CORRELATIONS BETWEEN THREE-DIMENSIONAL STRUCTURE AND FUNCTION OF IMMUNOGLOBULINS

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I. INTRODUCTION

Elaborate immune defense mechanisms protect vertebrates against attacks from environmental factors such as infections from viral and bacterial pathogens. The recognition of foreign molecules (antigens) and ensuing biosynthesis of specific proteins (antibodies) which bind the antigenic structures are the first steps towards their further processing and elimination from the vertebrate body. It is a well established fact that under suitable experimental conditions, almost any foreign chemical substance will be recognized and neutralized by the immune system with a high degree of specificity. In addition to the specificity of the adaptive immune response of vertebrates, earlier studies recognized that the response is frequently heterogeneous or degenerate, i.e., that a single, well-defined antigenic determinant (e.g., a dinitrophenyl [DNP] group attached to a carrier antigenic protein) will induce antibodies made up of different molecular species. This seemingly inexhaustible capacity to react to diverse antigenic challenges provided an attractive system to study the molecular and genetic bases of recognition and individuality, two major features of living organisms. These features are included in antibodies, molecules which thus became a fascinating model subject for biological and biochemical studies. The structural bases of the specificity and physiological function of antibody molecules have been extensively investigated using experimental approaches such as amino acid sequence determination, optical rotatory dispersion, circular dichroism, affinity labeling, electron microscopy, and many other physicochemical techniques. More recently, techniques that give further insights into three-dimensional structure and chemical conformation such as X-ray diffraction and nuclear magnetic resonance (NMR) have been successfully employed to provide essential information in the study of the molecular basis of biological recognition, individuality, and specificity. This review will be directed towards results obtained by X-ray diffraction analyses and to the correlation of these results with those obtained by other experimental approaches. Other reviews of this subject have also been published.[1-8]

A number of extensive and up-to-date reviews on the principles and techniques of X-ray crystallographic analysis of protein structure

are available.[9-12] The first experimental requirement for the solution of the three-dimensional structure of a protein by X-ray diffraction is the availability of large crystals (ideally 1 × 1 × 1 mm) which are highly ordered and diffract X-rays to high resolution. They should be stable under the monochromatic, characteristic radiation X-ray beam (usually CuKα) used for their analysis. In attempting to solve a protein structure *ab initio*, the problem of determining the phases of the scattered X-ray beams is solved by the introduction of heavy-atom ligands at a few, specific sites in the crystal lattice, a procedure which results in isomorphous heavy-atom derivatives. From measurements of the intensities of X-ray reflexions of native protein crystals and the reflexions from protein-heavy atom crystals, intensity and phase data can be obtained and used to calculate a Fourier series representation of the electron density in the crystal. At high resolution (e.g., 2 A) the Fourier map thus obtained allows the tracing of the polypeptide chain and, with a knowledge of the amino acid sequence, the placing of amino acid residue side chains. The three-dimensional structure obtained from crystallographic analysis has been shown to be very close to the one inferred to occur in solution for several proteins. The structure in the crystalline state is at least one of several possible conformational states. However, since X-ray measurements are made during prolonged periods of time (hours), only stable conformations can be analyzed. In some cases, such as that of oxyhemoglobin and deoxyhemoglobin, these conformations can be used to analyze other possible intermediate conformational states or structural transitions.[13]

A recent study attempts to give a quantitative picture of motion in protein molecules.[14] In this study, the dynamics of bovine pancreatic trypsin inhibitor, a small, globular protein with a chain length of 58 amino acid residues, has been simulated by solving the equations of motion for all atoms with an empirical potential energy function. This function contains terms for interactions associated with bond angles and lengths, hydrogen bonds, dihedral angles, and nonbonded (van der Waals) interactions. The coordinates obtained from crystallographic studies were used as the initial positions. The overall average temperature reached in the simulation was 295°K. The time-averaged positions of all atoms were close to those in the crystal structure with a root mean square deviation of 1.2 A for α-carbon atoms. The regions with defined secondary structure, α helix and β sheets, showed the smaller deviations. The largest deviations were observed at the N- and C-terminus, at an external loop of the polypeptide chain, and at exposed amino acid side chains. Some of the positional differences between the crystal structure and the time-averaged dynamical structure could be due to approximations in the empirical potential-energy function in which neither solvent effects nor H atoms were included. It is interesting to observe that concerted motions were detected. One of these involved an outside look which showed oscillatory motions as well as changes in shape.

II. POLYPEPTIDE CHAIN STRUCTURE OF IMMUNOGLOBULINS

Antibodies which belong to the class of serum proteins called α globulins or immunoglobulins are usually heterogeneous, a fact that can pose serious problems for structural studies such as X-ray diffraction and amino acid sequence determination. This problem has been overcome by studying homogeneous pathological immunoglobulins produced by monoclonal neoplastic lymphocytic cells in mice and man. These myeloma proteins, associated with the spontaneous occurrence of multiple myelomatosis and other pathological lymphoproliferative disorders in man and with experimentally induced tumors in mice, have been shown to be closely related to normal immunoglobulins and antibodies by a number of structural and functional properties. A striking example is that of the induced murine antiphosphorylcholine antibodies which have amino acid sequences identical to those of myeloma proteins. Human or mouse myeloma proteins can be obtained in large quantities and can be readily purified. In general, they exist as complete molecules although occasionally, only a portion of the molecule is present, the "light chain" most frequently. Human Bence-Jones proteins, which can be isolated from the urine of patients with multiple myelomatosis, are light chains which

display unusual thermal behavior; they precipitate at 40 to 60°C, redissolve at 95 to 100°C, and reprecipitate on cooling. (See Reference 15 for a detailed review of plasma-cell dyscrasias and pathological immunoglobulins.) Immunoglobulins (Ig) can be divided into the major classes or isotypes (characterized by their H chain type) shown in Table 1, which also includes their molecular weight and relative abundance in human serum. IgM, IgA, and IgG contain carbohydrates which are covalently attached to the molecule and are largely hexose and hexosamine molecules with smaller amounts of sialic acid and fucose.

As shown in Table 1, the IgG class is the most abundant in normal serum and is also the most commonly occurring isotype (or class) in myeloma immunoglobulins. The IgG class has been the most intensively studied. A diagrammatic representation of the structure of a human IgG is shown in Figure 1 which introduces some of the nomenclature that will be used in this review. The molecule consists of two identical "light" (L) polypeptide chains (each with a molecular weight of 20,000 to 25,000 and consisting of approximately 214 amino acid residues) and two identical "heavy" (H) polypeptide chains (each with a molecular weight of 50,000 to 55,000 and consisting of approximately 450 amino acid residues). Each chain can be divided into "homology regions" of variable (V) and constant (C) sequences containing approximately 110 amino acids. The L chain consists of regions V_L and C_L and the H chain consists of regions V_H, C_H1, C_H2, and C_H3. The four individual chains (two light and two heavy) are covalently linked by interchain disulfide bonds. Owing to the existence of many noncovalent interactions between the L and H chains, drastic chemical conditions (such as acid pH, exposure to urea, etc.) are required for the separation of this structure into individual polypeptide chains after reduction of the interchain disulfide bonds.

Earlier studies on the structure of immunoglobulins proceeded by attempts at cleaving the molecules into smaller fragments which could then be more easily characterized. Porter[16] found that controlled enzymatic digestion of rabbit IgG produces three fragments (see Figure 1), each with a molecular weight of approximately 50,000. Two of these fragments are identical and called the Fab fragments; the third is called Fc. Each Fab fragment consists of a complete L chain and the N-terminal half of an H-chain called Fd. The Fab fragment is so named because it retains the antigen binding activity of the parent molecule, although it can only behave as a monovalent antibody. No complement fixation activity can be observed in the immune Fab-antigen complex, indicating that the Fc region is required for complement fixation. Fab and Fc fragments can be obtained from immunoglobulins of different animal species. Controlled digestion of human or rabbit IgG protein by pepsin cleaves both H chains on the C-terminal side of the inter-heavy-chain disulfide bond(s) and produces a major fragment called $F(ab')_2$.[17] By subsequent reduction and alkylation of the inter-heavy-chain disulfide bond(s), a fragment called Fab′ (Figure 1) is readily obtained. The N-terminal portion of the H chain in this fragment is called Fd′ and is about ten amino acid residues longer than Fd

TABLE 1

Immunoglobulin Isotypes

Isotypes	Approximate mol wt	Heavy chain class and mol wt	Light chain class and mol wt	Total circulating immunoglobulin in serum of normal individuals (%)	Carbohydrates by weight (%)
IgM	900,000	μ; 60,000	κ or λ; 25,000	5—10	10—12
IgA	170,000—500,000	α; 60,000	κ or λ; 25,000	10—20	10—12
IgG	150,000	γ; 50,000	κ or λ; 25,000	70—80	2—3
IgD	180,000	δ; 50,000	κ or λ; 25,000	1	—
IgE	180,000	ε; 60,000	κ or λ; 25,000	1	—

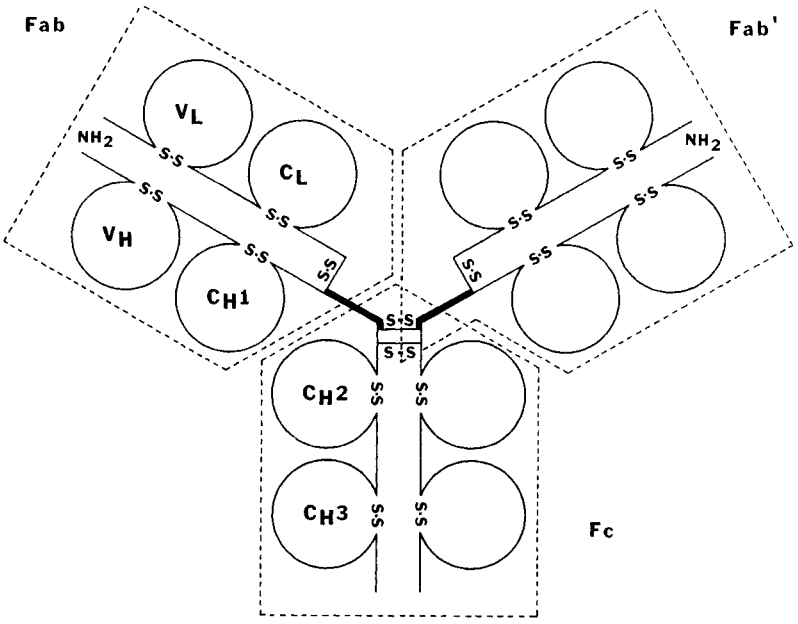


FIGURE 1. Diagram of a human immunoglobulin (IgG1) molecule. The light (L) chains (molecular weight about 25,000) are divided into two homology regions: V_L and C_L. The heavy (H) chains (molecular weight about 50,000) are divided into four homology region: V_H, C_H1, C_H2, and C_H3. C_H1 and C_H2 are joined by a "hinge" region indicated by a thicker line. Cleavage of the IgG1 molecule by papain generates Fab fragments (molecular weight about 50,000) consisting of an L and an Fd polypeptide chain and Fc fragments (molecular weight about 50,000). Cleavage by pepsin followed by reduction of inter-H-chain disulfide bonds generates an Fab′ fragment consisting of an L and an Fd′ polypeptide chains. Interchain and intrachain disulfide bonds and the N-termini of the L- and H-chains are indicated. (From Poljak, R. J., *Contemporary Topics in Molecular Immunology*, Vol. 2, Reisfeld, R. A. and Mandy, W. J., Eds., Plenum Press, New York, 1973, 2, chap. 1. With permission.)

in human IgG immunoglobulins. Similar Fab fragments have been obtained by the use of other proteolytic enzymes such as trypsin. All these proteolytic enzymes split peptide bonds in a region which appears to be openly accessible and which has been called the "hinge" (or "flexibility") region linking Fab to Fc. In Figure 1, the hinge regions are indicated by thicker lines.

The L chains of human IgG can be antigenically classified into two isotypes (or classes) called κ and λ, each characterized by a unique sequence in their C-terminal regions. Human IgM, IgA, IgD, and IgE also possess κ and λ light chains but their heavy chains are different and specific to each class. Amino acid sequence studies of human myeloma L chains have shown that L chains of the same class (κ or λ) consist of a C-terminal half of constant amino acid sequence (C_L) and an N-terminal half of variable sequence (V_L). Because of the genetic and functional implications, the patterns of variability of L-chain sequences have been extensively analyzed. It was observed that within a given class of L chains, there are sequences in the variable region which are very similar to each other and therefore constitute a "subgroup." Three such subgroups have been recognized in human κ chains and five have been proposed for human λ chains. All chains within a subgroup are very similar in sequence except at certain positions within V_L where extreme variability is observed.[18,19] Kabat and Wu proposed that these hypervariable sequences constitute the regions of the L-chain structure which are in contact with antigen, so that the presence of different sequences in these regions will modify the antibody specificity. Comparative studies on H chains of the same class have shown that the sequence of the regions C_H1,

C_H2, and C_H3 remain constant whereas the V_H region (Figure 1) displays variability. Just as in the L chain, the variable region of the H chain occurs at the N-terminal end of the molecule and is approximately 110 amino acid residues in length and contains hypervariable regions.[19,20]

H-chain sequences in the Fc region of rabbit IgG revealed another important structural features, namely, the existence of sequence "homology regions."[21] Two sequences are said to be homologous when chemically identical or related amino acids appear at corresponding positions in the two polypeptide chains, e.g., a serine in the first sequence and a threonine at the corresponding position in the second. Another criterion for homology between two sequences is to examine amino acid differences in terms of the minimum number of mutational events that are necessary to change the nucleotide sequence specifying the first chain to that specifying the second polypeptide chain. If the number of mutations is smaller than can be expected from random events, the sequences are said to be homologous. By either of the two criteria outlined above, one can define four constant homology regions: C_H1, C_H2, and C_H3 in the H chains and C_L in the L chains. The N-terminal variable regions V_L and V_H are homologous to each other but have a weaker homology to C_L, C_H1, C_H2, and C_H3. It is interesting to observe that the pattern of a single intrachain disulfide loop of similar length exists in each of these regions (Figure 1). In addition to their genetic implications, these findings also suggest that the existence of homology units greatly influences the overall three-dimensional folding of IgG molecules. Inspired by these and other observations, several L and H polypeptide chain folding schemes were proposed [22-25] which can be summarized by describing the tertiary structure of immunoglobulins as consisting of globular subunits, each corresponding to a homology region.

Electron microscopy has provided the first direct pictures of the general shape and structure of immunoglobulins. By using a divalent hapten (bis-N-DNP-octamethylenediamine, DNP: dinitrophenyl) as a link between anti-DNP antibody molecules, Valentine and Green[26] were able to deduce the general shape of an IgG molecule and the arrangement of the Fab and Fc regions from electron micrographs (Figure 2). When combined with antigen, the shape appears to be that of a letter "Y," the angle between the two Fab regions being dependent on the number of IgG molecules connected by the bis-DNP haptens. The flexibility required to obtain this variable separation is assumed to reside in the "hinge" region connecting Fab to Fc. Electron micrographs of an IgA protein produced by the (laboratory induced) mouse plasma cell tumor MOPC 315[27] indicated that the IgA structure consists of globular units or domains. In this study on IgA, a divalent bis-DNP hapten was also used, taking advantage of the fact that the MOPC 315 myeloma protein has the specificity of an anti-DNP antibody. No such globular subunits or domains had been consistently observed in electron micrographs of IgG.

Evidence has been obtained from fluorescence polarization and electron spin resonance studies[28] which indicates that immunoglobulins from frog and tortoise have a fairly compact general structure with no marked intramolecular rotational freedom, in contrast to the pronounced flexibility of mammalian IgG. A corresponding difference in flexibility can also be observed, but to a lesser extent, when comparing IgM molecules from these same species. As segmental flexibility increases in passing from lower vertebrate immunoglobulins to mammalian immunoglobulins, it has been suggested[28] that structural evolution of antibodies has in part been directed toward increasing this flexibility, thus favoring the interaction of the polyvalent antibody with foreign antigens. Indeed, flexibility in the antibody molecule appears to favor the precipitation of foreign antigen, facilitating its elimination. Due to experimental limitations, it is unclear whether IgE, whose general structure is more compact[29] is capable of forming a precipitate after antigen binding.[30] It has been shown by electron microscopy studies that the angle between the two Fab regions in carp macroglobulins does not vary;[31] these macroglobulins only give rise to a weak precipitation reaction.[32] In hen IgG, weak precipitation reactions[33] and limited flexibility[28] have also been observed.

