Conf-9111148--1

ANL/CP--74444

DE92 004723

Polymerization of Immunoglobulin Domains: A Model System for the Development of Facilitated Macromolecular Assembly [1]

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[1]This work was supported by the United States Department of Energy, Office of Health and Environmental Research, under Contract W-31-109-ENG-38.

ABSTRACT

We have recently determined that monoclonal immunoglobulin light chains (Bence Jones proteins) are capable of reversible polymerization at room temperature. This property, as exhibited by immunoglobulin light chains (normally a component of an intact antibody molecule), may have novel implications for the development of "molecular nanotechnology."

The polymerization capability of the immunoglobulin light chain is associated with the so-called variable domain of this molecule. The variable domain is a durable, compact beta-sheet structure of molecular weight approximately 12,000. Humans are capable of producing thousands of different light chain variable domains. Most of the primary sequence variation is limited to one portion of the molecule, that portion associated with the contribution of immunoglobulin light chains to the recognition and binding of thousands of different antigens by antibodies.

As a consequence of these variations, different light chains polymerize with different degrees of avidity, from negligible to extensive. The polymerization process depends on solution parameters such as pH. Thus, polymerization might be induced at one pH and suppressed or reversed at another. In some cases, Bence Jones proteins *in vivo* form an insoluble rigid assembly known as amyloid, which might result from lateral docking of two or more polymerized filaments.

The three dimensional structure of these proteins is well known through several crystallographic studies of antibody-like light chain dimers. Likewise, amino acid sequences of hundreds of these proteins have been obtained. Immunoglobulin molecules are readily produced in large quantities in *E. coli*; they are suitable for systematic site-specific mutation that provides for rational engineering to incorporate useful recognition and physical-chemical features. Combinations of molecules of appropriate specificities could assemble into structures of predeter-

mined three-dimensional forms and properties.

These features suggest that Bence Jones proteins represent a powerful model system within which to develop empirical rules relevant to a technology of protein-based "construction." Development of these rules will require the combined efforts of biophysical and crystallographic studies, protein engineering, and molecular modeling.

1. INTRODUCTION

The antibody molecule is the detection device of the immune systems of vertebrate organisms, binding to bacteria, viruses, and other "non-self" molecules. Attachment of antibodies labels these inappropriate elements as "foreign" and initiates further immunological responses for their removal. Evolution has provided an efficient detection system by devising a diverse antibody repertoire capable of recognizing an estimated millions of complementary molecular structures (for reviews, see Chothia *et al.* 1989; Davies *et al.* 1990).

A large contribution to this diversity is accomplished by the fact that the antigen binding site of an antibody is constructed by the combination of two independently synthesized polypeptide chains, a "light" chain and a larger "heavy" chain. Therefore, to a first approximation, the number of physiologically possible different structures of antigen binding sites is determined by the product of the number of different light chains and heavy chains the organism is capable of producing.

The antibody light chain, which is the subject of our discussion, is a protein composed of two independent "β-barrel" domains, each consisting of 110–120 amino acids, for a combined molecular weight of *ca.* 23,000. The domain located at the aminoterminus of the protein is encoded by a large number of gene exons, in contrast to the limited number of exons that encode the carboxyterminal domain. Additional amino acid substitutions in the amino-terminal domain occur as a result of mutations that accumulate during the lifetime of the organism. Consequently, the amino acid sequences of the amino-terminal domains are very heterogeneous, whereas the sequences of the carboxy-terminal domains are comparatively conserved. These domains are known as the variable domain and the constant domain, respectively. A similar organizational pattern is found for the heavy chain. In the intact antibody, the juxtaposition of the variable domains of both light and heavy chain account for the diversity of the antibody repertoire.

Antibodies are synthesized and secreted by a specialized blood cell known as a plasma cell; an individual plasma cell can produce only one kind of antibody. Multiple myeloma is a cancer of the immune system which results in the formation of tumors of identical plasma cells. Because the synthesis of excess light chains is a normal attribute of the production of antibodies, the presence of these tumors, which are plasma cell clones, generates monoclonal antibody and free monoclonal light chains not combined with heavy chains. The light chains are sufficiently small to be filtered through the kidney and appear in the urine of the patient.

