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EDITED BY

F. J. DIXON

Division of Experimental Pathology
Scripps Clinic and Research Foundation
La Jolla, California

HENRY G. KUNKEL

The Rockefeller University
New York, New York

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PREFACE

It is always difficult in any branch of science to assess the relative significance of individual advances. It is a task best left to historians who have the advantage of a retrospective purview. Current developments in immunology are a good case in point. Key information in diverse areas is accumulating at such a rapid pace that there is scarcely time to take stock of the situation and obtain a true evaluation of significance. The papers in Volume 21 relate directly to four of the forefronts that have developed particularly rapidly. In contrast to other areas, there can be little doubt about their long-range significance.

Dr. Poljak's review certainly represents a landmark study. His work on the X-ray diffraction analysis of the Fab fragment of a human myeloma protein has provided the first real insight into the three-dimensional structure of the Ig molecule. Four globular units sharing a basic pattern of polypeptide folding make up the two units from the light chain and the two from the heavy chain. These are connected by linear stretches of polypeptide chains. It is of special interest that the hypervariable portions of the two regions are both positioned at an exposed end of the molecule offering the greatest opportunity for variability at the combining site.

Dr. Kindt reviews the long series of interesting studies of rabbit allotypes including some of the exciting recent developments. He is a real leader in the field and his vast knowledge of the intricacies of the system is readily apparent. The rabbit system has held particular interest for a number of years because of the presence of genetic markers in the V regions; similar readily detected antigens have not been found in other species. He discusses his new and very rational theory that multiple structural genes control each V region and that an insertional mechanism into a relatively constant C region is involved.

The article by Dr. Weigle dwells on the details of control mechanisms involved in antibody production. The phenomenon of cyclical production of antibody after a single injection of antigen is striking and apparently considerably more common than is usually thought. It is clear that antibody itself plays a primary role in this regulation, but other added factors, such as suppressor T cells and secondary idiotypic antibodies, also may play a role under specific circumstances. The intriguing concept is presented that the cycling phenomenon

plays a key role in the conservation of memory cells and thus assures continued persistence of antibody.

The paper by Drs. Coutinho and Möller represents a very complete review of the mechanism of B-cell activation for the production of antibody. The work with mitogens particularly by these investigators has changed very considerably the earlier concepts. It now appears that the mitogenic effect, which they term polyclonal B-cell activation, is an integral part of antigen activation, particularly where the antigen itself is mitogenic as well. The authors assemble considerable evidence that this is the case with T-cell independent antigens. These questions with respect to T-cell dependent antigens remain unresolved and obviously must await further knowledge concerning the mechanism of action of the various T cell factors influencing B cells.

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X-Ray Diffraction Studies of Immunoglobulins¹

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I. Introduction

The most direct approach to the study of the detailed three-dimensional structure of macromolecules is the method of single-crystal X-ray diffraction. The determination of the structure of several enzymes and nucleic acids to atomic resolution achieved within the last 10-15 years is an outstanding demonstration of the potential of this approach. Recent developments in methods and techniques have facilitated the determination of more complex structures such as those of multimeric protein molecules.

Once the overall multichain structure of immunoglobulins became established, about 12 years ago, it was hoped that amino acid sequence determinations, and eventually X-ray diffraction studies, would provide useful models for the correlation of the function and structure and the genetic mechanisms that control the variability and specificity of antibodies. However, the fact that immunoglobulins were demonstrably heterogeneous seemed to present an insurmount-

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able obstacle for X-ray diffraction studies since crystallization, a necessary first step, had always been associated with the presence of a homogeneous preparation of a single molecular species. Just as in the case of sequencing studies, this problem was resolved by the use of myeloma immunoglobulins which provided homogeneous material in sufficient quantities to permit crystallographic studies to atomic resolution.

This review treats almost exclusively results obtained in the investigation of the three-dimensional structure of a human Fab' fragment currently under study in the author's laboratory. Results of X-ray diffraction studies which are under way in several other laboratories will be briefly mentioned or described in much less detail.