Affinity labeling experiments have provided further insight into the location and topography of antigen binding sites. In these experiments, a haptenic group is specifically (reversibly) bound and covalently attached to an amino

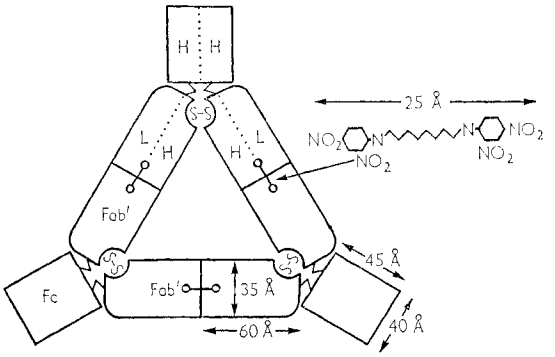


FIGURE 2. Diagram of a trimer of IgG molecules linked by bifunctional hapten molecules observed by electron microscopy. (From Valentine, R. C. and Green, N. M., *J. Mol. Biol.*, 27, 615, 1967. With permission.)

acid side chain on the antibody molecule by means of a chemically reactive group on the hapten. In principle, amino acid side chains that are part of the combining site in antibody molecules, or are close to it, can be specifically labeled and identified. Using a number of different reactive haptens and antibodies (and also myeloma proteins that behave like antibodies), it became evident that the antigen binding site was confined to the V_L and the V_H regions. Amino acid side chains in or close to the regions of hypervariable sequence in L and H chains were labeled,[22,34,35] which supported the hypothesis that these regions contribute to (or determine) the antigen binding site of antibodies. Synthetic antigens have been used as an experimental tool for analyzing the specificity of antibodies, the role of immunodeterminant groups in the antigen-antibody reaction, and the dimensions of the combining sites. With different antigens, the most exposed end of an immunodeterminant group has consistently been found to make the larger contribution to the energy of the binding reaction.[36-38] The dimensions of the binding site have been determined to be of the order of 35 × 15 × 10 A using probes consisting of antigenic polysaccharides and polypeptides on induced specific rabbit antibodies.

III. THREE-DIMENSIONAL STRUCTURE OF LIGHT-CHAIN DIMERS

High-resolution crystallographic analyses have been reported for a human chain dimer[39] and for human κ-chain V_L fragments.[40,41] In addition to these studies on crystalline L chains, crystal structure studies of Fab fragments have also provided detailed structural models of human and murine immunoglobulin L chains.

A. Human λ Chains: the Mcg Bence-Jones Dimer

The Mcg human myeloma immunoglobulin (IgGl [λ]) and its constituent λ chains, secreted as an urinary Bence-Jones protein, have been intensively studied by X-ray diffraction and amino acid sequencing techniques.[39,42] An electron density map at a nominal resolution of 2.3 A has been calculated and interpreted in terms of an atomic model[43] for the λ-chain dimer. The amino acid sequence of the Mcg L chain has also been determined.[42] The Mcg protein is perhaps unusual in that it contains a deletion of 15 residues in the hinge region of its H chain. This deletion includes the cysteine residues which form interchain disulphide bonds with an L chain and with another H chain (see Figure 1). The Mcg L chains are covalently bonded to each other by an interchain disulfide, an unusual bond in human IgG molecules. Amino acid substitutions at three positions in the constant (C) region of this λ chain have also been detected in human serum samples, indicating that this chain belongs to a distinct isotype or subclass of human λ chains.[44]

A 3.5-A resolution map allowed the tracing of the polypeptide chain which is illustrated in Figures 3 and 4. Subsequent work[43] has extended the resolution of this structure determination and resulted in a three-dimensional model in which all amino acid side chains can be assigned to electron density features of the Fourier map. Some of the more striking features of this structure will be briefly discussed in the following paragraphs.

The intrachain disulfide bonds in the V and C regions were identified as features of higher electron density than the rest of the molecule. The interchain disulfide bond corresponded to a region of lower electron density, a feature which was attributed in part to the fact that this bond is near the surface of the molecule and consequently, has more freedom of motion. Two globular regions, one containing the two V-region sequences and the other containing the two C-region sequences, were traced in the Fourier map. They are joined by two strands of

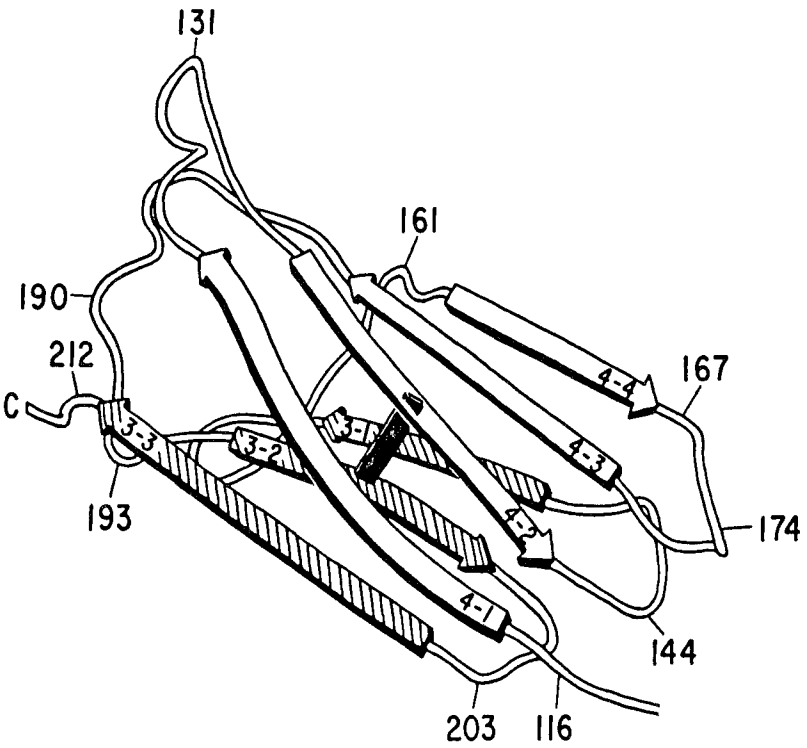


FIGURE 3. Schematic representation of the polypeptide chain backbone of the C_L homology subunit showing the two β sheets. One β sheet consists of four strands of antiparallel polypeptide chains, 4-1 to 4-4, shown by white arrows. The second β sheet consists of three strands shown by striated arrows, labeled 3-1 to 3-3. The intrachain disulfide bond linking the two sheets is shown as a black bar. (Reprinted with permission from Edmundson, A. B., Ely, K. R., Girling, R. L., Abola, E. E., Schiffer, M., Westholm, F. A., Fausch, M. D., and Deutsch, H. F., *Biochemistry*, 13, 3816, 1974. Copyright by the American Chemical Society.)

polypeptide chain (called "switch" regions since at these positions there is a change from variable sequences [V] to constant sequences [C]) which are exposed to the solvent. The accessible location of the switch regions explains the easily obtained proteolytic cleavage of L chains into V and C domains.[45,46] One of the most interesting aspects of this structure is that although both L chains in a dimer have identical amino acid sequences, the angle between the major axes of the V and C homology regions are different in the two chains, about 70° in one chain and 110° in the other. In this respect, the L-chain dimer resembles the Fab structure and indicates that the switch regions are sites of flexibility with potential functional significance. The V and C regions are very similar in three-dimensional structure, mainly consisting of two roughly parallel sheets of β structure (to be described for Fab structures). However, residues 48 to 60, which include a region of hypervariable sequence in the V subunit, have no structural equivalent in the C subunit. There is a cavity with a diameter of approximately 15 A and a depth of 10 A towards the amino terminal end of the V domain, which has been described as resembling a truncated cone.[43] Hypervariable segments of both L chains delineate this cavity, which is the equivalent of the antigen binding site contained in the Fab regions of antibody molecules. On the basis of this similarity, it was proposed that L-chain dimers might have acted as primitive antibodies.[43] However, the site differs from that of the Fab fragments by being wider, more symmetrical, and in leaving a channel towards the interior of the dimer which has been proposed[43] as an extension of a putative antibody combining site.

In the Mcg dimer, the V_L-V_L interactions and those between the two C_L regions are different

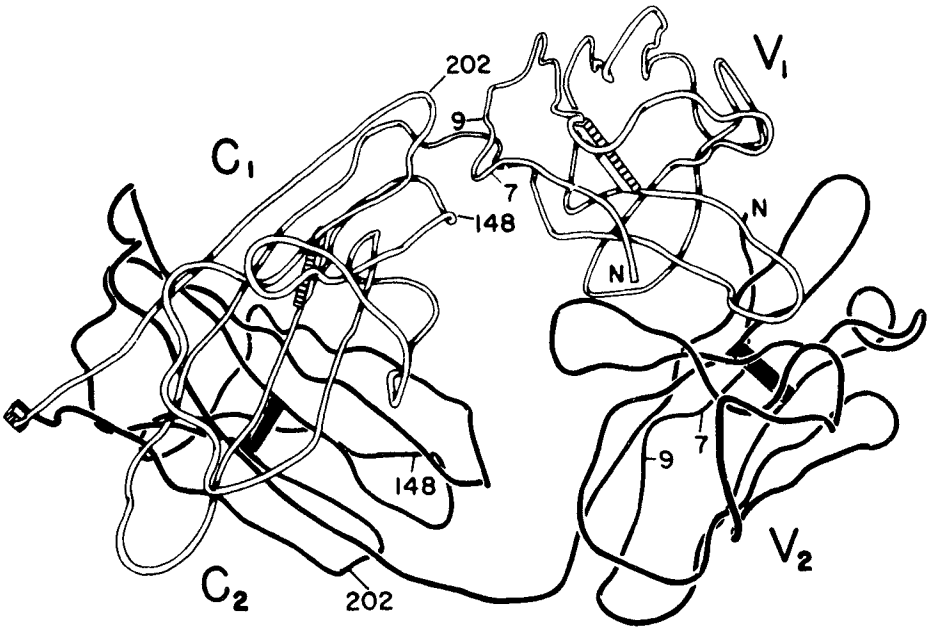


FIGURE 4. Tracing of the polypeptide chains in the Mcg L-chain dimer. This figure shows the different conformation assumed by the two identical chains. Note that in one of the chains, residues 9 and 202, and 7 and 148 are at much shorter distance than in the other chain. (Reprinted with permission from Schiffer, M., Girling, R. L., Ely, K. R., and Edmundson, A. B., *Biochemistry*, 12, 4620, 1973. Copyright by the American Chemical Society.)

in spite of the fact that V and C regions share a common structure. In the C regions, the β sheets, consisting of four antiparallel polypeptide chain strands, closely interact with each other to form the dimer. The other β sheets, containing three antiparallel strands, face the solvent. The V_L-V_L interactions involve different residues. This conformation can be described as "rotational allomerism"[47] in which the V regions are rotated relative to the C regions. Although these contacts resemble those observed in Fab fragments, the interactions between V_L and V_H are closer than those in the V regions of the L-chain dimer, giving rise to a more compact V_L-V_H domain.

B. Ligand Binding

Several ligands were diffused into crystals of the Mcg L-chain dimer. They included an iodinated derivative of 1-fluoro-2,4 dinitrobenzene, DNP derivatives of lysine and leucine, 5-acetyluracil, menadione, caffeine, theophylline, colchicine, ε-dansyl lysine, and phenantroline.[43] Low-resolution difference Fourier maps were calculated to locate the binding sites. These were found to occur in the cavity described above which is homologous to an Fab combining site. Some ligands penetrate beyond the cavity into the solvent channel between the V_L-V_L contacts.

Although binding constants of 10^4 to 10^5 l/mol have been reported for some of the L-chain ligands,[43] it is difficult to evaluate the physiological significance of the binding activity. It should be kept in mind that the ligands investigated are highly reactive substances, that L-chain dimers are not detected in normal serum, and finally, that the Mcg IgG protein does not seem to bind the L-chain ligands.

C. Structure of V_κ Fragments

The structures of two V_κ dimers have been reported.[40,41] Bence-Jones protein Rei yielded a crystalline V_κ dimer whose structure was determined to 2.8-A resolution.[40] This work established the presence of two β sheets in each of the V_κ monomers as described above for the λ-chain dimer. Approximately 50% of all amino acid residues were found to form part of the β sheets surrounding a hydrophobic interior filled with invariant or semi-invariant residues. In agreement with circular dichroism data indicat-

ing a small percentage of helical folding in immunoglobulin L chains, only one turn of helical conformation (a distorted α helix) was detected in the structure. Conserved glycine residues, at positions 16, 41, and 57, are involved in hairpin turns of the polypeptide chain. A large cavity, surrounded by the hypervariable regions of both V_κ monomers, resembles the antibody combining site of Fab fragments. The fact that the V_κ dimer has a three-dimensional structure closely resembling that of the λ-chain dimer described above indicates that the V regions can associate independently of interchain contacts in the C regions.

The crystal structure of a second V_κ dimer, Au, has been determined in the same laboratory.[41] An interesting feature of this structure is that Tyr 49 assumes a different conformation than that of the identical residue observed at that position in V_κ Rei. Thus, as a result of the influence of neighboring sequences, a given residue may contribute to different conformations at the combining site.

IV. THREE-DIMENSIONAL STRUCTURE OF Fab FRAGMENTS

The three-dimensional structures of two Fab fragments have been determined to high resolution. The Fab′ fragment from a human IgG1 (λ) has been studied by crystallographic techniques to a nominal resolution of 2.0 A.[48-50] The amino acid sequences of the L chain[51] and the V_H region of IgG New[52] have been determined. A second Fab fragment obtained from the mouse myeloma McPC 603 IgA protein has been studied to a nominal resolution of 3.1 A.[53,54] Part of the amino acid sequences of its L (κ) chain and of the H (α) chain have also been determined.[55] Some aspects of these structures will be reviewed below with particular emphasis placed on the human Fab structure only because of the author's direct involvement with the determination of its structure.

A. The Structure of Fab New

Fab and Fab′ fragments from human IgG1 (λ) myeloma proteins yield crystals[56-59] which are suitable for X-ray diffraction analysis. Crystals of the Fab and Fab′ fragments give identical diffraction patterns, indicating that they have the same three-dimensional structure.[58,59] A difference in the diffraction pattern would be expected on the basis that Fab′ is about ten amino acid residues longer than Fab (in its Fd′ chain). However, the high resolution studies of Fab′ New[49,50] indicated that the C-terminus of the Fd′ chain does not contribute significantly to the diffracted X-ray intensities, probably due to random motion at the hinge region of the Fd′ chain. Because of this feature, crystalline Fab and Fab′ are equivalent, and consequently, the Fab designation will be used here although the actual structure determination was pursued on Fab′ New crystals.

Fab New crystals give diffraction patterns in which reflexions extend to a resolution of 2.0 A, rendering these crystals an excellent subject for structural studies. Fourier maps at nominal resolutions of 6, 2.8, and 2.0 A were obtained and interpreted.[48-50]

The 6.0-A resolution map of Fab New showed two discrete domains (see Figure 5) containing the V_L and V_H (V domain) and the C_L and C_H1 homology regions (C domain).[48] The two polypeptide chains, L and Fd′, were assigned to two continuous, independent stretches of electron density which originate in one of the globular subunits and following a region of globular folding within that subunit, extend to the other subunit through a narrow bridge of polypeptide chain exposed to solvent. The overall pattern was determined to be that of a tetrahedral arrangement of four globular subunits. Two of these subunits correspond to the L chain homology regions V_L and C_L and the other two to the V_H and C_H1 homology regions of the H chain.

A 2.8-A Fourier map was interpreted[49] using the complete amino acid sequence of the L chain,[51] that of C_H1,[24] and a tentative sequence for V_H. A complete tracing of the polypeptide chains including amino acid side chains was achieved. The tracing of part of the V_L region caused considerable delay until it was realized, by simultaneous interpretation of the Fourier map and the amino acid sequence data, that the L chain is seven amino acid residues shorter than a typical human λ chain. This deletion (see Figure 6) appears unique to V_λ New. Although no breaks in the continuity of the polypeptide chain through the electron density were detected, the density corresponding to the C-terminal region of the L chain and the C-terminal

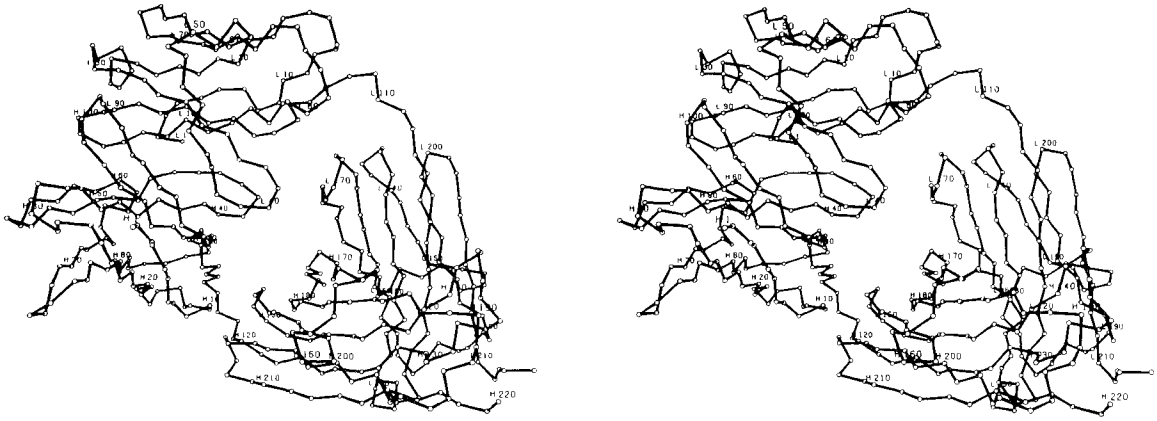


FIGURE 5. α-Carbon backbone of Fab New; stereo pair. L, light chain; H, heavy chain. Numbers designate positions of amino acid residues as given in Figure 10. (From Saul, F. A., Amzel, L. M., and Poljak, R. J., *J. Biol. Chem.*, in press. With permission.)

of Fd′ was lower than in the rest of the molecule. The C-terminal of the Fd′ chain could not be traced in the map beyond Cys 220, which participates in the interchain disulfide bond with L chain Cys 213. This feature of the electron density map explains why no difference can be detected between the X-ray diffraction patterns of Fab and Fab′ crystals.