Myeloma-derived light chains, known as Bence Jones proteins (Jones 1847, 1848), provided much of the early structural and genetic insight into the molecular biology of antibodies (for review, see Stevens *et al.* 1991). Free light chains are capable of self-association, or dimerization, mediated through interaction of their variable domains (Maeda *et al.* 1976, 1978; Stevens *et al.* 1980; Stevens and Schiffer 1981). The affinities of dimerization of the monoclonal proteins produced by individual patients vary, reflecting differences in the amino acid sequences. We have recently determined, that under appropriate conditions of pH, ionic strength, and ionic composition, many light chains exhibit higher order aggregation. The propensity for aggregation, and the optimal conditions for aggregation, are determined by the amino acid sequence of each individual protein. The three-dimensional structures of several dimeric Bence Jones proteins have been determined (Schiffer *et al.* 1973,1989; Epp *et al.* 1974, 1975; Fehlhammer *et al.* 1975; Colman *et al.* 1977; Furey *et al.* 1979,1983; Abola *et al.* 1980; Chang *et al.* 1985; Ely *et al.* 1985, 1989). These studies have shown, that although the interactions between monomeric β-domains may be altered by solvent conditions (Chang *et al.* 1985; Stevens *et al.* 1988; Ely *et al.* 1989; Schiffer *et al.* 1989), or amino acid determinants (Furey 1979,1983), the underlying three-dimensional structure of the β-domain is highly conserved (Figure 1). Analyses of the structure of one antibody light chain in a third solvent sys-

tem, as well of the structures for three new light chains, are currently in progress (M. Schiffer, unpublished information). In this report, we discuss the potential of systematic study of this extremely diverse family of structurally homologous proteins, and variants produced by molecular biology methods, to provide new insight into mechanisms for the control of assembly of polypeptide subunits.

2. MATERIALS AND METHODS

2.1 Proteins.

Purified human antibody light chains were generously provided by Dr. Alan Solomon, Department of Medicine, University of Tennessee Medical Center, Knoxville. Proteins were prepared as described previously (Solomon 1985).

2.2 Chromatography.

Self-association properties of antibody light chains were evaluated by size-exclusion chromatography, a technique by which reversible association is unambiguously revealed through differences in the elution behavior of protein samples applied to the column at different initial concentrations (Stevens 1980, 1989; Stevens *et al.* 1986; Myatt *et al.* 1991). Chromatography columns (3 mm $\times$ 25 cm) were packed with Superose 12 (Pharmacia). Columns were run at a flow rate of 60 μl/min. Sample size was 5 μl. Protein elution was monitored by absorbance at 214 nm.

2.3 Simulation.

The simulation under development for analysis of the affinity and kinetics of interactions responsible for antibody light chain polymerization is similar to those we have previously described for analysis of other macromolecular interactions (Stevens 1980,1986,1989). The chromatography column was represented as a finite array of imaginary cells; the monomer, dimer, oligomer composition of each cell was calculated; migration of each constituent was accomplished by a Gaussian distribution in which the mean represented the average velocity of the constituent (cells/cycle) and the standard distribution introduced dispersion of the sample.

Unlike the previous simulations, the current work involves two linked interac-

tion reactions. The first reaction is that between two monomers (M) to form one dimer (D)

$$M + M \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} D \tag{1}$$

Dimers are the fundamental building block for oligomer (O) formation.

$$D + D \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} O_2 \tag{2}$$

Higher order oligomers are formed by the addition of dimeric subunits.

$$D + O_{n-1} \xrightarrow{k_2} O_n \tag{3}$$

Dissociation of oligomers occurs with equal probability at each dimer–dimer junction within the polymer. Therefore, equimolar production of each of the possible dissociation products occurs.

$$\frac{(n-1)}{2} \cdot O_n \underset{k_{-2}}{\rightarrow} D + O_2 + O_3 + \ldots + O_{n-1} \tag{4}$$

This model implicitly postulates that monomer formation can only occur by dissociation of dimers; thus, oligomer formation effectively stabilizes dimers.