II. Polypeptide Chain Structure of Immunoglobulins and Antibodies

The reader is referred to previous extensive reviews of this subject such as those of Edelman and Gall (1969), Milstein and Pink (1970), Krause (1970), Hood and Prahl (1971), Natvig and Kunkel (1973). Figure 1 shows the diagrammatic structure of a human IgG1 molecule and introduces some of the nomenclature that will be used in this review. The variable homology regions, indicated as V_H and V_L in Fig. 1 occur toward the N-terminal of the heavy (H) and light (L) polypeptide chains and each include about 110 amino acid residues. The constant homology regions C_L , C_{H1} , C_{H2} , and C_{H3} also include about 110 amino acid residues each. The amino acid sequences of V_H and V_L are highly homologous and so are those of C_L , C_{H1} , C_{H2} , and C_{H3} . The homology between the V and C regions is weaker, although some features such as intrachain disulfide bonds, illustrated in Fig. 1, are common to all of them. It is well known that the V regions display a variability of sequence that is not observed in C regions. Comparison of V_L sequences indicates that this variability is more pronounced in certain regions which are hypervariable in sequence (Wu and Kabat, 1970; Kabat and Wu, 1971). Similar hypervariable regions have been detected in V_H sequences (Kabat and Wu, 1971; Capra and Kehoe, 1974).

III. Techniques of Crystallographic Analysis

Several extensive reviews on this subject are available (e.g., Dickerson, 1964; Holmes and Blow, 1966; Matthews, 1974). A shorter review including preliminary results of X-ray diffraction studies of immunoglobulins has also been published (Poljak, 1973).

Briefly stated, X-ray diffraction analysis of single crystals can provide a high-resolution model of a molecule by a Fourier series

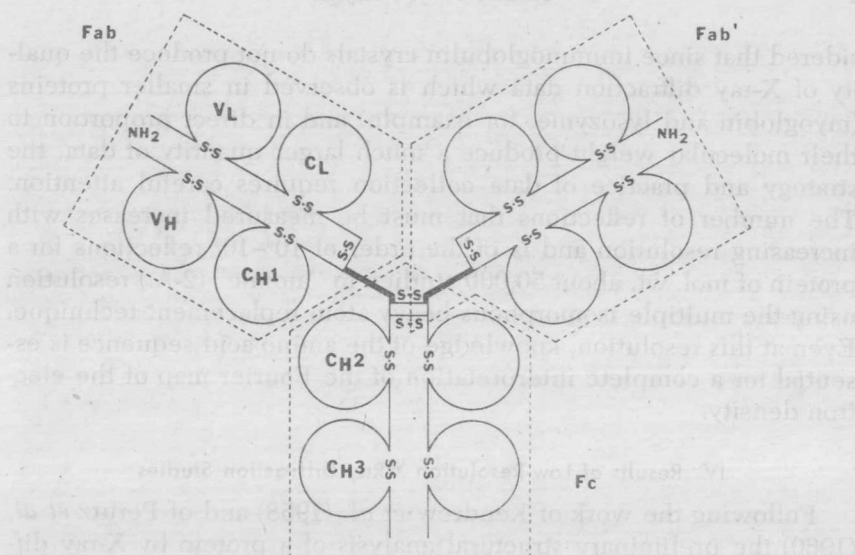


FIG. 1. Diagram of a human immunoglobulin (IgG1) molecule. The light (L) chains (mol. wt. about 25,000) are divided into two homology regions, V_L and C_L . The heavy (H) chains (mol. wt. about 50,000) are divided into four homology regions, V_H , C_H1 , C_H2 , and C_H3 . The C_H1 and C_H2 are joined by a "hinge" region indicated by a thicker line. Cleavage of the IgG1 molecule by papain generates Fab fragments (mol. wt. about 50,000) consisting of an L and an Fd polypeptide chains, and Fc fragments (mol. wt. 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. (Reproduced from Poljak, 1973, with kind permission of Plenum Press.)

