes of the ring are well accounted for by the torques acting on the ring due to nonbonded interactions with the surrounding matrix atoms. Many of these torques are sharply peaked functions of time, suggesting that collisional descriptions may be appropriate in first-order analytical models for the barrier-crossing dynamics (see also Section III.C.4). The torque impulses are similar to those that occur when the ring oscillates about its equilibrium orientation. The ring is driven over its rotational barrier not as the result of an unusually strong collision, but as the result of a transient decrease in the frequency and intensity of collisions which would tend to drive the ring away from the barrier. This observation suggests that small, transient packing defects play a role in initiating ring rotation.[207]
3. The local density of the matrix atoms was somewhat different for the two sets of transition states. For the five transition states of the first set, the average number of heavy atoms within 5 Å of a Tyr 35 ring carbon is consistently close to 20 (excluding atoms directly bonded to the ring). The corresponding numbers for the five transition states of the second set and for the X-ray structure are 17 and 21, respectively. Moreover, frictional effects were somewhat more evident in the first set of trajectories than in the second. This suggests a distinction between "congested" transition states (in which the local density is high and the ring suffers many collisions as it crosses the barrier) and "uncongested" transition states (in which the opposite is true).

4. Apart from χ^2_{35} (the dihedral angle which changes as the ring rotates), the fluctuations in the dihedral angles of Tyr 35 show no obvious correlation with the ring motion. On the other hand, certain bond angles in Tyr 35 do show appreciable variations (5 to 10°) which appear to be correlated with the ring motion. The pattern of these deformations indicates that they serve to relieve nonbonded repulsions between the ring and the Tyr 35 backbone as the ring rotates.

a. Rhodopsin

The only other attempt at modeling the dynamics of a reactive process in proteins is that concerned with the excited state isomerization of retinal in opsin.[133] Because so little is known about the details of the binding site and the nature of the isomerization process, (see Section II.B.6) a highly simplified model was used for the protein. The calculation determined "semiclassical" trajectories of an excited 11-cis retinal chromophore, represented by an all-atom empirical potential function, in a rigid, sterically restrictive model of the rhodopsin binding site. It was found that the steric constraints favored an essentially one-dimensional reaction path on which the cis bond of the chromophore is propagated rapidly along the retinal by a "bicycle pedal" isomerization process. From these studies it was concluded that prelumirhodopsin, the first metastable intermediate of visual excitation, probably contains a strained all-trans retinal molecule. The isomerization was found to occur very rapidly (in $0.2 - 0.6 \times 10^{-13}$ sec) due to the lack of the torsional barrier in the potential and the small moment of inertia of the bicycle pedal motion. Further work on this problem is clearly required, though it is possible that the rigid active site approximation is reasonable for these short times, which are comparable to elementary collision times inside proteins.[26] In most active-site events, barriers are likely to exist which will lead to a greater involvement of the protein motion in the substrate dynamics.

6. Limitations of the Model

Several limitations of the model used in the molecular dynamics studies were discussed in the initial report.[88] The limitations concern the nature of the energy function used for the protein and the neglect of solvent. Subsequent simulations have been performed on BPTI[26,89] using somewhat different parameter sets for the bonds, bond angles, and dihedral angles, but with the same Lennard-Jones, electrostatic, and hydrogen bond parameters. Small differences in the localized high-frequency motions result from the bond and bond angle parameter changes, as expected. The distributions of dihedral angle fluctuations are somewhat altered for small fluctuations ($\leq 5°$) as a result of the changes in dihedral angle parameters; these changes have little effect on the distributions of larger fluctuations in the dihedral angles because the energetics of such fluctuations are dominated by nonbonded interactions. The rms atomic displacement fluctuations and the variations of these fluctuations from residue to residue are very similar to the results reported earlier;[88] the only significant differences are smaller rms fluctuations in the N- and C-terminal residues in the more recent simulations, which result from more careful equilibration (relaxation of X-ray structure stresses and kinetic energy smoothing) prior to the actual simulation. Moreover, the time correlation functions for atom displacement fluctuations show only minor changes, as was discussed before in connection with the analysis of frictional effects in the tyrosine fluctuations. These results, together with recognition of the very short range of the hydrogen bonds and the relatively slowly varying character of the electrostatic potential, suggest that the most interesting features of the picosecond-time-scale dynamics are relatively insensitive to details of the potential function except for the scale of the Lennard-Jones parameters and, to a lesser extent, the dihedral angle parameters.