The interpretation of a 2.0-A resolution map was based on the model obtained from the 2.8-A map. However, the complete determination of the amino acid sequence of V_H[52] and crystallographic refinement of the model[60] allowed a more precise fit of the amino acid sequence to the electron density.

The overall dimensions of Fab are 80 × 50 × 40 A. A central cleft divides Fab into two globular regions, V and C, of about equal size. These two domains are connected to each other by two strands of polypeptide chain, as shown in Figure 5. The V domain contains the variable regions of the H and L polypeptide chains, V_H and V_L. The limits between the V and C homology regions can be defined from the model. The sequence -Val-Ser-Ser- (116 to 118, see Figure 10) which is shared by γ and μ human H chains marks the C-terminus of V_H, and following a sharp bend in the polypeptide chain, the sequence -Ala-Ser-Thr- (119 to 121) marks the N-terminus of C_H1. In the L chain, the sequence -Val-Leu-Arg- (107 to 109, Figures 6 and 10) corresponds to the C-terminus of V_L and the residues -Gln-Pro-Lys- (110 to 112) constitute the N-terminus of C_L. Thus, in the three-dimensional model, there is a clear separation between V and C homology regions. The C domain contains the constant C_L and C_H1 regions of the L and Fd′ chains. The fact that the amino acid sequence of C_H1[24] closely corresponds to the electron density map can be taken as another independent verification that the major features of that sequence are correct. Each of the four homology subunits can be enclosed in a parallelepiped of 40 × 25 × 25 A. The angle between the major axes of the C_L and V_L homology subunits is greater than 90° (100 to 110°), whereas the corresponding angle between the C_H1 and V_H subunits is less than 90° (80 to 85°, see Figure 5).

The C_H1, C_L, V_H, and V_L subunits are strikingly similar in their three-dimensional folding. This is expected from the homology of their amino acid sequences and is in agreement with the proposal of gene duplication as the mechanism which originated vertebrate immunoglobulin genes.[21] The structure of the V and C homology subunits consists of two β-pleated sheets formed by strands of antiparallel polypeptide chain. These two roughly parallel β sheets surround a tightly packed interior of hydrophobic side chains, including the intrachain disulfide bond which links the two sheets. More than 50% of the C_H1 and C_L residues are included in these sheets (see Figure 7). However, although the V_L and V_H subunits share the basic immunoglobulin fold (illustrated in Figures 3 to

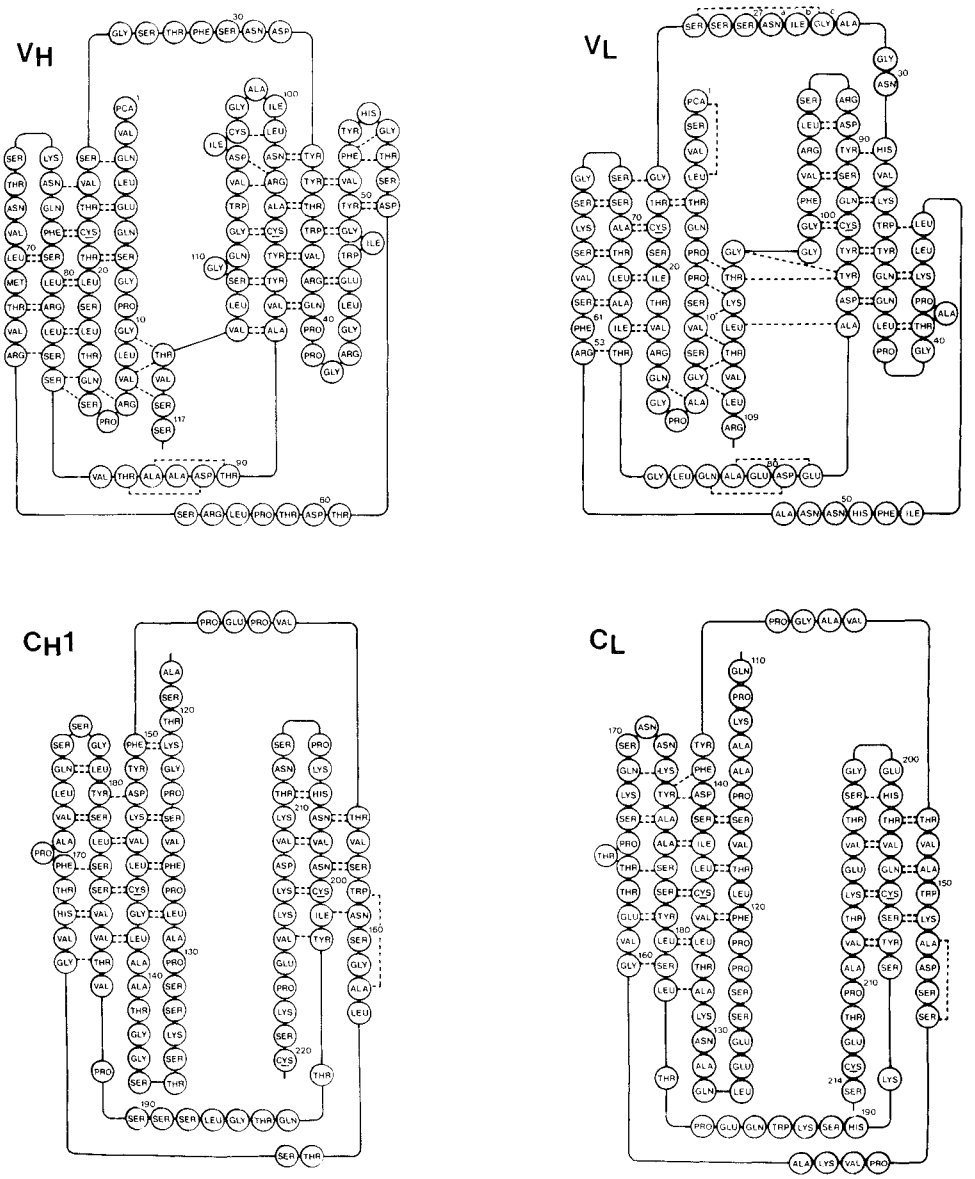


FIGURE 6. Diagrammatic representation of the structure of the homology regions V_H, V_L, C_H1, and C_L of Fab New. Hydrogen bonds between main-chain atoms are shown by broken lines. The two hydrogen-bonded clusters correspond to the β sheets of each homology subunit. Half-cystine residues which participate in intrachain and interchain disulfide bonds are underlined. (From Saul, F. A., Amzel, L. M., and Poljak, R. J., *J. Biol. Chem.*, in press. With permission.)

5, 7, and 8) of the C_H1 and C_L subunits, they include an additional length of polypeptide chain in the form of a loop not present in C_H1 and C_L (see Figure 8). The deletion of seven amino acids in the V_L sequence of IgG New (see Figure 6) results in a shortening of this additional loop of polypeptide chain, thus making the V_L structure of IgG New more similar to that of the C_H1 and C_L subunits.

The L and H chains of all immunoglobulins are covalently linked by a disulfide bond (Figure 1), with a few exceptions such as those of some human IgA molecules where two L chains are linked to each other by a disulfide bond.[61]

The L-chain cysteine residue that contributes to this bond is at the C-terminus of the chain in human κ chains and penultimate to the C-terminus in human λ chains (see Figure 9). In different isotypes of H chain and in different animal species, the cysteine residue that completes the S-S bond is either at position 214 or at about position 131 (Figure 9). The bonding scheme illustrated in Figure 9a applies to human IgG1[62] immunoglobulins such as IgG New. The interchain disulfide bond illustrated in Figure 9b, in which the H-chain cysteinyl residue occurs at position 131, is found in human IgG2, human IgG3, human IgG4,[63] human IgM,[64] rabbit IgG,[65] guinea pig IgG2a,[66] and in mouse IgG2a and IgG2b.[63] In the three-dimensional model of Fab′ New, the H chain Cys 214 is approximately 6 A from L chain Cys 213 to which it is linked by a disulfide bond. However, position 131 in C_H1 also occurs at a distance of 6 A from L chain Cys 213, so that its replacement by a cysteinyl residue could lead to an alternative interchain disulfide bond as found in the immunoglobulin molecules listed above.

Unusual intrachain disulfide bonds that have been observed in several immunoglobulins can be explained on the basis of the model of Fab New.[49] One of these bonds is the intra-H-chain disulfide observed in rabbit IgG, linking the polypeptide chain between residues 131 and 221.[65] An unusual disulfide bond which is observed in the V_H region of a human γ1 chain from IgG Daw[67] can also be explained by the close spatial proximity (about 6 A) of the homologous residues in Fab′ New. Perhaps the most interesting interchain disulfide bond that has been reported is the one that links V_L position 80 to C_L position 171 in rabbit antibodies of restricted heterogeneity.[68,69] A comparison of the sequences of these rabbit κ chains and the human λ chain from IgG New indicates that Cys 80 and Cys 171 in rabbit κ chains correspond to Ala 79 and Asn 172, respectively, in IgG New. The side chains of V_L Ala 79 and C_L Asn 172 face each other, and the distance between their α-carbon atoms is about 6.0 A. This is compatible with the presence of a disulfide bond linking the two homology subunits as observed in some rabbit κ chains. Thus, the Fab model provides an adequate structural framework for the various patterns of interchain and intrachain disulfide bonds that have been established by sequence analyses, therefore, giving further support to the postulate the κ and λ chains have the same overall three-dimensional structure and that the V_H and C_H regions of different classes of H chains (α, γ, μ, etc.) also have the same overall pattern of polypeptide chain folding. Further support for this postulate was given by the results of the crystallographic analysis of the V_κ Rei dimer[40] and the murine McPC 603 IgA-Fab fragment[54] in which the overall three-dimensional folding of the polypeptide chains is the same as that observed

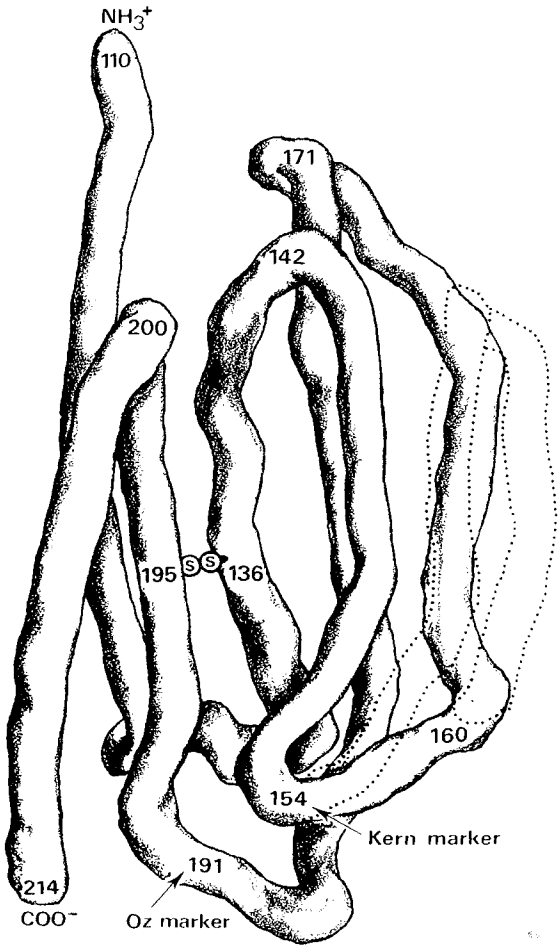


FIGURE 7. Model of the basic immunoglobulin fold. The solid trace follows the folding of the polypeptide chain in C_H1 and C_L. Broken lines indicate the additional loop of polypeptide chain characteristics of V_H and V_L homology regions. The numbers designate L-chain amino acid residues as given in Figures 8 and 10. (From Poljak, R. J., Amzel, L. M., Avey, H. P., Chen, B. L., Phizackerley, R. P., and Saul, F., *Proc. Natl. Acad. Sci. U.S.A.*, 70, 3305, 1973. With permission.)

```
                                        10                                  20
PCA-SER-VAL-LEU-THR-GLN-PRO-PRO-SER-VAL-SER-GLY-ALA-PRO-GLY-GLN-ARG-VAL-THR-ILE

                            27 27a 27b 27c          30
SER-CYS-THR-GLY-SER-SER-SER-ASN-ILE-GLY-ALA-GLY-ASN-HIS-VAL-LYS-TRP-TYR-GLN-GLN

         40                                     50
LEU-PRO-GLY-THR-ALA-PRO-LYS-LEU-LEU-ILE-PHE-HIS-ASN-ASN-ALA ___ ___ ___ ___

                60                                      70
___ ___ ___ ARG-PHE-SER-VAL-SER-LYS-SER-GLY-SER-SER-ALA-THR-LEU-ALA-ILE-THR

                  80                                    90
GLY-LEU-GLN-ALA-GLU-ASP-GLU-ALA-ASP-TYR-TYR-CYS-GLN-SER-TYR-ASP-ARG-SER-LEU-ARG

                 100                                   110
___ ___ VAL-PHE-GLY-GLY-GLY-THR-LYS-LEU-THR-VAL-LEU-ARG-GLN-PRO-LYS-ALA-ALA

                 120                                   130
PRO-SER-VAL-THR-LEU-PHE-PRO-PRO-SER-SER-GLX-GLX-LEU-GLN-ALA-ASN-LYS-ALA-THR-LEU

                 140                                   150
VAL-CYS-LEU-ILE-SER-ASP-PHE-TYR-PRO-GLY-ALA-VAL-THR-VAL-ALA-TRP-LYS-ALA-ASP-SER

                 160                                   170
SER-PRO-VAL-LYS-ALA-GLY-VAL-GLU-THR-THR-THR-PRO-SER-LYS-GLN-SER-ASN-ASN-LYS-TYR

                 180                                   190
ALA-ALA-SER-SER-TYR-LEU-SER-LEU-THR-PRO-GLU-GLN-TRP-LYS-SER-HIS-LYS-SER-TYR-SER

                 200                                   210
CYS-GLX-VAL-THR-HIS-GLU-GLY-SER-THR-VAL-GLU-LYS-THR-VAL-ALA-PRO-THR-GLU-CYS-SER
```

FIGURE 8. The amino acid sequence of the L chain from IgG New. An insertion at positions 27a, 27b, and 27c and gaps at positions 53 to 59 and 96 to 97 are introduced to maximize homology with other human chains.

in the human Fab fragment. At the present stage of the structural analysis of immunoglobulins, the postulate that all immunoglobulins have a common pattern of three-dimensional structure can be accepted with reasonable confidence.

Some of the variable and constant features of V_L sequences can be discussed in terms of the three-dimensional structure of V_λ New. Hairpin bends in the polypeptide chain of V_λ New occur around positions 14 to 15, 27 to 30, 39 to 40, 67 to 68, and 92 to 93, and an approximate 90° bend around residues 75 to 76 (see Figures 5 and 7). Except for the bend at positions 92 to 93 (a hypervariable region), all others involve a Gly residue that is constant in human λ-chain sequences. Most of these bends also involve a constant or nearly constant Pro-Gly or (Ser,Thr)-Gly sequence. A similar conclusion has been obtained from the study of a crystalline V_κ fragment.[40] Glycine residues also contribute to a constant sequence Phe-Gly-Gly-Gly

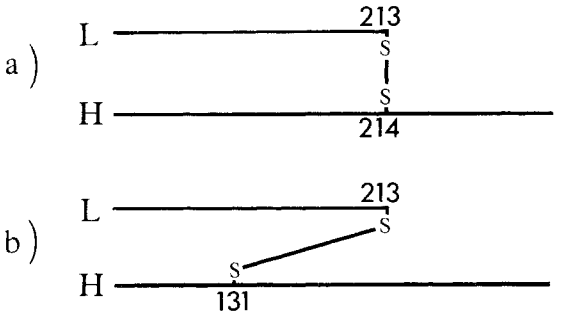


FIGURE 9. Diagrams of two different patterns of interchain disulfide bonds linking H and L chains of immunoglobulins.