representation of the electron density, calculated from experimental diffraction data and plotted as a contour map. The conformation obtained from X-ray diffraction studies has been shown to be very close to that inferred to exist in solution for several proteins (see review by Matthews, 1974). Since X-ray measurements are made during prolonged periods of time (hours), only stable conformations can be analyzed, for example, at the beginning and at the end of a conformational change, or at both stages if they can be obtained under controlled conditions (for example, oxy- and deoxyhemoglobin crystals). The development of data-gathering instruments, such as computer-controlled diffractometers and large memory high-speed computers and their peripheral equipment, have made the study of larger, more complex macromolecules easier than it would have been only a few years ago. Even with these technical developments it should be con-

sidered that since immunoglobulin crystals do not produce the quality of X-ray diffraction data which is observed in smaller proteins (myoglobin and lysozyme, for example) and in direct proportion to their molecular weight produce a much larger quantity of data, the strategy and practice of data collection requires careful attention. The number of reflections that must be measured increases with increasing resolution and is of the order of 10^4 – 10^5 reflections for a protein of mol. wt. about 50,000 studied to "atomic" (2-Å.) resolution using the multiple isomorphous heavy atom replacement technique. Even at this resolution, knowledge of the amino acid sequence is essential for a complete interpretation of the Fourier map of the electron density.

IV. Results of Low-Resolution X-Ray Diffraction Studies

Following the work of Kendrew *et al.* (1958) and of Perutz *et al.* (1960) the preliminary structural analysis of a protein by X-ray diffraction methods is first attempted at low (usually 6-Å.) resolution. This approach is followed because low-resolution studies are capable of providing a general model of the quaternary and tertiary structure of the protein under study and can be completed in a much shorter time than is required for higher-resolution analyses. In addition, low-resolution studies provide a critical test of the experimental techniques and the mathematical and computational procedures which will be used in subsequent (longer) high-resolution studies.

Fragments Fab and Fab' from human myeloma proteins were crystallized by Rossi and Nisonoff (1968) and by Rossi, Choi, and Nisonoff (1969). X-Ray diffraction patterns of the crystalline Fab and Fab' fragments (Avey *et al.*, 1968; Humphrey *et al.*, 1969) from IgG New gave identical intensity patterns indicating that they have the same structure. It would normally be expected that, since Fab' is about 10 amino acid residues longer than Fab (in its Fd' chain), its X-ray intensity patterns should reflect this difference. Subsequent high-resolution studies indicated the C-termini of the Fd' and Fd chains do not make any significant contribution to diffracted X-ray intensities, probably due to random motion of the ends of the polypeptide chains. From this it follows that Fab' New and Fab New are equivalent for crystallographic studies.

Another crystalline fragment, Fab Hil (Rossi and Nisonoff, 1968; Humphrey *et al.*, 1969) also proved suitable for X-ray diffraction studies. However, since it contains two Fab molecules per asymmetric unit of the crystal lattice, its study requires about twice the number of intensity measurements than are required for the study of Fab New. Both fragments, New and Hil were obtained from human

IgG1 (λ) myeloma proteins of allotype Gm(1⁺, 3⁻, 4⁻, 5⁻) (Rossi and Nisonoff, 1968).

The 6-Å. resolution study of Fab' New (Poljak *et al.*, 1972) showed that the Fab' fragment consists of two discrete globular domains, V and C₁, containing the V_L and V_H and the C_L and C_H1 homology regions, respectively. Despite the limitations of a low-resolution study, the two polypeptide chains, L and Fd', were found to be two, continuous, independent stretches of electron density, which originate in one of the globular subunits and, after a region of globular folding in that subunit, extend to the other subunit through a narrow outer bridge of polypeptide chain (Fig. 2). The overall scheme is that of a tetrahedral arrangement of four globular subunits, two of which (V_L and C_L) are formed by the L chain and the other two (V_H and C_H1) by the Fd' chain. This type of arrangement is