Clearly, further work on the potential function is required to improve the reliability of dynamical results.

The neglect of solvent is expected to have a major effect only at the molecular surface, where hydrogen bonding, hydrophobic effects, reduction of the electrostatic interaction, and collisions with solvent molecules could alter the structure and dynamics of exposed groups. To some extent, these surface alterations will affect the internal dynamics; e.g., some low-frequency modes may involve distortions of the protein surface which would be subject to external damping in solution. However, a variety of results suggest that short-time (picosecond) local structural fluctuations in the protein interior are rather insensitive to the absence of solvent. A recent simulation of an alanine dipeptide dissolved in water, with explicit consideration of the dynamics of both the dipeptide and the aqueous solvent (195 water molecules), showed that the picosecond fluctuations in the internal degrees of freedom of the dipeptide were very similar to those obtained from a vacuum simulation.[208] This is consistent with the observed similarity of the Raman spectrum of peptides in crystals and in solution.[224] The results suggest that the local high-frequency motions in proteins are not sensitive to solvent, although larger fluctuations on a longer time scale certainly would be.

A limitation of the molecular dynamics calculations which has not been considered previously concerns the use of classical mechanics to calculate the protein motions. Since a protein is, in fact, a quantum mechanical system, it is necessary to discuss the corrections which should be applied to the classical results. To simplify the analysis of quantum effects, we assume that the molecule exhibits harmonic vibrations. Although anharmonic contributions to the motions are certainly important, the magnitude of the quantum corrections should be adequately described in the harmonic limit. Further, anharmonic coupling is likely, if anything, to reduce the importance of quantum effects on the atomic dynamics.

One property for which quantum corrections can be important is the molecular heat capacity. In quantum statistical mechanics, the partition function and therefore the heat capacity of a harmonic mode depends on the frequency of the mode and the temperature of the system.[209] At 300 K, modes with frequencies below 230 cm^{-1} have vibrational levels which are closely spaced relative to kT; these modes are excited to a sufficient degree that the associated heat capacity is close to the classical limit (i.e., $C_v \geq 0.9k$). Higher frequency modes are less excited and have smaller heat capacity contributions; at 300 K, modes with frequencies greater than about 700 cm^{-1} yield heat capacities of less than half the classical value. Thus, the heat capacity calculated by classical mechanics overestimates the true heat capacity of the system. In the absence of a normal mode calculation of the BPTI vibrational frequencies, it is possible only to make a rough estimate of the quantum correction to the classical heat capacity of the protein. Normal mode calculations on small peptides suggest that a given residue will have between 10 and 20 localized high-frequency modes with an average quantum-mechanical heat capacity on the order of k/2 per mode (hydrogen atoms, whose vibrations make a small contribution to the room temperature heat capacity, are not explicitly included in the analysis of the peptides). Assuming that similar modes occur in the protein, the classical heat capacity will be too large by about 5 k to 10 k per residue, or 0.5 k to 1.0 k/atom. With this correction, the heat capacity per atom calculated in the 100-psec simulation would be reduced from 3.1 k to a value between 2.1 k and 2.6 k atom; this is in good accord with calorimetric measurements.[52] Another effect which could influence the heat capacity calculations would be transitions of the protein from one harmonic well to another, resulting in variations of the zero-point vibrational energy, which is not included in the classical calculations. However, such transitions would probably cause significant changes only in low-frequency modes, which contribute little to the zero-point energy.