3. RESULTS AND DISCUSSION

3.1 Biomedical significance of light chain aggregation.

The initial impetus for the study of biophysical aspects of antibody light chains that did not pertain to their immunochemical function resulted from a biomedical enigma. Many multiple myeloma patients experience complications that arise from tissue and organ deposition of these proteins (Glenner 1980a,b;

Tubbs *et al.* 1981; Solomon 1982,1986; Solomon *et al.* 1991; Gallo *et al.* 1989; Stevens *et al.* 1991). These phenomena are idiosyncratic: not all patients experience complications and the physiological sites and expression of the pathologies vary among the patients who do experience light chain-related problems. Therefore, the relative contributions of host factors and protein-specific physicochemical factors are unclear.

Although both patient- and protein-related factors are probably involved to varying degrees in each individual's case, evidence for a causal relationship between protein structure and the expression of the complication was obtained with the finding that in cases of multiple myeloma associated with the production of the rare light chain subgroup, λ_{VI}, amyloid seems to be invariably present (Solomon *et al.* 1982) . Amyloid is a stable less commonly polymer formed by other light chains, and by several other proteins which share the β-sheet structure characteristic of light chains. This suggested that the ability of light chains to polymerize, exhibited strikingly by λ_{VI} proteins, might be shared in varying degrees by other light chains as determined by individual amino acid substitutions. Thus, we used size-exclusion chromatography to examine the aggregation tendencies of light chains under various conditions of pH, ionic strength, and buffer composition.

3.2 Effect of solution conditions on light chain aggregation.

Figure 2 compares the aggregation properties of a Bence Jones protein produced by a patient who experienced renal failure, a frequent outcome of the production of light chains. The upper panel demonstrates that the size distribution of the light chain sample is effectively independent of concentration when tested under neutral pH conditions (50 mM phosphate buffer, 100 mM NaCl, pH 7.2), indicating that under these conditions, the interaction between light chain dimers is negligible. The lower panel displays the behavior of the same protein under a different set of solution conditions, 30 mM acetate buffer at pH 4.5

(250 mM NaCl). Under these conditions, a shift to a significantly earlier elution position is found, revealing the presence of concentration-dependent aggregation of these light chains that is apparently induced by the increase in the proton concentration in the solvent. The elution position at minute 16 corresponds to a molecular weight appropriate for a light chain tetramer, presumably formed by the association of two dimers; the clear resolution between the apparent tetramer and the lower molecular weight components eluting later suggests that the dissociation rate of the tetramer is slow (Stevens 1989). Variable domain fragments of this protein are also capable of forming tetrameric complexes under the same conditions (data not shown). At the highest protein concentration, aggregates that eluted at approximately minute 10 were observed. Because the molecular weight exclusion limit of a Superose 12 column is on the order of 200,000–300,000, it can be inferred that polymers comprising at least five light chain dimers were present in this sample and that they did not dissociate during the ten minutes required to transit the column.

Figure 3 indicates that low pH is not obligatory for the formation of high molecular weight light chain oligomers. In this instance, the light chain, obtained from a patient in which amyloid was present, demonstrated concentration-dependent aggregation under the neutral isotonic conditions in which the protein depicted in Figure 2 exhibited little or no polymerization. A significantly higher percentage of the Fig. 3 protein (compared to the light chain shown in Figure 2) eluted at the exclusion limit of the column, demonstrating a relatively higher propensity for the formation of oligomers. Moreover, the continuum of eluted protein that appears between the excluded volume position and the position corresponding to the dimer, the constituent considered the protomer for polymer formation, suggests a faster dissociation rate constant than that evident for the light chain in Figure 2.

The data shown in Figures 2 and 3 demonstrate the solvent-dependency of

light chain aggregation and indicate that noncovalent polymers of extended size can be formed. These data are consistent with earlier findings of Neet and Putnam (1966) which indicated that at elevated temperatures, light chain polymers of molecular weight in excess of 10^6 could be formed. To date, we have examined antibody light chains from more than 40 myeloma patients. Approximately one-half of these protein samples have exhibited polymerization tendencies under one or more of the solvent conditions in which they have been tested (Myatt *et al.*, manuscript in preparation).