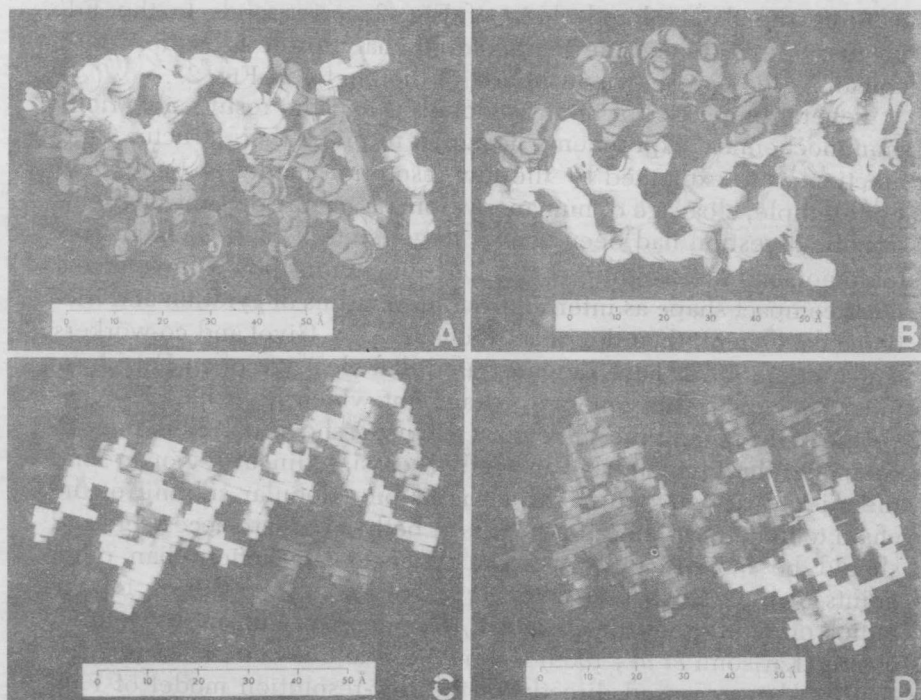


FIG. 2. Four different views (A-D) at 90° from each other, of the 6-Å. resolution model of Fab' New. The two continuous chains of electron density described in the text are shown in white and gray. Two globular domains, V and C₁, separated by a cleft and joined by two easily accessible connections are clearly seen. Photographs C and D clearly show the approximate tetrahedral symmetry of the molecule. (Reproduced from Poljak *et al.*, 1972, with kind permission of Nature.)

roughly similar to that observed in hemoglobin (Perutz *et al.*, 1960) where four globular subunits are arranged in tetrahedral configuration, although in hemoglobin there are four independent (two α , two β) polypeptide chains.

The presence of discrete globular domains V and C_1 of Fig. 2, had already been demonstrated by Green *et al.* (1971) in a study by electron microscopy of the dinitrophenyl (DNP)-binding murine IgA, myeloma protein, MOPC 315. Furthermore, Green and colleagues inferred that each globular domain contained a homology region of both the L chain and the H chain. As expected, the detailed symmetry of the arrangement is more clearly indicated in the 6-Å. resolution X-ray diffraction model of Fab' New. However, the assignment of the V ($V_L + V_H$) and C_1 ($C_L + C_H1$) domains from the 6-Å. resolution study, made on the basis of the occurrence of a cavity-shaped region at one end of the molecule, was not confirmed by correlation with the high-resolution model to be described below. From this correlation, the dark-colored chain of Fig. 2 corresponds to the Fd' polypeptide chain and the light-colored chain to the L chain. The V domain is the one shown at the left of the model in Fig. 2.

Several properties of immunoglobulins and the results of various immunochemical experiments designed to probe their structure and function were explained by the low-resolution model of Fab' New. For example, cleavage of human myeloma L chains by controlled enzymatic digestion had been shown to generate V_L and C_L fragments (Solomon and McLaughlin, 1969; Karlsonn *et al.*, 1969) which have a more compact shape as inferred from their radius of gyration and frictional coefficient, than the intact L chain. Also, Givol and co-workers (Inbar *et al.*, 1972) have demonstrated that cleavage of an Fab' fragment by pepsin can generate a fragment which he called F_V , consisting of the V_L and the V_H regions. Figure 2 shows that both the Fd' chain and the L chain have an easily accessible linear region around the middle of their length which joins one globular subunit of the chain to the other. These midpoints of both chains are accessible and should be more readily cleaved by enzymatic action than other points of the chains. The average value of 16 Å. obtained for the radius of gyration of the C_L and V_L fragments of human myeloma L chains (Karlsonn *et al.*, 1969) is very close to the value of 15 to 20 Å. that can be measured directly in the low-resolution model of Fab' New. Close inspection of the low-resolution structure appeared to indicate that the L and the Fd chains share a common overall structure such that they could pair with each other as in Fab, or form a dimer with a homologous chain (an L-chain or an H-chain dimer).