It is important also to consider the effects of quantum corrections on the distribution of momentum and displacement fluctuations in the protein. This is most readily done by expanding the quantum-mechanical canonical partition function in powers of Planck's constant, $\hbar$; the first term in this expansion is the classical partition function and the next nonvanishing term (of order $\hbar^2$) may be used to estimate the quantum corrections.[209] For an harmonic mode, one finds that the widths of the momentum and displacement fluctuation distributions given by a classical calculation are smaller than in the corresponding quantum calculation at the same temperature; the differences depend on the frequency of the mode and the temperature of the system. To a first approximation, the classical fluctuation distributions for a given mode at a temperature T are equivalent to quantum distributions at a lower temperature T_q; the magnitude of the correction for the given mode can be expressed in terms of a relative temperature difference

$$\Delta T/T = (T - T_q)/T \approx \frac{1}{12}\left(\frac{\hbar\omega}{kT}\right)^2$$

where ω is the angular frequency of the mode.[209] A characteristic frequency for the displacements in BPTI can be estimated from the rms atom position fluctuations by treating the atoms as independent (Einstein) oscillators and applying the equipartition theorem;[210] with the calculated fluctuation per Cartesian component of $[(x-\langle x\rangle)^2]^{1/2} = 0.43$ Å and an effective mass of 14 amu, the frequency is about 50 cm^{-1}. At 300 K, modes of this frequency are subject to corrections of about $\Delta T/T = 0.004$. The corresponding quantum correction to the classical rms displacement fluctuation for an atom depends on the shape of the effective potential well which limits the atomic motion; for harmonic wells, the typical rms fluctuation of 0.75 would be increased by only 0.2%, while the corrections would be still smaller for potentials approaching a square well. Higher frequency motions (e.g., bond length fluctuations) are subject to larger corrections (e.g., $\Delta T/T = 1.9$ for a vibration at 1000 cm^{-1}). However, many interesting internal coordinate fluctuations also have very small corrections (e.g., $\Delta T/T = 0.004$ for aromatic ring torsional oscillations at a frequency of $\sim$50 cm^{-1}). The results of this analysis suggest that the dynamical character of the interesting (lower frequency) structural fluctuations in proteins will be given to satisfactory accuracy by classical calculations.

7. Summary and Future Directions

We conclude this section by briefly summarizing the available molecular dynamics results and outlining possible future developments. It is now clear that direct simulation of dynamical events on the picosecond time scale is feasible for an isolated protein. Application of such simulation methods to BPTI shows that the structural fluctuations in this protein are sizable; particularly large fluctuations are found where steric constraints due to molecular packing are small (e.g., in the exposed side chains and external loops), but substantial mobility is also found in the protein interior (e.g., torsional fluctuations of aromatic rings). Local atomic displacements in the interior of the protein are correlated in a manner which tends to minimize disturbances of the global structure of the protein. This leads to fluctuations that are larger than would be permitted in a rigid polypeptide matrix.

To a first approximation, the effective dynamical subunits of the protein are the relatively rigid segments (e.g., amide groups, aromatic rings, isopropyl groups of valine) which are linked together by single bonds that permit larger torsional fluctuations. With the rigid segments, high-frequency motions associated with the harder internal coordinates (bond lengths, bond angles) occur; such fluctuations do not make significant direct contributions to the net atomic motions but appear to play an important