(positions 99 to 102) which is not part of a bend. The constant character of this sequence in all λ chains can be explained by the following observations:

1. Phe 99 is located in an internal, interchain hydrophobic pocket that includes the homologous constant Trp 108 in V_H and is re-

lated to Phe 99 by a local pseudo twofold axis of symmetry. Therefore, it can be assumed that Phe 99 (and Trp 108) makes an interchain contact that is important for V_L-V_H assembly.

2. Gly 100 in V_λ (or Gly 109 in V_H) is tightly packed between the intrachain disulfide bond and Leu 4 (a constant residue in V_L and V_H).
3. Gly 101 is relatively close to the constant Gln 6, although there is room here for a side chain as observed in V_κ (Gln 101) or in V_H (position 110).
4. Gly 102 (111 in V_H) is very close to a constant aromatic residue (Tyr/Phe, 86 in V_λ or 95 in V_H) which occupies most of the space available in this region; thus, only a Gly residue can be accommodated.

Some other residues that are constant in V_λ or that show only limited variability, such as Tyr 35, Gln 37, Ala 42, Pro 43, and Asp 84, are involved in close contacts with the V_H subunit. Other constant residues such as Gln 37, Glu 82, Tyr 85 in V_L, and Tyr 142 in C_L make internal hydrogen bonds. In addition to the residues just discussed, most of the nonpolar, hydrophobic amino acids that occur in the interior of the structure between the two β sheets are invariant or are replaced by other hydrophobic residues. They are Leu 4, Gln 6, Val 10, Val 18, Ile 20, Cys 22, Val 32, Trp 34, Leu 46, Phe 61, Val 63, Ala 70, Leu 72, Ile 74, Leu 77, Ala 83, Tyr 85, Cys 87, Ser 89, Val 98, Thr 103, Leu 105, and Val 107. Thus, residues that appear to have an important structural role, such as those at bends and those which contribute to intra- and intersubunit bonds, are found to be invariant or semiinvariant.

In the C_L subunit, Ser 154 and Lys 191, which correlate with the serologic nonallelic human λ-chain markers $Kern^-$ and Oz^+, respectively, appear on the surface of the molecule approximately 8 A from each other (see Figure 8). The *Inv* allotypic markers of human κ chains have been shown[70] to involve Ala/Val and Val/Leu substitutions at positions 153 and 191, respectively, which closely correspond to the positions of the $Kern^-$ and Oz^+ markers in human λ chains. Replacements at positions 153 and 191 in κ chains will alter the antigenic determinants of the molecule that are recognized by antiallotypic antisera. Since the distance between the α-carbon atoms of the homologous residues in Fab′ New is approximately 8 A, replacements involving both positions can be simultaneously recognized by a single antiallotypic antibody molecule.

B. Comparison of the Structure of the Homology Subunits

A homology subunit is defined here as the globular unit of three-dimensional structure containing the amino acid sequence of a homology region. The word domain is most frequently used with the same meaning; however, a three-dimensional domain consists of V_L + V_H (V), C_L + C_H1 (C_1), $(C_H2)_2$ (C_2), and $(C_H3)_2$ (C_3) for IgG molecules.

As stated above, the homology subunits of Fab share a basic immunoglobulin fold. Amino acid sequence comparisons have been extensively used to align the primary structures of different immunoglobulin molecules and their homology regions. The alignment of V_H and V_L sequences with those of the C_H1 and C_L homology regions is less straightforward than alignments between V_H and V_L or C_L and C_H regions. This problem has been approached by matching their three-dimensional structures and aligning residues that occupy similar positions in the constant pattern or immunoglobulin fold of the homology subunits.[50] A quantitative analysis of this homology has recently been made.[60] A similar analysis has been reported in a comparison of the structures of superoxide dismutase and the Fab fragment from McPC 603 IgA.[71]

Initial matrices relating the Cα coordinates of the homology subunits were obtained from a small number of structurally equivalent amino acids. The number of equivalences was then extended by an automatic search for stretches of polypeptide chain for which the distances between putatively equivalent Cαs were smaller than 3.8 A. Based on the extended equivalences, new matrices were calculated and the process was iterated until no changes in equivalences were observed. A summary of the results is presented in Table 2, which lists the number of Cαs occurring at distances of less than 1.5 and 3.0 A for the six possible pairings of subunits which were superimposed and compared by this process. The average value of the minimum base change necessary to exchange

the codons of the structurally equivalent amino acids is also given in Table 2.

As can be seen in Table 2, there is an even closer structural homology between V_H, V_L, C_H1, and C_L in Fab New than that observed for McPC 603 Fab,[71] probably reflecting the higher resolution of the Fab New model. Presumably, the Cα distances given in Table 2 could become smaller with further crystallographic refinement. The number of Cαs which superimpose with distances shorter than 1.5 and 3.0 A is larger when comparing V_H to V_L and C_H1 to C_L. Also, there is good (inverse) correlation between the number of Cαs that are structurally equivalent and the average minimum base change per codon. Furthermore, when a restrictive condition for structural equivalence is imposed ($d_{C\alpha-C\alpha} \leqslant 1.5$ A), the average base change per codon becomes smaller, reflecting a higher degree of conservation of amino acid sequences.

It should be emphasized here that amino acid sequence information is not used in the quantitative three-dimensional alignment procedure described above. However, this procedure leads to amino acid sequence alignments that clearly reflect the well-established homologies between the V_H and V_L and between the C_H and C_L regions of immunoglobulins (see Figures 6 and 10). The closest sequence similarity in Fab New occurs between $C_H1(\gamma)$ and $C_L(\lambda)$, although, as shown in Table 2, the structural similarity between V_H and V_L resembles that between C_H1 and C_L. In addition, Table 2 shows that there is considerable homology between the V and C regions. These results can be interpreted to indicate that all homology regions contain a basic core of amino acid residues with highly preserved three-dimensional structure. The chemical nature of these residues is also preserved as indicated by the correspondingly lower values of the average base change per codon. As stated above, these findings strongly support the postulate that a gene-duplication mechanism gave rise to the different homology regions of immunoglobulins.

The Cαs of the homologous sequences, -Phe-Gly-Gly-Gly- (99 to 102) in V_L and -Trp-Gly-Gln-Gly- (107 to 110) in V_H, can be closely superimposed as can Cα atoms immediately preceding and following those residues. Thus, this conserved conformation gives no evidence supporting the postulate[72] that the Gly residues could serve as a pivot to allow for optimal contacts between an antibody and its ligands. An alternative explanation for these constant, homologous V_H and V_L sequences has been discussed in the preceding section in terms of intersubunit (V_H to V_L) and intrasubunit contacts.

C. Quaternary Structure of Fab Contacts Between H and L Chains

The closer contacts between the homology subunits of Fab New are diagrammatically represented in Figure 11 by lines joining Cα atoms separated by a distance of 8 A or less.[60] This figure provides a description of regions of V_H, V_L, C_H1, and C_L in which there are higher density of contacts. Inspection of Figures 8 and 9 indicates that the interactions between V_H and V_L and between C_H1 and C_L are more extensive than those between V_H and C_H1 and between V_L and C_L. The fact that the V_H and C_H1 subunits

TABLE 2

Alignment of Alpha Carbon Coordinates of the Four Homology Subunits of Fab (New)

Subunits	Number of C_α pairs equivalenced with $d_{C\alpha-C\alpha} \leqslant 1.5$ A	Average minimum base change per codon for $d_{C\alpha-C\alpha} \leqslant 1.5$ A	Number of C_α pairs equivalenced with $d_{C\alpha-C\alpha} \leqslant 3.0$ A	Average minimum base change per codon for $d_{C\alpha-C\alpha} \leqslant 3.0$ A
V_H-V_L	56	0.98	81	0.97
C_{H1}-C_L	60	0.71	82	0.80
C_L-V_L	40	1.03	66	1.23
C_L-V_H	29	1.04	59	1.28
C_H1-V_L	27	1.04	58	1.24
C_H1-V_H	25	1.29	49	1.40

(From Saul, F. A., Amzel, L. M., and Poljak, R. J., *J. Biol. Chem.*, in press. With permission.)

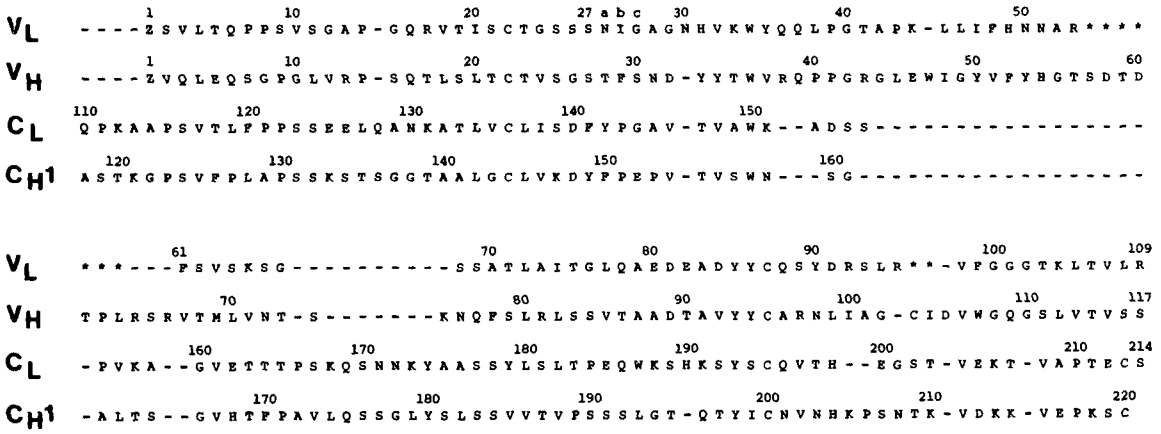


FIGURE 10. Alignment of the amino acid sequences of the V_H, V_L, C_H1, and C_L homology regions of IgG New obtained by comparison of their three-dimensional structures. Asterisks (*) indicate deletions in the V_L sequence (see Figure 8). Gaps introduced to maximize alignment of the three-dimensional structures are shown by dashed lines (----). (The one-letter code for amino acids is as given in Reference 144.) (From Saul, F. A., Amzel, L. M., and Poljak, R. J., *J. Biol. Chem.*, in press. With permission.)

(whose major axes make an angle smaller than 90°) interact more extensively than V_L and C_L

Intersubunit contacts between side- and main-chain atoms situated at a distance not larger than 1.2 times their van der Waals radii are given in Table 3. This table lists contacting residues and the number of close contacts that atoms from a given residue make with atoms of other residues. Evidently, amino acids with larger side chains have a potential to make more contacts with other amino acids, e.g., V_H Trp 107 makes 29 intersubunit contacts, Trp 47 makes 28 contacts, and Arg 43 makes 24 contacts.

The contacts between V_H and V_L are of particular interest in view of the fact that different H and L immunoglobulin chains can form structurally viable pairs. Three types of V_H-V_L contacts will be considered in this discussion: the contacts which are at the core of the contacting region and made by residues which are invariant or semi-invariant in V_H and V_L sequences, the contacts made by invariant or semi-invariant residues with hypervariable residues, and those made between hypervariable residues.

The core of the V_H-V_L contacting region can be described as determined by residues Val 37, Gln 39, Leu 45, Tyr 94, and Trp 107 in V_H and by residues Tyr 35, Gln 37, Ala 42, Pro 43, Tyr 86, and Phe 99 in V_L. These residues are structurally homologous with the exception that V_L Ala 42 has no clear correspondence in V_H due to a structural "insertion" (see Figure 10). These homologous V_H and V_L residues make numerous contacts with each other (about 50% of those listed in Table 3) or with other nonhypervariable residues. The rings of Trp 107 (V_H) and Pro 43 (V_L), at the center of the V_L-V_H contacting region, are nearly parallel and stacked on each other. The contact residues listed above are invariant or are replaced by homologous residues in V_L (κ and λ) and V_H sequences from different animal species. For example, Tyr 35, Gln 37, Pro 43, and Phe 99 appear constant in human L chains (κ or λ), and Gln 39, Tyr 94 (replaced by Phe in a very few cases), and Trp 107 (replaced by Phe or Tyr in a very few cases) appear nearly constant in human H chains. Ala 42, Tyr 86 in V_L, and Val 37 in V_H are more frequently replaced by homologous residues: Ser 42, Phe 86, and Ile 37. The invariant or nearly invariant nature of these residues of the main V_H-V_L contacting area provides a structural basis (together with interactions between C_H1 and C_L) for the properties of different H and L chains to recombine into new immunoglobulin molecules (see References 73, 74, and in particular, 75 for a recent review and experimental data on this topic).

A second type of contact listed in Table 3 is made between constant or nonhypervariable residues and hypervariable residues. For example, the side-chain atoms of V_H Trp 47, a con

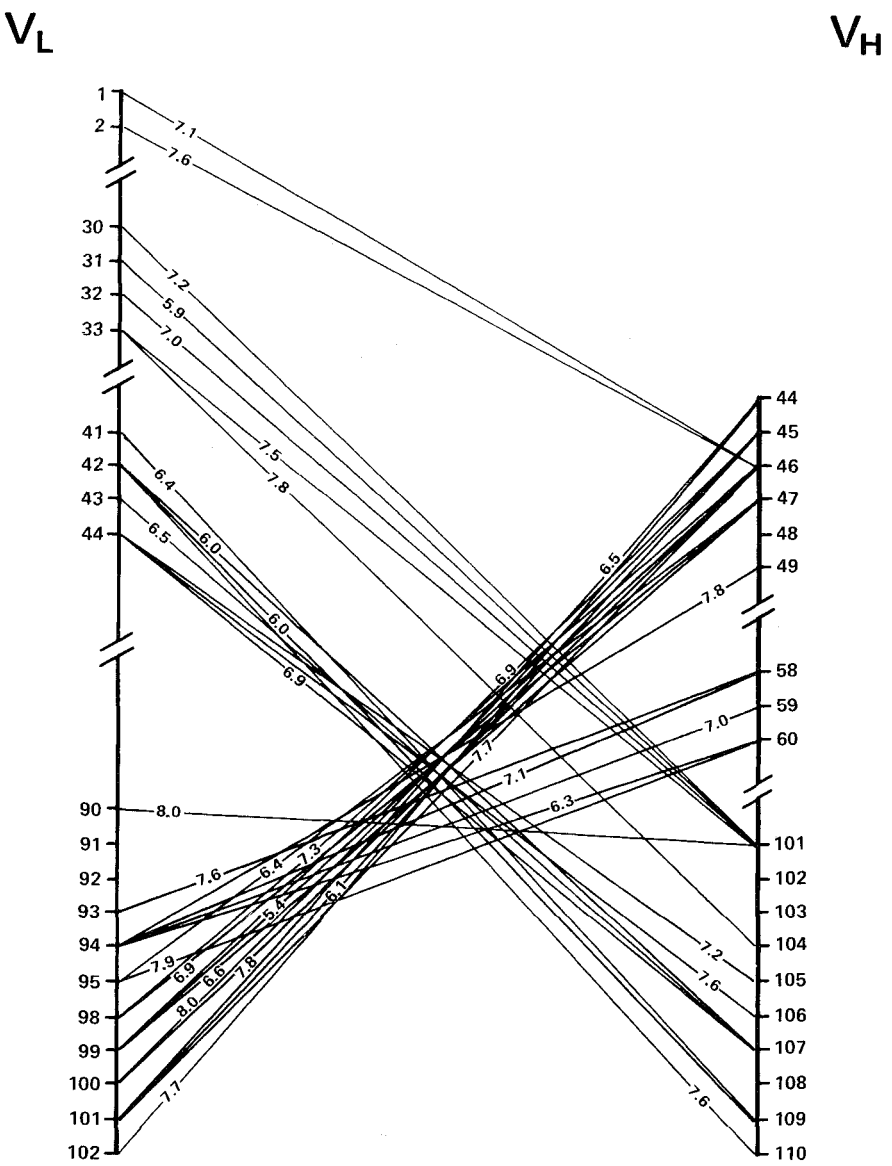


FIGURE 11A.

FIGURE 11. Contacts between α-carbon atoms of V_H, V_L, C_H1, and C_L at distances of 8 A or less in Fab New. Contacts are indicated by lines joining residues designated by their numbers in the sequences. The contact distances on the lines are given in angstroms. Note the extensive V_H-V_L and C_H1-C_L interactions and the fewer longitudinal contacts made by V_H-C_H1 and V_L-C_L. (From Saul, F. A., Amzel, L. M., and Poljak, R. J., *J. Biol. Chem.*, in press. With permission.)

stant residue in human, mouse, guinea pig, and most rabbit immunoglobulin sequences, make close contacts with Ser 93, Leu 94, and Arg 95 in the third hypervariable region of V_L. However, a large number of these contacts involve the peptide chain atoms of the V_L residues. Replacements in the V_L side chains will not necessarily alter the nature of these contacts. Similar contacts appear to be made by V_L Leu 45 (invariant or semi-invariant in human L chains) with the peptide chain at V_H hypervariable position 104. Contacts of this type could also be made from V_L Tyr 35 to the peptide-chain atoms of the fourth hypervariable region of V_H in chains of different length than V_H New.