FIG. 3. Schematic representation of the structure of an IgG molecule incorporating structural features determined for the Fab and Fc fragments (see text). (Reproduced from Poljak *et al.*, 1972, with kind permission of Nature.)

Amino acid sequence homologies first demonstrated by Hill *et al.* (1966) led to speculation that the homology regions of immunoglobulins would fold into basic globular subunits of similar three-dimensional structure (the structure specified by a primordial gene) which, suitably repeated, could account for the whole immunoglobulin structure. This speculation is clearly consistent with the low-resolution model of Fab' New. On the basis of this model and on the symmetry determined for the Fc fragment (Goldstein *et al.*, 1968) and for a whole IgG molecule (Sarma *et al.*, 1971), a schematic picture of an IgG molecule can be represented as shown in Fig. 3.

V. High-Resolution Studies

A 2.8-Å. resolution study of the Fab' New fragment (Poljak *et al.*, 1973) led to an atomic resolution model that correlated amino acid sequence data with tertiary structure. Subsequently, a 2.0-Å. resolution Fourier map has been calculated and an atomic resolution model was built based on this map and on the amino acid sequence (Poljak *et al.*, 1974). Models of an L (λ) dimer at 3.5-Å. resolution (Schiffer *et al.*, 1973) and of a V_{κ} dimer at 2.8-Å. resolution (Epp *et al.*, 1974) have been presented. A 4.5-Å. resolution Fourier map of the Fab' fragment from the phosphorylcholine binding protein McPC 603 has also been published (Padlan *et al.*, 1973). Complexes between Fab McPC 603 and a phosphorylcholine ligand (Padlan *et al.*, 1973) and Fab' New and several ligands (Amzel *et al.*, 1974) have been studied by X-ray diffraction methods. Some of the results obtained from these studies will be discussed below.

A. SHAPE, DIMENSIONS, AND SYMMETRY OF FAB'

The 2.8-Å. resolution Fourier map of Fab' New clearly indicated the molecular shape of Fab' and the arrangement of the L and Fd' polypeptide chains, which are essentially as described in the 6-Å.

resolution study (Figs. 4 and 5). The overall dimensions of the Fab' molecule are $80 \times 50 \times 40$ Å, and those of the homology subunits are $40 \times 25 \times 25$ Å. ("homology subunit" is used here to denote the globular unit of three-dimensional structure that contains the amino acid sequence of a homology region, V_L , V_H , C_L , or C_H1 , etc.). A centrally located cleft, openly accessible to solvent divides the molecule into two globular structural domains, V and C, which are linked by two "switch" region sequences. The structural subunits C_L and C_H1 in the C_1 domain interact more closely and are more tightly packed than the V_L and V_H subunits in the V domain. The angle between the