role in that small deformations of the harder internal coordinates are required to satisfy steric requirements engendered by the torsional displacements. The important structural fluctuations which arise from the torsional motions about the single bonds connecting the rigid segments have an irregular, Brownian appearance with superimposed high- and low-frequency oscillations; the latter appear to reflect elastic vibrations of sizable portions of the entire molecule. The Brownian character of the motion can be interpreted as resulting from frictional effects and random forces due to the fluctuating protein matrix acting on the rigid subunits. In cases where localized coordinates can be defined for the dynamical subunits, it is possible to quantitatively evaluate inertial and frictional contributions to the subunit dynamics by use of the Langevin equation. An example is given by the torsional motions of buried tyrosine rings of BPTI, for which the motional behavior has been shown to be roughly comparable to what would be expected for rings immersed in organic solvents. The motional behavior is dominated by the nonbonded interactions between the rings and their surroundings, and the calculations suggest that the modulation of the random forces has an impulsive rather than a smooth character. For other groups (e.g., ones involved in extensive hydrogen bonding) the random forces may vary more smoothly.

A molecular dynamics simulation of a native protein generates highly detailed information about its short time local motions. What we have presented here includes only a brief selection of the massive amounts of data available from one exploratory study on a small protein. It is to be hoped that the reported results do give some indication of the power of the method and its potential for the study of important problems.

It seems likely that the calculation of effective potential surfaces for specific motions in proteins will be applied to an increasing number of problems in coming years. These methods are relatively inexpensive, easy to implement, and yield much useful information on the energy requirements of specific motions and on the distribution of strain in proteins under specific perturbations. Such static approaches will be supplemented where necessary by molecular dynamics methods. Since full-scale calculations on proteins are rather expensive and limited to short intervals of time ($\leq$100 psec), a major goal of future work will be the development of dynamical methods adapted to specific problems.

In some problems, such as the initial stages of protein folding, it may be possible to use highly simplified potential functions in dynamical simulations since the detailed local motions are of less interest than are overall motions of the polypeptide chain. Some success has been achieved in such a study of the dynamics of BPTI,[211] using a simplified energy function similar to one described earlier.[212] Related functions, together with Brownian dynamics simulation methods,[213] have recently been applied to study polypeptide helix-coil transitions.[214]

In a study of local fluctuations in proteins (including reactive events) it may prove useful to separate the system into a part which is treated in detail (e.g., an active site plus substrate) and a part which is treated only approximately, either by use of a simplified dynamical model or by replacing it with a thermal bath having only statistical properties. The separation of a system into a detailed subsystem coupled to a stochastic bath has proved useful in other areas, as in the study of chemical dynamics on solid surfaces[215] and in theories of chemical reactions which proceed by complex formation.[216] In such an approach it would be desirable to limit the detailed subsystem to the atoms in or near the site of interest. Only atoms which have strongly correlated fluctuations would be included, with the remainder of the protein and the solvent surroundings constituting the thermal bath.

An approach that focuses on a small part of the protein may also be useful for developing methods for estimating the entropies of activation for barrier crossings involved in reactions. In some problems it may be possible to estimate the entropy

changes by assuming that only relatively localized low-frequency motions are altered by the barrier crossing and that their contribution can be evaluated by a local normal mode analysis.

Considerable work is needed to improve the molecular models and energy functions themselves. Most important is the development of methods to take account of the solvent. Although explicit treatments of the solvent molecules by a molecular dynamics approach[208] are essential to fully characterize their effect on the dynamics of the protein, alternative approaches based on an implicit model of the solvent may be useful. This could be done by replacing the potential functions for atoms near the protein surface by effective potentials of mean force, which account for the energetics of solvent interaction in an average way. The dynamical coupling of the protein surface atoms with the solvent might be modeled by using Brownian dynamics,[213,217] that is, by treating the solvent as a continuum characterized by empirical parameters (viscosity, dielectric relaxation time, etc.) and suitable stochastic fluctuations.

IV. CONCLUDING DISCUSSION

The experimental and theoretical work described in this review demonstrates that proteins under native conditions are molecules with considerable internal freedom, in which a wide variety of motions take place. These range from the local atomic fluctuations, through side-chain oscillations and group displacements involving secondary and other structural elements to large scale tertiary and quaternary rearrangements; concomitantly, the time scale of the motions ranges from pico to milliseconds or longer.