The third type of contact to be discussed here

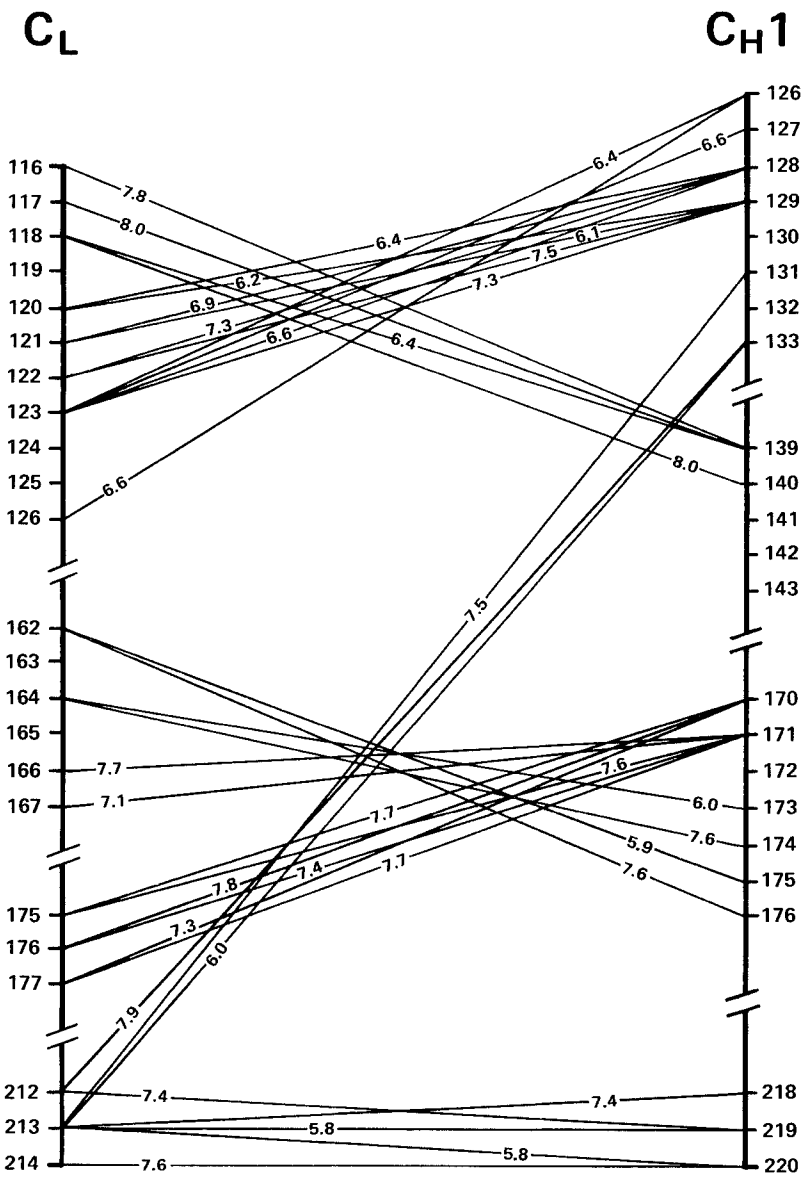


FIGURE 11B.

is that made between hypervariable residues, e.g., between V_H Asn 98 and V_L Arg 95. These contacts are more difficult to evaluate in general terms because (1) the location of some of the residues involved might be changed by further refinement to a larger extent than those of most other residues in the sequence and (2) it is possible that in other immunoglobulins, replacements by different amino acid chains at these positions could be accommodated by small displacements of the hypervariable peptide loops. Consequently, these "idiotypic" interactions are more difficult to assess. However, they could perhaps explain the preferred reassociation observed between complementary H and L chains derived from a single immunoglobulin molecule.[75] Most of the contacts discussed above consist of van der Waals interactions between hydrophobic side chains. However, a few H bonds can be indicated: V_H

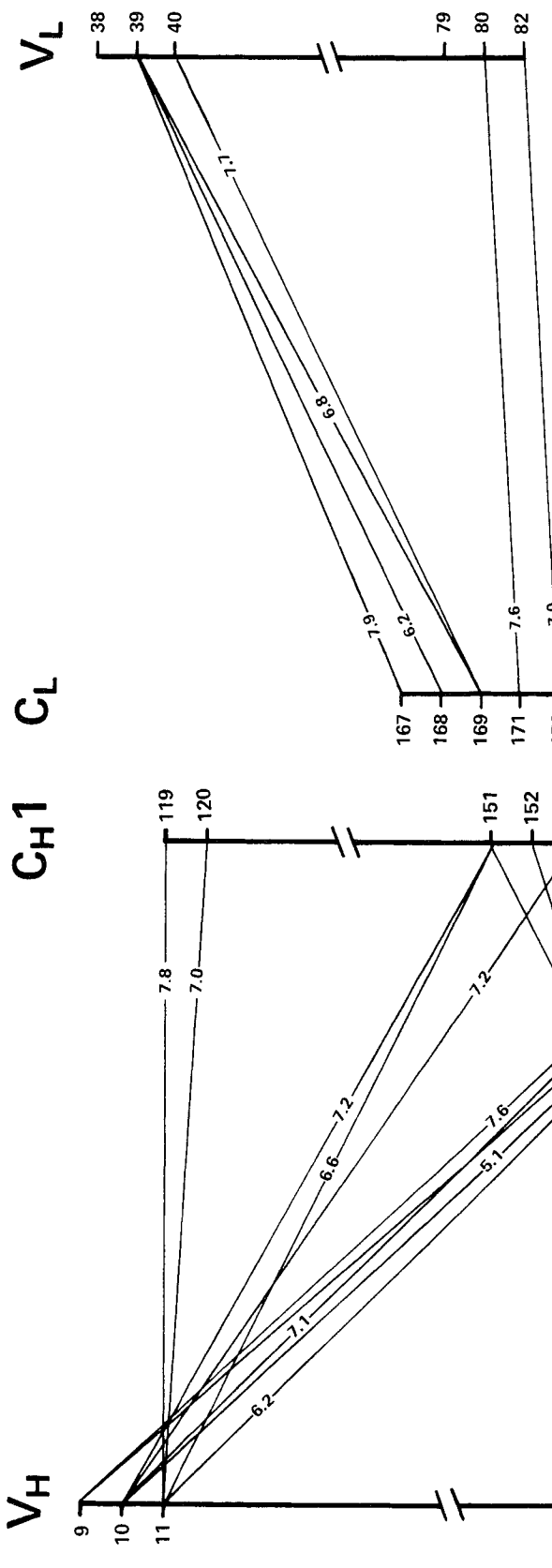


FIGURE 11C.

FIGURE 11D.

TABLE 3

Intersubunit Contacts[a]

V_H	V_L	Number of contacts
VAL 37	PHE 99	1
GLN 39	GLN 37	5
ARG 43	ASP 84	14
ARG 43	TYR 86	7
ARG 43	GLN 37	3
LEU 45	TYR 86	4
LEU 45	PHE 99	2
GLU 46	PHE 99	3
TRP 47	ARG 95	19
TRP 47	LEU 94	8
TRP 47	SER 93	1
ASP 58	SER 93	2
ASP 60	LEU 94	3
THR 61	LEU 94	1
TYR 94	ALA 42	6
ASN 98	ARG 95	8
LEU 99	ARG 95	3
ALA 101	TYR 90	6
ALA 101	HIS 31	7
ALA 101	LYS 33	2
GLY 102	LYS 33	6
ILE 104	TYR 35	3
ILE 104	GLN 88	3
ILE 104	LEU 45	2
TRP 107	PRO 43	21
TRP 107	ALA 42	2
TRP 107	PHE 99	4
TRP 107	TYR 35	2
C_H1	V_H	
ALA 118	LEU 11	2
SER 119	LEU 11	2
THR 120	LEU 11	3
PHE 150	LEU 11	3
PHE 150	THR 114	1
PRO 151	LEU 11	2
PRO 151	THR 114	2
GLU 152	LEU 112	4
PRO 153	LEU 112	7
C_L	V_L	
GLN 110	GLU 32	1
LYS 168	PRO 39	5
ASN 172	GLU 82	7

TABLE 3 (continued)

Intersubunit Contacts[a]

C_H1	C_L	Number of contacts
PHE 126	GLU 126	14
PHE 126	GLU 125	1
PHE 126	SER 123	3
LEU 128	PHE 120	20
LEU 128	VAL 135	2
LEU 128	PRO 121	1
ALA 129	PHE 120	8
ALA 129	PRO 121	2
LYS 133	GLU 212	1
THR 139	THR 118	3
THR 139	LYS 206	2
ALA 141	PHE 120	6
LEU 142	PHE 120	4
GLY 143	PHE 120	5
LEU 145	TYR 179	1
LEU 145	VAL 135	1
LYS 147	GLU 126	1
LYS 147	LYS 131	3
LYS 147	THR 133	2
PHE 170	LEU 137	10
PHE 170	ILE 138	4
PHE 170	SER 177	4
PRO 171	SER 167	2
PRO 171	ALA 175	1
VAL 173	TYR 179	6
GLN 175	GLU 162	7
SER 176	GLU 162	8
LEU 182	TYR 179	2
SER 183	TYR 179	6
SER 183	VAL 135	1
SER 183	LEU 137	1
VAL 185	LEU 137	3
VAL 185	PHE 120	3
LYS 218	CYS 213	2
SER 219	GLU 212	2
SER 219	CYS 213	6
CYS 220	CYS 213	7

[a] The number of interatomic distances not larger than 1.2 times the van der Waals radii (C-C ≤ 4.32 A, O-O ≤ 3.65 A, N-N ≤ 3.72 A, C-D ≤ 3.98 A, and C-N ≤ 4.02 A) are listed.

(From Saul, F. A., Amzel, L. M., and Poljak, R. J., *J. Biol. Chem.*, in press. With permission.)

Gln 39 to V_L Gln 37, and V_H Asn 98 to V_L Tyr 90 and/or V_L Arg 95. Also, an ion pair is formed between V_H Arg 43 and V_L Asp 84.

In the Fab New model, the contacts between V_H and V_L are very close (Table 3), giving rise to a compact dimer. No haptens or even solvent molecules can be accommodated between V_H and V_L beyond the combining site, a situation which is different from that described for an L-chain dimer.[43]

As shown in Table 3, the interactions between C_H1 and C_L are extensive. The core of the

contact area between C_H1 and C_L is defined by C_H1 residues Leu 128, Ala 129, Gly 143, Leu 145, and the structurally homologous C_L residues Phe 120, Pro 121, Val 135, and Leu 137. These residues appear to be invariant or nearly invariant in the H and L sequences from different animal species. Most of the other contact residues such as C_H1 residues Phe 126, Pro 127, Thr 139, Lys 147, Phe 170, Pro 171, Val 173, Gln 175, Ser 181, Val 185, and Lys 218, and C_L residues Thr 118, Ser 123, Glu 125, Glu 126, Lys 131, Thr 133, Thr 164, Ser 177, Tyr 179, and Lys 206 are also invariant or replaced by homologous residues in the immunoglobulin chains from different animal species. In the contact area, the central location of C_H1 Leu 128 and C_L Phe 120 is reflected by the large number of contacts (twenty) they make with each other and with many other residues (see Table 3). As pointed out by Novotny and Franek,[76] the amino acid sequence of the four-stranded β-pleated sheet is more conserved than the rest of the C regions in different animal species, leading to a dendrogram (or genealogic tree) of distorted evolutionary distances. This observation can be analyzed in terms of the structural model presented here as follows. The four-stranded β sheets of C_H1 and C_L contain side chains which make intrasubunit contacts and in particular, contain all or nearly all of the contact residues between C_H1 and C_L. Evidently, mutational events leading to amino acid replacements at these positions would have to occur in a complementary pattern in both C_H1 and C_L in order to preserve tertiary and quaternary immunoglobulin structure; consequently, they would be expected to occur at a slower rate than mutations in other regions of C_H1 and C_L.

As can be seen in Table 3, the region immediately preceding the interchain disulfide bond does not provide close contacts between C_H1 and C_L. In addition, the two strands of polypeptide chain that come together at the interchain disulfide bond do not closely interact with the rest of C_H1 or C_L. This region can be described as having a loose conformation with a lower electron density in the Fourier map. These structural features are in agreement with the notion of segmental flexibility residing around this part of the immunoglobulin structure and in the immediately adjacent hinge region of the H chain.

As mentioned above, the Fab structure can be described as a tetrahedral arrangement of homologous subunits covalently linked in pairs (V_L to C_L and V_H to C_H1) by linear stretches of polypeptide chain ("switch" regions) bent to a greater extent in the Fd chain than in the L chain.[49,54] Furthermore, two identical L chains in a dimer assume different conformations[38] such that one chain appears similar to the L chain of the Fab fragment whereas the other L chain of the dimer resembles the Fd chain. Although no gross conformational changes have so far been observed in Fab fragments, these findings suggest that the Fab fragment could display flexibility in the switch regions allowing a conformational change to take place by a hinge-like movement at one or both switch regions. Since a disulfide bond linking V_L to C_L has been found in some rabbit IgG molecules, the flexibility of the more open L chain may be more limited than that of the H chain. An "opening" of the Fd chain, as illustrated in Figure 12, would lead to a relative movement of the structural subunits exposing some of the V_H and C_H1 side chains that were not previously exposed. This topic will be reconsidered in some of the following sections.

V. THREE-DIMENSIONAL STRUCTURE OF THE Fc FRAGMENT

Although several laboratories have studied crystalline human and rabbit Fc fragments, a detailed structure has been obtained only recently.[77] Human Fc crystals were prepared from pooled human serum IgG by digestion with plasmin. Although the material is not completely homogeneous because it originates from a mixture of different classes and allotypes, cleavage by plasmin between Lys 222 and Thr 223 gives an Fc fragment containing a central part of the hinge region sequence -Cys (226) -Pro-Pro-Cys-Pro-. The structure has been determined to a resolution of 3.5 A using multiple isomorphous replacement and molecular replacement techniques. The electron density map was interpreted in terms of the known amino acid sequences of the C_H2 and C_H3 homology regions of human IgG. Although the resolution of this study and the quality of the Fourier map do not allow the precise location of every feature of the fragment, its overall

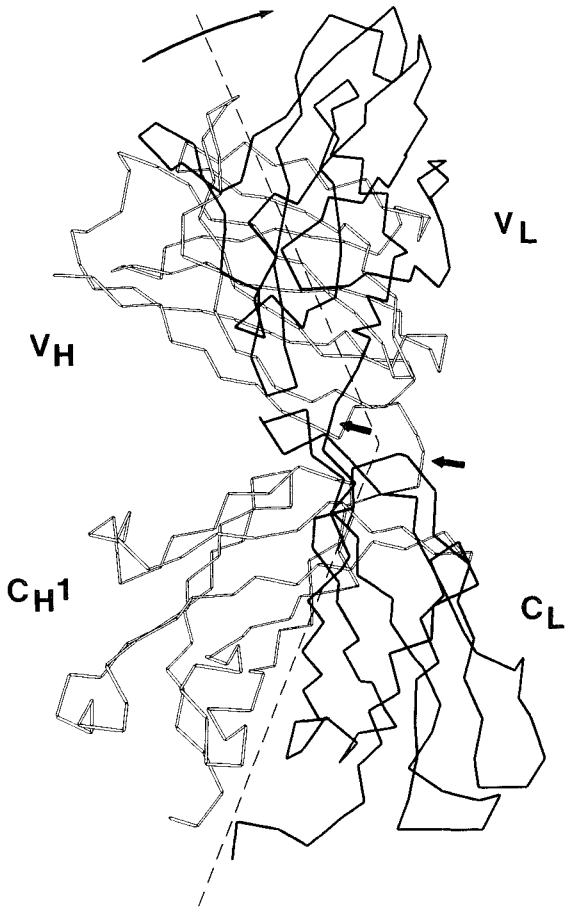
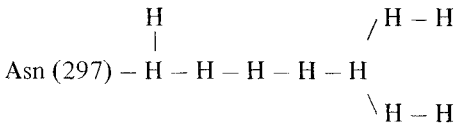


FIGURE 12. A diagram of the α-carbon backbone of Fab New. The L chain is traced by a solid line, the H chain by an open line. Approximate twofold axes of symmetry relating V_H to V_L and C_H1 to C_L are shown by broken lines. Two short arrows indicate the switch regions of both chains. The longer arrow indicates a relative motion of the V (V_H + V_L) and C_1 (C_H1 + C_L) domains. Compare with Figure 14.

structure is definitely established. The structure has been described[77] as having the shape of a "Mickey Mouse" with the C_H2 domains forming the ears and the C_H3 domains the round, globular head (see Figure 13). The tertiary structure of C_H2 and C_H3 conforms to the immunoglobulin fold. A loosely folded segment of polypeptide chain extending from Ser 337 to Gln 342 connects the two domains. This segment is exposed to solvent and consequently, to proteolytic attack by enzymes. The C_H3 domains interact very closely in a pattern which is similar to the C_H1-C_L interactions observed in Fab fragments. The C_H2 show no interaction with each other. The N-terminus, including the sequence -Cys-Pro-Pro-Cys-, appears to be disordered since it cannot be traced in the map; the segment which follows it in the N-terminal sequence may be in close contact with part of the carbohydrate chain attached to Asn (297). This sequence and the carbohydrate chains lie in between the C_H2 domains (see Figure 13). The structure of the carbohydrate chains is somewhat tentative, but their general location and overall conformation are clear. The electron density assigned to carbohydrate is compatible with a branched chain of the general type:



where H is a hexose unit. This chain would be longer than has been observed in myeloma proteins. The attachment site (Asn 297) is at an accessible turn of the polypeptide chain compatible with posttranslational addition of the sugar moiety by specific transferase enzymes.