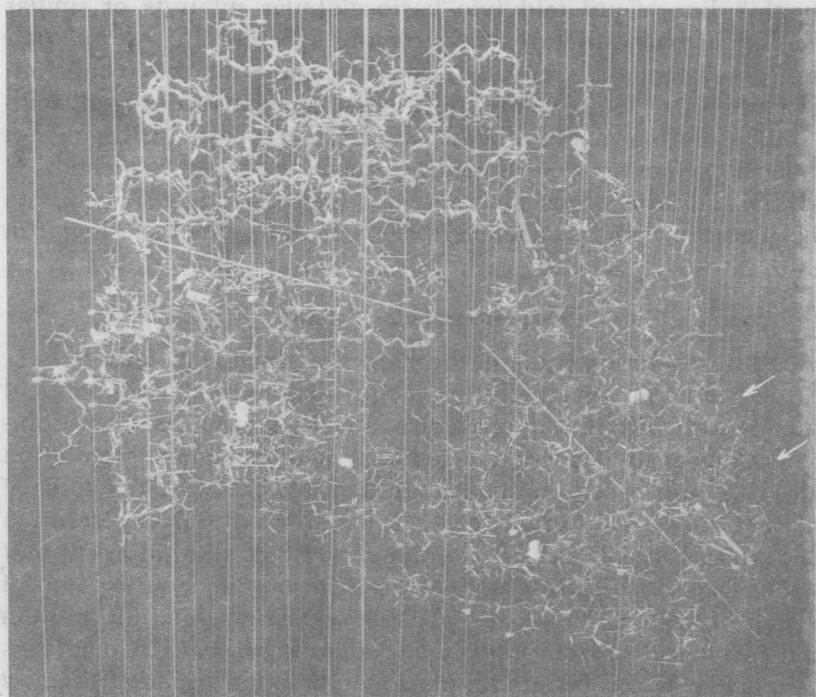


FIG. 4. Model of Fab' New based on a 2.8-Å. resolution Fourier map. The V (left) and C_1 (right) domains are separated by a central cleft. Labels show the "switch" region at the midpoints of the L (upper) and Fd' (lower) chains. Approximate local twofold axes of symmetry are indicated by two white rods. The four intrachain disulfide bonds and the interchain disulfide bond are marked by white spheres. White tapes connect the α -carbon positions of residues that form disulfide bonds in other immunoglobulin molecules (see text). The tags at the left end of the model indicate the hypervariable positions of the L chain (round tags) and the H chain (rectangular tags). Arrows at the right end of the model point to Ser 154 and Lys 191 (Kern and Oz serological markers, respectively). This view of the molecule closely corresponds to photograph A of the low-resolution model in Fig. 2.

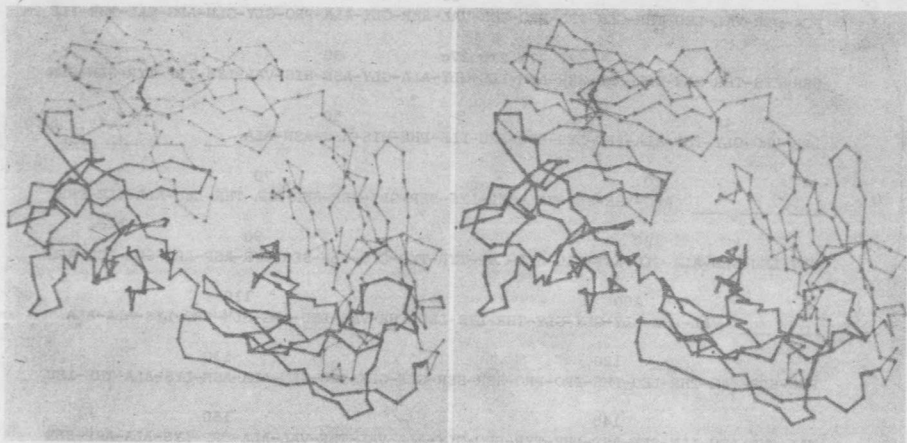


FIG. 5. Stereo pair drawing of the α -carbon backbone of the Fab' structure oriented as shown in Fig. 4. The thicker trace corresponds to the Fd' polypeptide chain. (Inexpensive stereo viewers to help achieve a three-dimensional effect from this figure can be obtained from the Taylor-Merchant Corporation, New York, N. Y. 10036.)

major axes of the C_L and V_L homology subunits is greater than 90° (100° – 110°), whereas the angle between the axes of the C_H1 and V_H subunits is smaller than 90° (80° – 85°), see Figs. 4 and 5. As a result, the Fd' chain is more folded over itself, displaying a closer association between the V_H and C_H1 subunits than is the case for the V_L and C_L subunits of the L chain. It is interesting to observe here that the L (λ)-chain dimer Mcg (Schiffer *et al.*, 1973) does not crystallize as a dimer of conformationally identical chains. One of the L chains of the dimer assumes a conformation that appears very close to that of the L chain from IgG New. The second L chain from the Mcg dimer, although identical in sequence to the first, assumes a conformation closer to that of an Fd' chain, with an angle smaller than 90° between the major axes of its V and C subunits.

As indicated in Fig. 4, approximate twofold axes of symmetry relate C_L to C_H1 and V_L to V_H .

B. AMINO ACID SEQUENCE OF THE L AND H CHAINS FROM IGG NEW

High-resolution Fourier maps of protein structures are usually interpreted by a correlation of the electron-density features with the amino acid sequence of the protein under study. Extensive sequence work which has been carried out with different im-