The presence of such motional freedom implies that a native protein at room temperature samples a range of conformations. Most of these are in the general neighborhood of the average structure, but at any given moment an individual protein molecule is likely to differ significantly from the average structure. This in no way implies that the X-ray structure, which corresponds to the average in the crystal, is not important; rather, it suggests that fluctuations about that average are likely to play a role in protein function. Both conformational and energy fluctuations with local to global character are likely to be important. In a protein, as in other nonrigid condensed systems, structural changes proceed through fluctuations, and larger structural changes arise from correlated fluctuations. Perturbations, such as ligand binding, that produce tertiary or quaternary alterations do so by introducing forces that bias the fluctuations in such a way that the protein makes a transition from one structure to another. Alternatively, the fluctuations can be regarded as searching out the path or paths along which the transition takes place.

In what follows we shall briefly consider some of the areas where fluctuations in structure and energy may be important in the biological activity of proteins. Since in a protein (as in any polymeric system in which rigidity is not supplied by cross-linking due to covalent bonds) significant fluctuations cannot be avoided; they must have been taken into account in the evolutionary development. Although the existence of the fluctuations is now well established, our understanding of their biological role in specific areas is rather limited. Nevertheless, it is worthwhile to review some of the possibilities, since they will be subjects of intensive research in the coming years. We shall treat a number of functions of globular proteins including storage and transport of atoms, molecules, and electrons; binding involving enzyme inhibitors, hormone receptors, antibodies, and protein-nucleic acid interactions; and catalytic action in enzymes. In all of these functions the inherent flexibility of proteins and the concomitant motions are likely to be of importance.

For the transport protein hemoglobin there is more evidence concerning the role of motion than for any other protein. The structural changes that occur on ligand binding

and their relation to the allosteric mechanism are well documented. A detailed reaction path for the transmission of tertiary alteration from the oxygen binding site at the heme to the surface of the subunit has been determined by a combination of experimental and theoretical analyses (see Section III.A.2). What has not yet been done is to show in corresponding detail the coupling between these tertiary structural changes and the relative stabilities of the two quaternary structures. It is clear, nevertheless, that an important role of the quaternary structural change is to transmit information over a longer distance than could take place by tertiary structural changes alone; the latter are generally damped out over rather short distances unless amplified by the displacement of secondary structural elements or domains. The detailed dynamics of the allosteric mechanism has not yet been investigated; in particular, the barriers along the reaction path have not been analyzed nor has the importance of the fluctuations in the activated processes involved been determined.

For the storage protein, myoglobin, there are small structural changes on ligand binding, but their signficance is not known. What has been demonstrated, as described earlier (Section III.A.3), is that fluctuations in the globin are essential to the binding process; that is, the protein matrix in the liganded and unliganded X-ray structures is so tightly packed that there is no sufficiently low energy path for the ligand to enter or leave the heme pocket. Only through structural fluctuations in certain bottleneck regions can the barriers be lowered sufficiently to obtain the observed rates. Energy minimization has been used to investigate the displacements involved and the resulting barrier magnitudes, but supplementary dynamic studies are needed to analyze the activation entropies and rates of ligand motion across the barriers.

There are many proteins and peptides in which the transport of substances is through the molecule rather than via overall translation as in hemoglobin. The most obvious cases are membrane systems, in which fluctuations are likely to be of great importance in determining the kinetics of transport. For channels that open and close (e.g., gramicidin) as well as for active transport involving enzymes (e.g., ATPases), fluctuations, in some cases highly correlated ones, must be involved. At present, structural details and studies of the motions are lacking, but this is an area where dynamic analyses are likely to be made in the near future.