VI. STRUCTURE OF IgG MOLECULES

A low-resolution crystallographic study of a complete human IgG molecule (IgG Dob) has been reported.[78] This IgG, a cryoglobulin, crystallizes spontaneously on cooling. Unfortunately, the X-ray diffraction pattern does not extend to high resolution and the crystals are damaged by relatively short exposures to X-rays, making the crystallographic study difficult even at 6-A resolution. Two globular regions were isolated from the map. One of these regions surrounds a twofold axis of symmetry and was therefore assigned to the Fc part of the molecule. The other globular region should correspond to the Fab part of the molecule. Of four possible ways of assembling a complete IgG Dob molecule from the globular Fab and Fc regions, one was chosen as a most likely structure. In this model, the molecule is T shaped. No other structural feature could be inferred from the map. Concerning its T shape, it should be considered that Dob may not be a typical IgG molecule since it contains a deletion in its hinge region,[79] a factor which together with crystal lattice forces may influence the shape of the molecule. Further studies of IgG Dob are under way.

As mentioned above, the crystalline McG

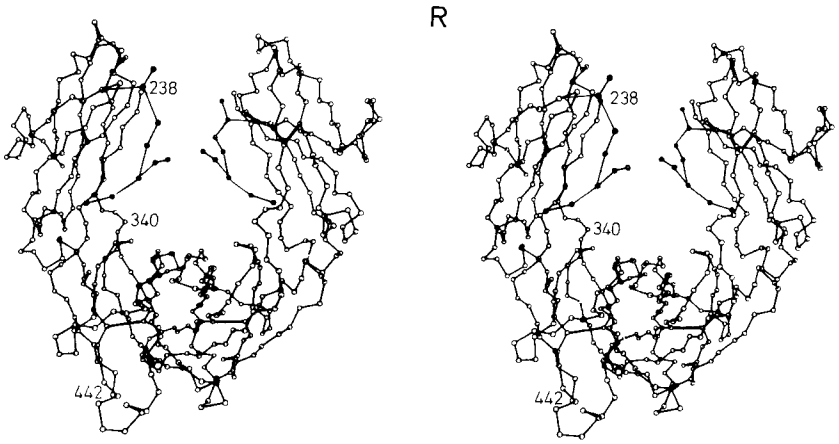


FIGURE 13. Stereo-pair drawing of the α-carbon (○) backbone of human Fc and the carbohydrate hexose units (•). (From Huber, R., Deisenhofer, J., Colman, P. M., Matsushima, M., and Palm, W., in *The Immune System,* 27th Mosbach Colloquium, Springer-Verlag, Berlin, 1976, 26. With permission.)

IgG protein is under crystallographic study. McG IgG is also affected by a deletion in its hinge region. Only preliminary results have so far been reported from the crystallographic study of its structure.[81,136]

A third crystalline human myeloma IgGl protein (Kol) has been studied by X-ray diffraction techniques to a resolution of 4 A.[81] This study is of particular interest due to the fact that IgG Kol appears to possess a normal hinge region, a feature that could allow the study of the normal structural relations between the Fab and Fc parts of the molecule. The structural analysis of IgG Kol[80] revealed two interesting features: no electron density could be assigned to the Fc region which must be disordered in the IgG Kol crystals and the Fab arms of the molecule differ from the crystalline Fab fragments in quaternary structure.

The electron density corresponding to the Fab regions of the molecule could be traced to residues 213 and 209 in H and L chains, respectively, and in a tentative way, beyond these points down to the hinge region sequence -Cys-Pro-Pro-Cys-Pro- (residues 226 to 230). The Fc region cannot be traced out or even be assigned a general area in the unit cell without overlap problems. However, the tight packing around the hinge peptide in the crystal structure requires that this peptide be rather extended. From this interpretation, it follows that the C_H2 domains cannot come close to the Fab region or, in other words, there are no contacts between C_H1 and C_H2 except for those arising from the continuity of the peptide chain.

The second interesting feature mentioned above in the structure of IgG Kol is related to the quaternary structure of its Fab region. This feature can be described as a "bending of the elbow" around the switch regions to make the V_H-V_L domain more colinear with the C_H1-C_L domain than is observed in the Fab New and the Fab McPC 603 fragments (see Figure 14). The contacts between V_H and V_L and those between C_H1 and C_L do not seem to be affected by the straightening of the Fab regions. Thus, it is mostly the longitudinal contacts between V_L and C_L and, in particular, those between V_H and C_H1 (see Section IV) which are changed by this rearrangement of the V and C domains. This rearrangement is schematically illustrated in Figures 12 and 14.

VII. THREE-DIMENSIONAL STRUCTURE OF IgM

Although no crystallographic analysis of the three-dimensional structure of IgM has yet been attempted, Feinstein[82,83] has used the information obtained from X-ray analysis of IgG fragments, from comparisons of amino acid sequences, and from electron microscopy to build a plausible model for IgM. This topic has been reviewed[7,83] and will be briefly summarized here.

A schematic representation of the polypep-

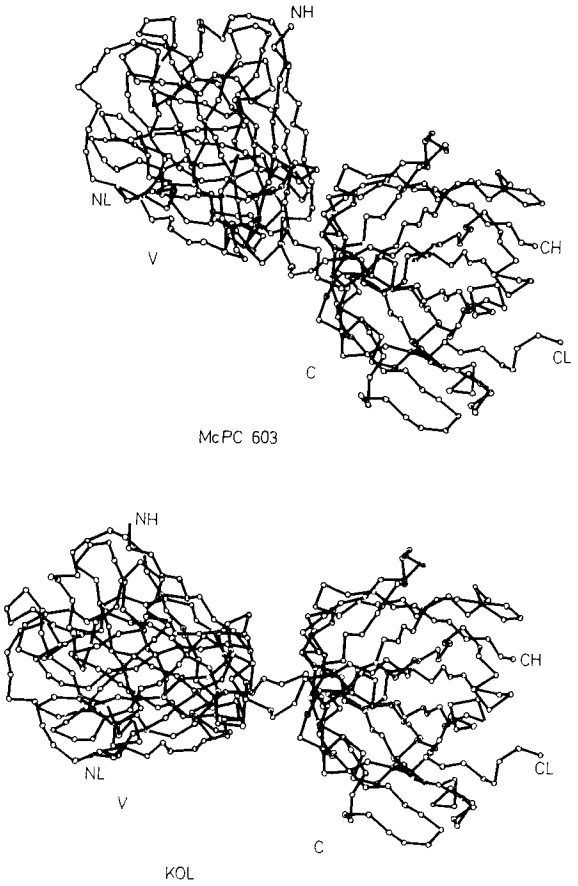


FIGURE 14. The α-carbon backbones of the V and C_1 domains from McPC 603 Fab and IgG Kol. Note the different arrangement of the V and C_1 domains in the two structures; compare with Figure 12. (From Huber, R., Deisenhofer, J., Colman, P. M., Matsushima, M., and Palm, W., in *The Immune System*, 27th Mosbach Colloquium, Springer-Verlag, Berlin, 1976, 26. With permission.)

tide chain structure of IgM is shown in Figure 15. Although basically similar to IgG, an IgM 7S subunit includes a μ H chain which, in comparison with an H chain, contains an additional homology subunit (V_H, C_H1, C_H2, C_H3, and C_H4). Comparisons of the amino acid sequences of γ and μ chains[84] indicate that $C_\mu3$ is closer to $C_\gamma2$. Both regions contain a homologous sequence (Asn-Ser-Thr, 297 to 299 in $C_\gamma2$; Asn-Ala-Thr in $C_\mu3$) which is an attachment site for a carbohydrate moiety. In contrast with IgG, a single (19 S) IgM molecule can activate complement.

Model building on the basis of the V_H-V_L and the C_H1-C_L regions of Fab New and on amino acid sequence, and electron microscopy information led Feinstein to propose a model illustrated in Figure 16. The precise structural bases for the aggregation of IgM 7S monomers and the role of the J chain and the $C_\mu4$ homology regions in the structure of the 19S IgM polymer cannot be spelled out in this model and are basically omitted in Figures 15 and 16. Note that as in the case of the C_H2 domains of IgG, their homologous C_H3 domains in IgM do not interact as closely as the rest of the V and C domains.

VIII. STRUCTURAL SIMILARITIES BETWEEN IMMUNOGLOBULINS AND OTHER PROTEINS

High-resolution crystallographic analyses have shown significant similarities in the basic three-dimensional folding patterns of many proteins. This topic has been recently reviewed elsewhere[12,85] and will be covered very briefly in the present review.

Four general types of similarity in three-dimensional structure can be described. The first type is that of a family of proteins having similar or related functions and which share different degrees of sequence homology, including, at times, a prostethic group or some invariant amino acid residues at their active centers. Examples of these are the proteins of the myoglobin-hemoglobin family,[86] the cytochrome C family,[87] the serine proteases[88] of the trypsin-chymotrypsin family, and the acid proteases of the pepsin family.[89]

A second type of structural similarity is found in proteins such as lactic acid dehydrogenase, malate dehydrogenase, liver alcohol dehydrogenase, glyceraldehyde phosphate dehydrogenase, adenylate kinase, flavodoxin, etc. with a nucleotide bonding domain of similar three-dimensional structure but which differ in the remaining part of their peptide chain folding.[90]

A third type of similarity is that detected in structural domains within a protein such as the carp muscle calcium-binding protein[91] and in immunoglobulins as previously described in this review. The hypervariable regions of immunoglobulins illustrate a feature which is frequently found when comparing the three-dimensional structure of proteins with similar folding: loops connecting neighboring elements

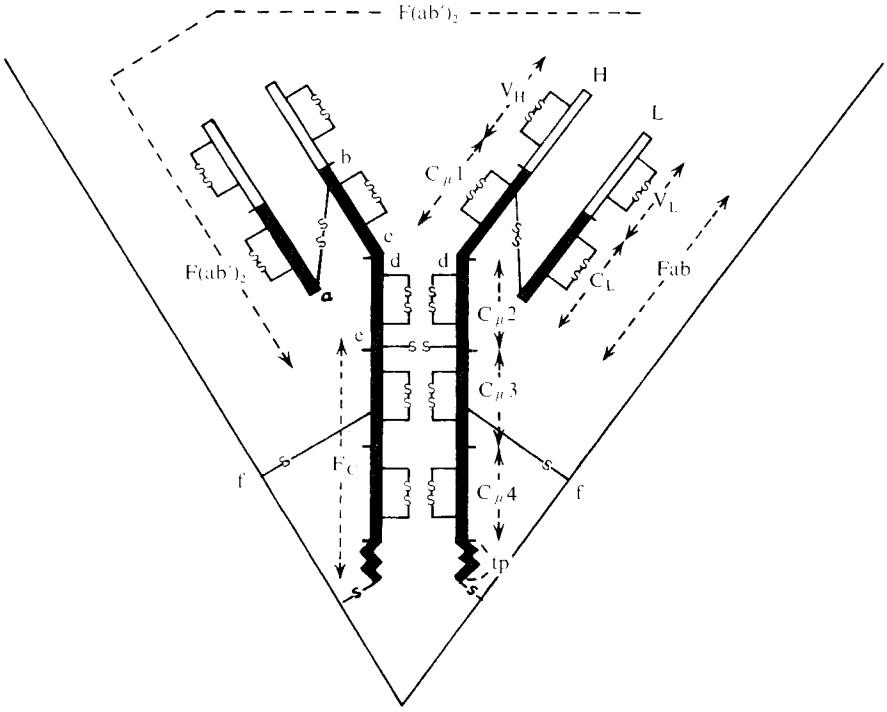
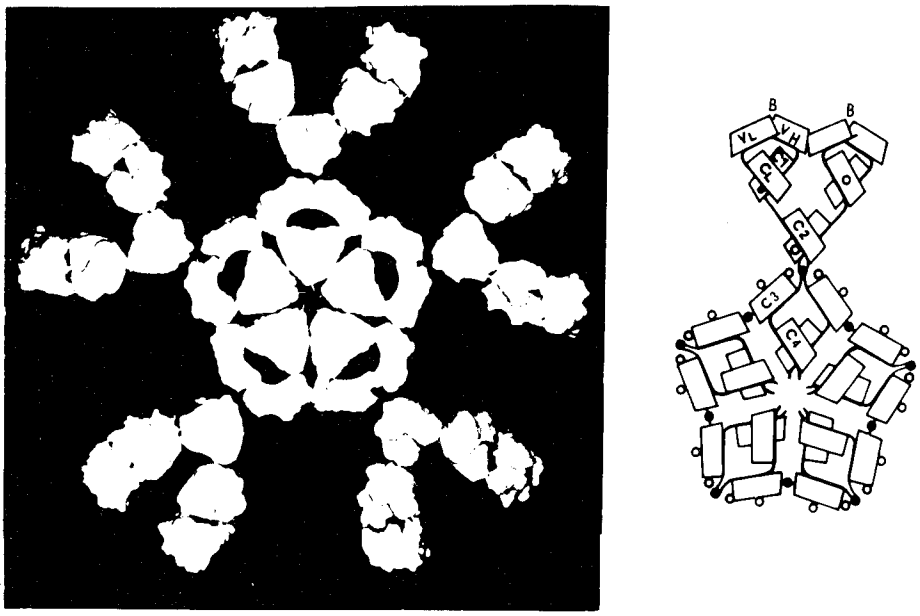


FIGURE 15. Diagram of the polypeptide chain structure of L and H chains in an IgM molecule. Only one of the five H_2L_2 monomers is shown. (Courtesy of Dr. A. Feinstein.)



16A 16B

FIGURE 16. (A) Model of IgM incorporating the known features of the three-dimensional structure of IgG Fab and Fc fragments. (B) Diagram of the model showing only one of the five $F(ab)'_2$ arms. •, Interchain disulfide bond; O, site for carbohydrate attachment. (Courtesy of Dr. A. Feinstein.)

of secondary structure may vary in length by insertions or deletions. However, the elements of secondary structure connected by these variable loops, such as β-pleated sheet strands or helical segments which make the "core" of the structure, do not vary in conformation.

A fourth type of similarity is of the type recently established between the bovine Cu, Zn superoxide dismutase subunit, and immunoglobulins.[71] No amino acid sequence homology can be detected between these proteins. Superoxide dismutases are intracellular metalloenzymes that process superoxide radicals into O_2 and H_2O_2. Thus, they are not related to immunoglobulins by their function. The structural similarity is diagrammed in Figure 17. Superoxide dismutase contains an additional, N-terminal strand of β-sheet structure. The remaining strands, labeled A to G in Figure 17, correspond to the similarly labeled strands of an immunoglobulin C homology region. In superoxide dismutase, there are two extended polypeptide chain loops connecting strands C to D and F to G (see Figure 17) which contribute to the Cu and Zn metal binding sites and are essential for enzymatic activity. These loops are topologically equivalent to the second and third hypervariable regions of V_L or the second and fourth hypervariable regions of V_H which contribute to the antibody combining site. The intrachain disulfide bond connecting the two β sheets of an immunoglobulin domain is not present in superoxide dismutase. Also, the two superoxide dismutase subunits that constitute a dimer make contacts which are not similar to those made by the C or the V domains in immunoglobulins.