In electron-transport proteins such as cytochrome c, protein flexibility is likely to play two roles in the electron transfer. The evidence presently available favors a vibronic-coupled tunneling mechanism for transfer between cytochrome c and other proteins, although outer-sphere mechanisms have not been fully excluded.[6,218] In the vibronic-coupled tunneling theory, tunneling processes which would be energetically forbidden for rigid proteins become allowed if the appropriate energies for conformational distortions are available. A fit of experimental data to the theory[6] indicates that the important fluctuations are characterized by an average frequency on the order of 250 cm^{-1}. Also, the transfer rate is a sensitive function of donor-acceptor distance and may be greatly increased by surface side-chain displacements which allow for the closer approach of the interacting proteins.[218]

For proteins involved in binding, flexibility and fluctuations enter into both the thermodynamics and the kinetics of the reactions. For the rate of binding of two macromolecules (protein-antigen and antibody, protein-inhibitor and enzyme), as well as for small multisite ligands, structural fluctuations involving side chains, hydrogen-bonding groups, etc., can lead to lowering of the free energy barriers. Furthermore, dividing the binding process into successive steps for which flexibility may be needed can increase the rate.[219] The required fluctuations are likely to be sufficiently small and local that they will be fast relative to the binding and therefore not rate limiting.

The relative flexibility of the free and bound ligand has to be considered in the overall thermodynamics of the binding reaction. If the free species have considerable

flexibility and fluctuations are involved in the binding step as described above, it is likely that the bound species will be less flexible and so a significant entropic destabilization will result. Thus it has been suggested that for strong binding in cases where the rate is not important, relatively rigid species are desirable. This would reduce the conformational entropy decrease and could lead to a very favorable enthalpy of binding if there is high complementarity in the two binding sites. However, it must be remembered that some flexibility and increase in the conformational space available to the bound species has a stabilizing effect which partly compensates for the loss of translational and rotational entropy on binding.[220] Thus, the balance between flexibility and rigidity will be determined by the function of the binding in each particular case. A classic example of very strong binding is given by BPTI, an unusually stable and rather rigid protein whose geometry is such as to fit well into the trypsin active site. However, even for this relatively well-studied case, neither the atomic details of the binding reaction nor the origin of the thermodynamic parameters has been worked out. For protein hormones and receptors, the important factors are less clear though it is generally presumed that binding leads to conformational change in the receptor. There are relatively rigid hormones like insulin and highly flexible ones like glucagon. Many of the transmitter substances are fairly flexible, though, because of their small size, fewer degrees of freedom are involved. A flexible molecule like glucagon with different structures in solution when bound to the receptor (random coil vs. α-helical) might serve via multisite interactions to pull the receptor from one conformation to another while itself undergoing a structural change. However, the mechanism and dynamics of such processes have yet to be worked out.

The flexibility that has been found to exist in the immunoglobulin is likely to be important. In addition to some local motional freedom at the binding site, the existence of inter- and intradomain flexibility may make antibodies better able to bind antigens of different sizes as well as to distort appropriately to interact with complement. The time scales associated with the various possible motions (10^{-12} to 10^{-6} sec) are likely to be rapid with respect to the binding reaction.

In the function of proteins as catalysts, there is the greatest possibility of contributions from motional phenomena. The role of flexibility per se has often been discussed, particularly from the viewpoint of structural changes induced by the binding of the substrate.[190] In addition to cooperative effects due to quaternary alterations, analogous to those for hemoglobin, there are a variety of results that can arise from the perturbation of the tertiary structure. One example is the ordered binding of several substrates (or effectors and substrates), with the first molecule to bind altering the local conformation so as to increase or decrease the subsequent binding of other molecules. The occurrence of large-scale changes, such as the closing of active-site clefts by substrate binding, as in certain kinases, has been interpreted in terms of catalytic specificity, alteration of the solvent environment of the substrate, and exclusion of water that could compete with the enzymatic reaction. In large enzymes with more than one catalytic site or in coupled enzyme systems, conformational freedom may be important in moving the substrate along its route from one site to the next.

The flexibility of the substrate binding site in enzymes can result in effects corresponding to those already considered in receptor binding. In the enzyme case there exists the often-discussed possibility of enhanced binding of a substrate with its geometry and electron distribution close to the transition state; for this to occur, conformation fluctuations are essential. Entropic effects also are likely to be of significance, both with respect to solvent release on substrate binding and possible changes in vibrational frequencies that increase the vibrational entropy of the bound system in the enzyme-substrate complex or in the transition state. There are also indications that the inactivity of enzyme precursors can result from the presence of conformational free-

dom in residues involved in the active site. The entropic cost of constraining them in the proper geometry for interacting with the substrate may be so high that the activity is significantly reduced relative to that of the normal enzyme where the same residues are held in place more rigidly. Such a control mechanism has been suggested for the trypsin, trypsinogen system, and for other proteins.[42]

As to the time dependence of fluctuations and structural alterations, there are a variety of possibilities to be considered. In the binding of reactants and release of products, the time course of fluctuations in the enzyme could interact with the motion of the substrate. The opening and closing fluctuations of active-site clefts, which have been estimated to decay in a time on the order of 10^{-11} sec for lysozyme, may be modified by interactions with the substrate as it enters or leaves the binding site. Further, as described for activated processes in the interior of a protein (see Section III.C.5), fluctuations could play an essential role in determining the effective barriers for the catalyzed reactions. If the substrate is relatively tightly bound, local fluctuation in the enzyme could couple to the substrate in such a way as to significantly reduce the barriers. Further, if such coupling effects exist, specific structures could have developed through evolutionary pressure to enhance the required fluctuations. Frictional effects that occur in the crossing of barriers in the interior of the protein could act to increase the transition state lifetime and so alter the reaction rates relative to those predicted by conventional rate theory.

It has been suggested that large-scale fluctuations in the enzyme contribute to their catalytic functions.[20] Most likely appear to be effects resulting from charge fluctuations either due to counterions[19] or motions of charged side chains.[28] These are estimated to take place on a 10^{-6} - to 10^{-9} - sec time scale and could, in principle, couple with the reaction of substrates involving ionic species. Less likely are mechanisms in which structural fluctuations at the active site are assumed to be tightly coupled to the protein surface, which is perturbed by particular patterns of collisions with the solvent.[221] While collisions of solvent molecules with the protein surface undoubtedly play an important role in exciting and damping structural fluctuations, the tight coupling assumption is unlikely to be correct. In proteins, as in other normal materials, high-frequency structural fluctuations have a localized character, so that a collision at the protein surface is not expected to have a significant mean effect at a distant active site; any such effect would be swamped by the intrinsic thermal fluctuations of the protein. An alternative and more likely possibility is that the energy released locally in substrate binding is utilized directly for catalyzing its reaction, perhaps by inducing certain fluctuations. Whether such an effect occurs would depend on the rate of dissipation of the (mainly) vibrational energy and the existence of patterns of atoms and interactions to channel the energy appropriately. It will be of great interest to determine whether any of the rather speculative possibilities outlined here for the role of energy of structural fluctuations in enzymatic reactions can be documented theoretically or experimentally for specific systems.

In the present discussion of the biological role of flexibility and fluctuations, as in the other parts of this review, we have not considered the problem of protein folding. In the folding process, fluctuations and concerted motions must play an essential role in searching out the native structure. The different time scales of such motions may well be important in determining the rate of the folding process. Further, internal diffusion and diffusion through solvent of portions of the protein are certain to be involved.[222] Thus, the topic of protein-folding is one that needs to be considered from a dynamic viewpoint. Unfortunately, the vast range of configurational space that has to be examined, the many barriers that are likely to be present, and the long time scale of the overall process make it very difficult to study the detailed motions of the atoms

involved in the folding process. Nevertheless, some progress in this most challenging area of protein dynamics can be hoped for in the not too distant future.

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