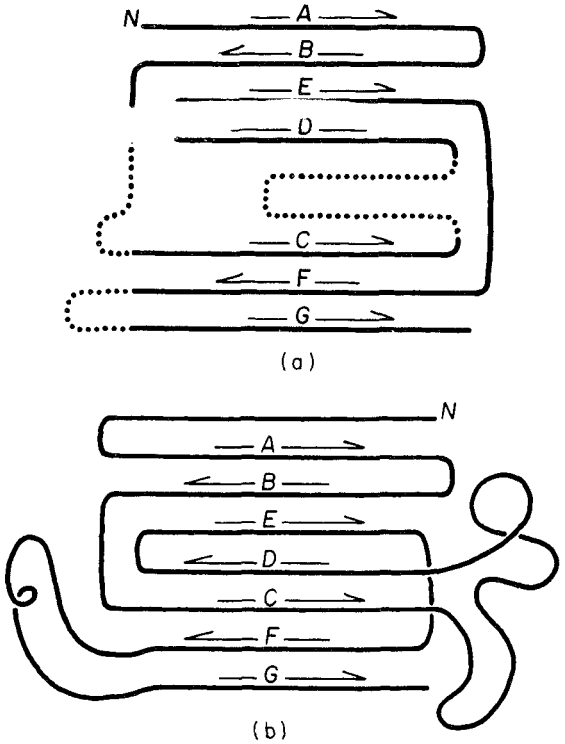


FIGURE 17. Diagrams of the structures of (a) an immunoglobulin V_H or V_L region and (b) a subunit from the Cu, Zn-superoxide dismutase enzyme. The structurally homologous strands of polypeptide chain which are part of β sheets in both structures are designated A to G. Arrows give the N to C direction of the polypeptide chains. Hypervariable regions are shown by dotted lines. (From Richardson, J. S., Richardson, D. C., Thomas, K. A., Silverton, E. W., and Davies, D. R., *J. Mol. Biol.*, 102, 221, 1976. With permission.)

The similarity in the three-dimensional structures of superoxide dismutase and immunoglobulins can be explained by the following hypothetical alternatives: a common although distant evolutionary origin or convergent evolution towards a stable three-dimensional folding. As pointed out by Richardson and colleagues,[71] the process of protein folding is not well enough understood to judge on the merits of the second alternative. However, there are many other different types of β structure and none show the similarity discussed here. A common evolutionary origin is the most appealing explanation of the observed similarity in three-dimensional structure.

Histocompatibility antigens have been postulated to share an evolutionary origin with immunoglobulins.[92,93] The amino acid sequences of proteins encoded by the mouse H-2 histocompatibility complex or those of the HLA system in humans have not yet been extensively analyzed to allow a meaningful search for sequence homologies. However, the amino acid sequence of β_2 microglobulin,[92] a polypeptide chain associated with the heavy chains of histocompatibility antigens, has been determined. This sequence is highly homologous to those of the C regions of immunoglobulins, indicating that the overall chain folding of β_2 microglobulin is similar to the folding of the C regions of immunoglobulins. This homology extends to the amino acid chains by which C_L and C_H1 interact. Recently, β_2 microglobulin has been de-

tected in a spontaneously occurring complex with a γ-chain fragment in the urine of a patient with plasma cell leukemia.[145] The complex could be dissociated in 7.5% n-propanol, indicating noncovalent, hydrophobic interactions with the γ chain fragment. This fragment appears to originate from the Fc region of an IgG protein. The sequence homology and the ability of β_2 microglobulin to interact with immunoglobulins and histocompatibility antigens suggests that these proteins share the basic immunoglobulin fold. The structural similarities would be in agreement with a common genetic origin and subsequent steps of gene translocation and duplication.

Amino acid sequence homologies between rabbit and human C-reactive protein and a distant although significant homology between these proteins and immunoglobulins have recently been reported.[94] The C-reactive protein, initially defined by a unique, Ca-dependent precipitating reaction with pneumococcal C-polysaccharide,[95,96] is a serum protein which is highly elevated (up to 10^3-fold) during acute inflammatory reactions. This protein acts as an agglutinin and an opsonin and can activate the complement system, properties which are reminiscent of those of specific antibodies.[97-99] Although the sequence homology with immunoglobulins appears weak, structural similarities may indeed exist. However, since the reported sequence analysis extends only to position 50, it is hard to decide on the merits of this proposal on purely structural grounds.

IX. THE ANTIBODY COMBINING SITE

A. General Description

The definition of the molecular basis of specificity and recognition by antibody molecules resides in the identification and characterization of the combining site. Evidence obtained from amino acid sequence analyses and from affinity labeling studies was used to tentatively identify the segments of polypeptide chain which are involved in the definition of antibody combining sites. Most of the sequence studies were performed on homogeneous myeloma proteins of unknown specificity. Affinity labeling studies were performed on antibodies and on myeloma proteins of known binding specificity. Recent reviews of this topic are available.[1,8]

The presence of regions of "hypervariable" sequence was first recognized by Kabat and Wu[18,19] who made use of a statistical analysis of L-chain sequences. In these sequences, residues around positions 30, 50, and 95 were designated as hypervariable.[19] A similar conclusion was obtained by comparison of human H chain sequences.[20] These findings led to the postulate that the hypervariable regions of both the H and L chains contribute in some way to the conformation of the antigen combining site of immunoglobulin molecules.

Affinity labeling studies were performed in several antibodies and immunoglobulins with the purpose of identifying the antigen complementarity regions.[100-105] These experimentally involved studies could be briefly summarized as showing that in general, the affinity-labeled amino acids occur at, or adjacent to, the hypervariable regions of the H and L chains.

Definitive evidence that the hypervariable regions of the H and L chains determine the conformation of antigen combining sites was obtained in the study of Fab New.[49] The hypervariable regions were located at adjacent bends of the H and L chains, defining a crevice or pocket at one end of the Fab fragment. A very similar arrangement of hypervariable regions was observed in the Fab fragment of the murine IgA McPC 603 myeloma protein. The human L-chain dimer Mcg[39] and the human V_κ dimer Rei[40] show similar arrangements of hypervariable regions in the L chains. The combining site of Fab New extends over a large area surrounding a central groove or pocket which is 15 A long, 6 A wide, and 6 A deep. Residues 27 to 30 of the L chain (first L-chain hypervariable region) form the "upper" end of the groove (see Figure 14), while H-chain residues 55 to 60 (second H-chain hypervariable region) and 30 to 33 (first H-chain hypervariable region) form the "lower" end. The "sides" are formed by L-chain residues 90 to 95 (third L-chain hypervariable region) on the left and H-chain residues 102 to 107 (third H-chain hypervariable region) on the right. The pocket is predominantly lined by the side chains of these residues of the V_L and V_H regions. Therefore, it is evident that amino acid replacements at these

positions will alter the conformation and, consequently, the specificity of the antibody combining site. Furthermore, hypervariable regions of immunoglobulins contain insertions and/or deletions that alter the length of the polypeptide chain in these regions. Sequence modifications of this type have a very significant effect on the size and shape of the combining site. For example, the L chain (κ) of murine myeloma protein McPC 603 is longer than other human and murine κ and λ chains due to an insertion of six amino acids in its first hypervariable region (as in human $V_{\kappa III}$ sequences). Partly due to this insertion, the structure of the Fab fragment of McPC 603[54] has a "deeper" combining region than Fab New. H-chain sequences also show a variable number of amino acids in their hypervariable regions. In particular, the length of the third hypervariable region of V_H has been found to range from 13 to 20 amino acids when counted from Cys 96 to Trp 107. In Fab New, the loop defined by this region has a different overall conformation than that found in equivalent regions of other homology subunits; it is bent towards the local twofold axis of symmetry, which relates V_H and V_L, forming a narrow groove at the combining site. Varying the number of amino acids in this region probably results in drastic changes in the size and shape of this groove.

In conclusion, the pattern of insertions and deletions observed in the sequences of hypervariable regions will be a major determinant of the general dimensions of the active site. In addition, these variations complemented by single amino acid replacements will define a chemical environment within the active site which is characteristic of a given immunoglobulin molecule. In this way, it is possible to accommodate a large number of antibody specificities while retaining a constant overall pattern of three-dimensional folding.

The description of the combining site of immunoglobulins presented in the preceding paragraphs can be used to assign a structural and functional meaning to the notion of "subgroup" in κ and λ chains. Since "subgroups" correlate with the presence of insertions and/or deletions in the hypervariable regions (the first hypervariable region in particular), they correspond to different conformations of the active site. For example, human κ chains of subgroup III ($V_{\kappa III}$) have insertions of up to six amino acids in their first hypervariable region. These chains will define a "deeper" active site (such as that observed in McPC 603) than those defined by either a κ chain of subgroup I ($V_{\kappa I}$) or a human λ chain (such as that in IgG New). It is interesting to observe that murine λ chains appear to lack the pattern of insertions and deletions found in the hypervariable regions of murine κ chains and in human κ and λ chains. Thus, it can be expected that murine λ chains will make a smaller contribution to the diversity of antibody specificities so that the expression of λ chains or the number of λ-chain genes in mice would be limited in relation to κ chains as reflected in the κ/λ serum ratio.[106]

A system of mouse myeloma λ chains has been proposed as a model for the expression of somatic mutation and antigen selection mechanisms acting sequentially on a single V_λ germ line gene.[106] Based on the close homology in amino acid sequence between murine and human myeloma chains and on the available three-dimensional models of the human λ chains New and Mcg, the mouse λ-chain variants have been analyzed to ascertain the possible functional significance of the reported amino acid substitutions.[107] Of eighteen BALB/c chains that have been sequenced, twelve are identical (λ_0), four have one amino acid replacement (λ_1), one shows two replacements (λ_2), and another shows three replacements (λ_4).[108] All these replacements fall within one of the three segments of hypervariability of L chain sequences in a region of the three-dimensional structure where there are no apparent constraints in the nature of the amino acid side chains which project out into the solvent. However, when these replacements were placed on the three-dimensional model of the combining site, they did not all appear to have a significant role in terms of possible hapten or antigen binding activity. Although this analysis was based on comparing human and mouse sequences which are highly homologous and of the same length in their hypervariable regions, it could be argued that it is not conclusive because it is based on a static three-dimensional model which could be altered to give new conformations in solution. This possibility was

considered but, within the framework of the available three-dimensional structures, it did not seem plausible that changes in conformation in the hypervariable regions or close to them would have a significant effect on the contribution of most of the murine λ chain replacements to the combining site.[107] It appears from this analysis that the replacements observed in the mouse λ chains are not important in contributing to contacts with antigens at the combining site or in otherwise modifying the conformation of that site to give rise to new antigen binding specificities. In contrast, the very maxima of hypervariability plots obtained from human and murine myeloma L-chain sequences occur at positions which appear to have an important role in determining the conformation of the combining site and close contacts with haptens and antigens.

B. X-Ray Diffraction Studies of Fab-hapten Complexes

It is important to point out that the crystallographic studies reviewed here were performed using human and murine myeloma proteins, not induced antibodies. However, myeloma proteins have been shown to bind a variety of haptens and antigens in reactions that closely resemble those of induced antibodies. Myeloma proteins that have been screened for hapten binding activity have frequently been found to bind some ligands with association constants comparable to those of induced antibodies (10^4 to 10^5 l/mol and higher). Furthermore, these binding reactions give linear Scatchard plots with intercepts that show that two haptens are bound to one immunoglobulin molecule. These and other criteria indicate that hapten binding by myeloma proteins can be directly correlated to antibody-hapten reactions. However, as expected for small ligands, structural studies of haptens bound to immunoglobulin molecules show interactions involving only a fraction of the amino acids present in the combining site. This was true, for example, in the study of the structure of the complex between the Fab fragment of murine IgA McPC 603 and the hapten phosphorylcholine.[53] Phosphorylcholine binds to McPC 603 with an affinity constant of 1.7×10^5 l/mol. A derivative of the hapten labeled with a heavy atom was diffused into McPC 603 Fab crystals, and its position was determined by a difference-Fourier map. Phosphorylcholine was found to bind to the combining region of the protein in the cavity defined by the hypervariable regions of the L and H chain. The interactions of the hapten with the combining site mainly involve Arg 52, Lys 54, and Glu 35 of the H chain in agreement with the presence of both a positive and negative charge on phosphorylcholine at neutral pH.

It is relevant to add here that murine antiphosphorylcholine antibodies, induced in several strains of inbred mice, share idiotypic determinants and amino acid sequences with well-characterized myeloma proteins which include McPC 603.[109] Thus, there can be no doubt that one is looking at a legitimate antibody combining site. Since phosphorylcholine is a constituent of bacterial cell walls, the physiological function of these antibodies is immediately apparent. A human myeloma, Walderstrom's macroglobulin (IgM), has recently been shown to bind phosphorylcholine.[110] The amino acid sequence of the N-terminal 36 residues of the (μ) H chain has been determined[111] and compared to that of the murine phosphorylcholine-binding myeloma proteins.[111] The sequences are remarkably similar. In particular, in the first hypervariable region, there is only one substitution, from Glu (mouse H chains) to Asp at position 35. This similarity in the sequence of a H-chain variable region in two different species has been presented as an argument in favor of the stable transmission of antibody genes throughout evolution.[111]

The human myeloma protein IgG New, whose crystalline Fab fragment was discussed in a preceding section, was screened for the binding of a large number of haptens.[112] Several compounds such as uridine, orceine, menadione, and others were found to bind to this protein with low affinity constants (approximately 10^3 l/mol). However, a γ-hydroxyl derivative of vitamin K_1 (vitamin K_1OH, see Figure 18) was found to bind with an affinity constant of 1.7×10^5 l/mol. Crystallographic studies on the complexes of these compounds and Fab New showed that all of them bind to the "upper" part of the combining site in the region surrounded by the third hypervariable region of the H chain and the first and third hypervariable regions of the L chain.[113] The naphtoquinone moiety of vitamin K_1OH is in

close contact with residues Tyr 90, Gly 29, and Asn 30 of the L chain and residues 100 to 102 of the H chain (see Figure 19). The phytyl chain extends over a larger area making contact with L-chain residues 47 (a constant Trp), 50, 58, and 101. Since the affinity constant for the binding of vitamin K_1OH (1.7×10^5 l/mol) is larger than that of menadione (approximately 10^3 l/mol), it was concluded that the increased affinity is due to the interaction of the phytyl chain with the combining site of Fab New.

An important observation made in the study of crystalline phosphorylcholine-Fab and vitamin K_1OH-Fab complexes was that no major conformational changes were observed after hapten binding.

C. Model Building

A possible, hypothetical approach to the study of the chemical basis of antibody specificity is that of model building. Amino acid sequences of myeloma proteins and antibodies can be built into three-dimensional models using the known structures of the Fab fragments and L chains discussed above. In these attempts, it is assumed that the basic immunoglobulin fold is not altered, a reasonable assumption. A more difficult problem is that of fitting the hypervariable region sequences, in

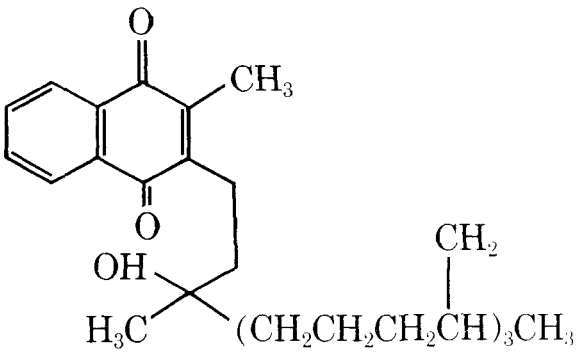


FIGURE 18. Vitamin K_1OH, a hydroxyl derivative of vitamin K_1.

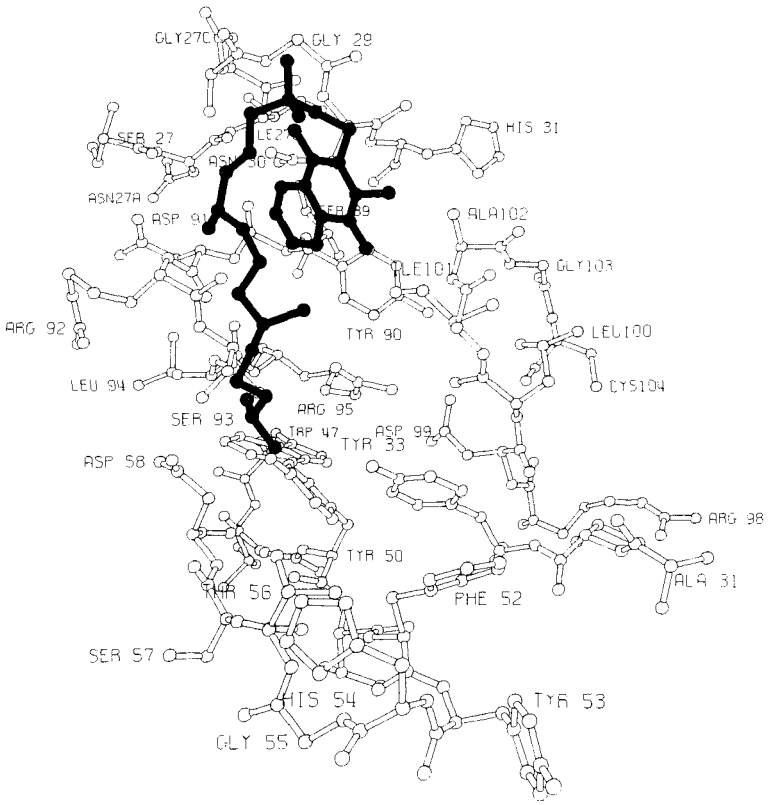


FIGURE 19. View of vitamin K_1OH bound to the combining site of Fab New. Amino acid residues are designated by numbers, as given in Figure 10.

particular, when deletions and insertions of amino acids occur. These sequences contribute to the conformation of the combining site which is to be determined by model building, e.g., on the basis of sequence homology between IgG New and MOPC 315, an IgA murine myeloma protein, it was pointed out that there is a high concentration of aromatic residues at the combining site of IgA MOPC 315.[50] The high density of adjacent hydrophobic and aromatic side chains that line the combining site correlates well with the observed specificity of MOPC 315 IgA for DNP and other ligands that include benzene and naphtalene aromatic rings in their structure. More recently, a combining site model of MOPC 315 IgA has been built on the basis of atomic coordinates derived from McPC 603 Fab and additional information derived from Fab New.[114] Models for rabbit anti-polysaccharide antibodies have been proposed using the same approach. Based on the known three-dimensional structures of H and L chains and amino acid sequences of myeloma proteins, Kabat et al.[115] have attempted to predict which amino acid side chains in those sequences will be specific for antigen-antibody contacts. A general criticism of these speculations is that they are based on a small number of three-dimensional structures which have not yet been refined to the desired precision in most cases and do not yet cover a wide enough range of combining site conformations to form the basis for model building. It is clear that more structure determinations of immunoglobulin combining sites and, in particular, those of induced antibodies of known specificity, are needed to provide a sound basis for model-building attempts.

D. Other Studies on Hapten Binding

Studies of hapten binding in cyrstalline hapten-Bence-Jones Mcg dimer complexes have been discussed in a preceding section. The combining site of MOPC 315 IgA has been intensively studied by Dwek and colleagues[116] using high-resolution nuclear magnetic resonance (NMR), electron spin resonance (ESR), chemical modification techniques, and the structure proposed by the model-building approach discussed above.[114] This work is very extensive and will be discussed briefly in this review.

Previous work led to the conclusion that about 50% of the binding energy of DNP ligands to IgA 315 was contributed by the hapten group.[117] Following this conclusion, two series of haptens with attached magnetic resonance probes were used in this study. One of the series consists of alkyl phosphonate groups attached to the DNP group leading to distinctive phosphorous signals that can be analyzed by NMR. The other series consists of five- or six-membered nitroxide rings attached to DNP which are analyzed by their ESR spectra. From studies of the nitroxide DNP-combining site complex, it was concluded that the DNP moiety is rigidly held at the combining site. Separate signals from each bound enantiomer of the five-membered nitroxide DNP hapten showed that the site was asymmetric on both sides of the DNP ring. ^{31}P magnetic resonance provided a probe of the electrostatic environment of the bound hapten. Altogether, these probes gave an estimate of the dimensions of the combining site, 6×9 A at its entrance with a depth of about 11 to 12 A. Interpretation of proton resonances in DNP-Fv complexes (Fv: $V_H + V_L$) leads to a model in which the DNP group interacts by stacking with a Trp ring which is identified as Trp 93 in V_L. Finally, the binding of DNP glycine was also studied to provide clues to the orientation of the hapten and its precise interactions with the amino acid side chains at the combining site. In addition, the NMR studies allowed precise positioning of contact residues which was used to refine the starting model. However, it appears that the refined coordinates obtained in this pioneering study of the combining site are still dependent on the starting coordinates obtained by model building. Given the wealth of structural and chemical data available about MOPC 315 IgA, it would be of great interest to determine the crystal structure of this protein and ligand-combining site complexes and to compare the results with those obtained in the NMR study.

Studies of the average intrinsic association constant (K_0) for hapten-antibody systems revealed no significant differences between IgG antibodies and the Fab fragments prepared from them.[118-121] These results indicate non-cooperativity between the antibody combining sites. (A different conclusion was reached in another study.)[122] The very rapid nature of the interactions between hapten and antibodies and

the need to use haptens that can be followed by a change in their absorption spectra or in fluorometric properties upon binding to antibodies have limited the number of kinetic studies of those interactions. However, studies on the kinetics of binding of DNP haptens to MOPC 315 IgA and its Fab fragment by a temperature jump-chemical relaxation method found no significant difference between their forward association rate constants, k_f.[118] Similar conclusions were obtained with anti-DNP[119] and antifluorescein rabbit antibodies.[120] In a more recent study, the ratios of the association and dissociation rate constants of high affinity antiouabain rabbit antibodies were also found to be in good agreement with the K_0 value determined at equilibrium.[121] The results of this kinetic study, which utilizes a different hapten and new techniques, agrees with previous studies showing no demonstrable cooperativity between the two antibody combining sites.

Several calorimetric studies provided additional information about interactions in antigen-antibody and hapten-antibody systems. Measurements of the enthalpy of binding of DNP lysine to pooled anti-DNP rabbit antibodies have been reported.[123] Despite the fact that the pooled antibodies were heterogeneous, with affinity constants ranging over more than one order of magnitude, it was concluded that the reaction was enthalpically driven ($\Delta H = -21$ kcal/mol). In another study,[124] pooled rabbit anti-DNP antibodies were also used. In this case, the antibodies were fractionated according to their affinity for DNP by precipitation with limiting amounts of antigen. By this procedure, five pools of antibodies were obtained with affinity constants covering a 17-fold range. The measured enthalpies for the antibody-DNP-lysine reaction for the five pools did not show significant differences. The average value for the enthalpy of binding in the reaction was -13.9 ± 0.4 kcal/mol. Since the affinity constants for the different pools varied from 2.24×10^7 to 1.37×10^6, the free energies and entropies of binding varied from -10.0 kcal/mol and -5.1 cal/mol/degree to -8.35 kcal/mol and -10.6 cal/mol/degree, respectively. Evidently, the reactions are enthalpically driven in all the pools and, in all cases, entropic effects favor the dissociation of the antibody hapten complexes. Furthermore, the differences in affinity for different antibody pools seem to be entirely due to changes in the entropy of binding. Several tentative explanations of these effects are proposed by the authors, but it is clear that more detailed studies, combined with three-dimensional structural information, are necessary to understand the thermodynamics of binding in antibody reactions.

E. Multispecificity of Antibodies

The capacity of myeloma immunoglobulins and antibodies to bind different ligands has been taken as support for the postulate that an antibody molecule can be multispecific. X-Ray crystallographic studies have shown that an L-chain dimer[43] and IgG New[113] can bind a number of ligands with different chemical properties at the combining site. It should be emphasized here that the physiological significance of these binding reactions is not clear. The general picture that emerges from the binding studies on Fab New[113,125] is that of a restricted multispecificity by which chemically related haptens would be bound at the same approximate site. From a study of the mouse myeloma protein IgA 460, it was concluded that DNP and menadione groups could be bound at nonoverlapping subsites of the combining site.[126] The reported distance of the binding subsites appears larger than would be expected on the basis of the conformation of other combining sites such as that of IgG New. The cross reactions between DNP and menadione (vitamin K_3) in early and late rabbit antibodies have recently been investigated.[127] The authors conclude that the observed cross reaction between DNP and menadione is to be expected in terms of the structural similarities between DNP and menadione. The notion of restricted multispecificity appears very plausible from studies of cross-reacting antibodies, the known structures of antibody combining sites, and the general chemical nature of hapten-antibody interactions. However, general multispecificity of antibody combining sites has not yet been established.

A related problem arises in trying to define the actual extent of the antibody combining site. The systems that have been explored thus far, i.e., the McPC 603 IgA and IgG New, are not enough to define the entire area (or volume) of the site, although they certainly have defined a central part of it. It will be necessary to inves-

tigate other hapten-antibody or antigen-antibody systems using techniques such as X-ray diffraction and NMR spectroscopy to answer this question.

X. EFFECTOR FUNCTIONS AND STRUCTURE OF ANTIBODY MOLECULES

The primary function of antibody molecules is usually defined as that of binding antigens. As discussed in preceding sections of this review, the molecular site for this function resides in the V (V_H + V_L) domain. Effector or secondary functions are known to reside in the Fc part of immunoglobulins. Among these functions, complement fixation and cytophilic reactions (attachment to cell membranes) are very important in different phases of immune responses.

There is considerable evidence that the $C_\gamma 2$ domain in IgG is the site of complement fixation. This topic has been extensively reviewed.[1,7,128] (The reader is referred to those reviews for a comprehensive summary of experimental results and their interpretations on this subject.) A number of antigen binding IgG molecules, possibly four or more,[129] seem to be necessary for the activation of complement, whereas a single IgM molecule bound to antigen will activate complement. The fact that heat-aggregated IgG Fc fragments are capable of complement activation has been used as evidence in favor of the notion that activation is simply due to aggregation of antibody molecules brought about by reaction with a polyvalent antigen. In the case of IgM, a simple shape change could account for complement binding.[7] A different view of the mechanism of activation is that of an allosteric transition or conformational change induced by antigen binding and transmitted through the V_H, V_L, C_L, and C_H1 domains, and the hinge region to the C_H2 domains of IgG. In the case of IgM, since C_H3 appears to contain the site of complement binding, the allosteric signal would be transmitted even farther to that domain.

Conformational changes affecting volume,[130,131] sedimentation coefficients, and flexibility[132-134] of IgG molecules after antigen binding have been reported. More recently, changes in circular polarization of luminescence of tryptophan residues in the Fc as well as in the Fab parts of antibody molecules have been presented as evidence for specific conformational changes induced by antigen binding.[135]

Based on the results of cyrstallographic analyses of Fab fragments and those of IgG Kol and human Fc, Huber and his colleagues have proposed[136] a structural model to account for a conformational change that could be transmitted from the antigen combining site to $C_\gamma 2$. The basic observations are the following:

1. The Fab arms of IgG Kol are in an extended (''relaxed'') conformation in which the pseudo twofold axes relating V_H to V_L and C_H1 to C_L are nearly parallel, whereas in the Fab crystal structures, there is a bending at the switch regions such that the corresponding axes make angles of about 120° (see Figures 12 and 14). Thus, there is intrasegmental flexibility at the switch regions (''elbow bending'') between V (V_H + V_L) and C_1 (C_H1 + C_L).
2. The peptide linking C_H2 to C_H3 in the crystal structure of the Fc fragment is not in an extended conformation. Stretching out of this peptide and a loosening of the C_H2-C_H3 contacts appear possible. The C_H2 domains are not closely packed, suggesting that they could have greater freedom of movement than V, C_H1, or C_H3 domains.
3. The hinge peptide appears to be folded back in between the C_H2 domains in the crystal structure of the Fc fragment.

A rigid or ''minimum disorder'' model of the whole immunoglobulin molecule built with Fab arms in the conformation observed in Fab crystals and with a retracted hinge peptide results in close contacts between C_H1 and C_H2. This ''rigid'' molecule is proposed as the hypothetical liganded antibody molecule. The liganded molecule is T shaped as is IgG Dob in which a deletion at the hinge region is assumed to force the T shape; this deletion is equivalent to a retraction of the hinge peptide. Antigen binding is assumed to induce this conformation in which the molecule has lost its inherent flexibility and in which longitudinal contacts from V_L to C_L, V_H to C_H1, C_H1 to C_H2, etc., contribute to a rigid structure. Complement fixation to the

liganded, rigid structure can take place because binding of the complement C1q factor is no longer hindered by Fab and possibly C_H3 motions, which transiently cover the C1q binding site. Furthermore, it is postulated that the formation of the longitudinal contacts might induce a structural change in C_H2, allowing complement binding to that domain. In support of this mechanism, Huber and colleagues point out that homologous segments mediate V_H-C_H1 and C_H2-C_H3 contacts. These hydrophobic contacts are made by C_H2 residues 247 to 253 and 310 to 314, and C_H3 residues 376 to 379 and 428 to 433 in human IgG (see Section IV for a more complete description of V_H-C_H1 contacts in a human immunoglobulin).

The structural model described above has attractive features and encompasses some known experimental facts. For example, crystalline Fab structures were not observed to undergo conformational changes after hapten binding,[54,113] in agreement with the idea that they had already assumed the rigid conformation of a liganded antibody molecule. In addition, it is known from hydrodynamic[137] and spectroscopic[138,139] observations and in agreement with electron microscopy experiments[26] that the inherent segmental flexibility of IgG is reduced upon antigen binding.

There are other observations which the postulated mechanism does not explain. For example, human IgG4 and mouse IgG1 do not activate complement although both have a shorter hinge region than human IgG1 or rabbit IgG. However, the Fc fragment from human IgG4 binds complement with high affinity.[140] Also, in the guinea pig system, antigen-antibody complexes involving IgG2 activate complement, whereas IgG1 antibodies do not. The most noticeable structural difference between these two immunoglobulin isotypes resides in the hinge region, which is four amino acid residues shorter in γ1 (from IgG1) than in γ2 chains.[141] Moreover, since IgG1 molecules appear more rigid and form larger aggregates with antigens than IgG2,[141] one would expect them to be in the conformation and concentration appropriate for complement activation. Evidently, the structural basis for the interaction of complement with antigen-antibody complexes is not fully understood.

Transmission of specific conformational signals from the antibody combining site to the putative site of complement fixation in C_H2, along a distance of about 100 A, is difficult to visualize. The longitudinal contacts between domains are few compared to those between pairing homology subunits (e.g., C_L-C_H1). Moreover, these contacts are formed only in the liganded model of antibody molecules so that they would be useful in stabilizing the rigid or liganded conformation rather than transmitting a conformational change. Concerning this topic, conformational changes in Fab and Fc regions of antibody molecules have been reported using measurements of the circular polarization of luminescence.[135] The results were interpreted to indicate changes in the environment of tryptophan residues. However, many of these tryptophan residues are invariant features of immunoglobulin structure and occur in the interior of the homology subunits next to the intrachain disulfide bonds which should be the most stable region of the structure. Undoubtedly, the environment of tryptophan residues will change after antigen binding due to an overall loss of flexibility in the antibody molecule. Lancet and Pecht have recently reported kinetic evidence for a hapten-induced conformational transition in the murine IgA protein MOPC 460.[142] This myeloma protein, which binds nitroaromatic haptens with relatively low association constants ($K = 10^4$, 10^5 l/mol), has been used as a model for hapten-antibody interactions. The mathematical analysis of the experimental data obtained in this work is involved. The difference between the free energy of ligand binding and the two postulated (allosteric) states of MOPC 460 IgA, T, and R is small. The authors point out that a larger value could be expected upon interactions with antigens involving more residues at the combining site. It would be interesting to repeat these experiments with homogeneous antibody molecules of higher hapten-binding association constants. An allosteric model of antibody activity as suggested in this study requires conformational changes that increase the binding affinity for a given ligand at the free combining site and promote complement fixation at another site(s).

In conclusion, it can be said that in spite of all the uncertainties and disagreements in experimental data discussed above, in a preceding

section, and by others,[1,7,128,143] the striking feature that the structural studies and, in particular, the recent crystallographic studies have revealed in immunoglobulin molecules is that of flexibility. In addition, the structure of the C1q complement factor with its collagen-like filaments suggests that flexibility is also a major factor in the interaction of C1q with antigen-antibody complexes. Segmental flexibility is a well-established fact in immunoglobulin structure; the major site of this property is the hinge region. X-Ray studies of Fab fragments suggest that this flexibility extends to the region around the interchain disulfide bond connecting H and L chains. In addition, an IgG molecule has other segments of inherent, more limited flexibility: the switch regions connecting V_H to C_H1, V_L to C_L, and possibly C_H2 to C_H3. Added to segmental flexibility (hinge region), intrasegmental flexibility at the switch regions should facilitate antigen binding by allowing an optimal fit between antigenic determinants displayed at varying distances, and the antibody combining site. Binding of antigen would be sufficient to decrease intrasegmental and, in particular, segmental flexibility, thus uncovering a putative site for C1q binding in the C_H2 domains. The longitudinal contacts discussed above would help stabilize the liganded conformation, further decreasing unstable molecule motions which would interfere with complement fixation. This would not be a specific allosteric mechanism since any ligand capable of binding to the antigen combining site would have the same effect. In different antibodies, complementarity-determining sequences would vary only under the selective pressure of antigen recognition. The genetic information for combining sites could be used in different isotypes irrespective of their secondary functions. Flexibility would be a molecular device to maximize antibody efficiency by accommodating the spatial requirements of varying distances between antigenic immunodeterminant groups.

Irrespective of the view that one may adopt at this time, it is clear that progress has been made in our understanding of the correlations between immunoglobulin structure and effector functions. However, much structural research remains to be done before a complete understanding of the molecular mechanisms of immune responses can be reached.

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