3.3 Hypothetical mechanism of light chain polymerization.

Figure 4 depicts our preliminary model for the interaction between light chain dimers that leads to oligomer formation. The rationale for the formation of this model arises from considerations of the properties of amyloid formed by antibody light chains and will be described in more detail elsewhere (Stevens *et al.*, manuscript in preparation). Fundamentally, the concave surface of the variable domain dimer, which contains that portion of the molecule that contributes to the diverse antigen binding capabilities of antibodies, is structurally complementary to the concave surface that is found at the opposite pole of the structure. Therefore, depending upon the amino acid composition of these two surfaces, which varies for each protein obtained from individual patients, different affinities of interaction are found as well as different optimal solution conditions for interaction. The constant domain of the light chain, which is connected to the variable domain through an extended flexible peptide chain, neither contributes to nor blocks the polymerization mechanism illustrated in Figure 4.

3.5 Light chains as a model system to study engineered protein assembly.

Figure 4 represents a testable hypothesis to account for the spontaneous assembly of antibody light chains into high molecular weight aggregates. Several considerations suggest that this family of proteins will provide a unique model

system within which to systematically study the structural basis of polypeptide assembly.

3.5.1 Structural data base. As indicated above, numerous crystallographic studies of antibody light chains of human origin have demonstrated the highly conserved nature of the three-dimensional structure of the β-barrel domain. This conservation of structure has been further established by crystallographic studies of the antigen binding fragments of monoclonal antibodies. Therefore, interpretation of the functional properties of any antibody light chain is facilitated by fundamental confidence in the knowledge of its basic three-dimensional structure, even in the absence of a detailed crystallographic study.

3.5.2 Diversity. Because the evolution of immunoglobulin light chains was driven by the requirement for the generation of diversity in the antibody repertoire, it is probable that the combination of amino acid sequence heterogeneity coupled with conserved basic three-dimensional structure can be equaled only by that of antibody heavy chains. Complete sequences of human light chain variable domains have been obtained from more than 140 patients (Kabat *et al.* (1987) and Solomon, personal communication). Assuming that the amino acid substitutions which have been documented to date all represent independent structurally permissible replacements, in excess of 10^{80} variant light chains can be constructed for study. Although a number of this magnitude is effectively meaningless, it assures that a broad spectrum of recombinant light chains is available.

3.5.3 Genetic engineering. The biomedical and biotechnological utility of monoclonal antibodies has motivated substantial progress in the cloning and bacterial production of antibody components in recent years (Boss *et al.* 1984; Cabilly *et al.* 1985,1989; Pluckthun *et al.* 1987; Better *et al.* 1988; Huston *et al.* 1988; Skerra *et al.* 1988; Condra *et al.* 1990; Glockshuber 1990a,b; Sharon

1990). These methods can be used to produced large quantities of antibody light chains of predetermined amino acid sequence. Engineered light chains will be used to identify the surfaces of the molecule involved in formation of polymers. Subsequently, light chains which differ by single amino acid substitutions will provide the experimental data for the development of new computational tools for the engineering of specific protein-protein interactions.

3.5.4 β-Domain assembly. The assembly capabilities of the β-domain structure are widely expressed biologically. These structures are the basic building blocks of the so-called immunoglobulin superfamily, which includes the several classes of antibodies, β-2 microglobulin, the T-cell receptors, secretory components, the major histocompatability components, cell adhesion molecules, and neurological cell receptors (Williams 1984; Hunkapiller and Hood 1986; Williams an Barclay 1988). Driscoll *et al.* (1991) have recently determined the structure of the CD2 cell adhesion molecule, finding it to be conformationally homologous to the light chain variable domain, and consistent with the idea that the interactions between adhesion molecules of two interacting cells are mechanistically the same as the interactions between two light chain variable domains or between a light and a heavy chain variable domain. The β structure also appears to be a common feature of many proteins that are capable of forming intractable amyloid fibrils. In addition to the antibody light chain, these include the antibody heavy chain, β-2 microglobulin, transthyretin (prealbumin), and serum amyloid-A protein. Reversible assembly of β-domain subunits is a well-characterized feature of the protein capsids that shelter the RNA or DNA genomic component of viruses.

3.5 Role of computer simulation. The ability to systematically engineeer and utilize polypeptide subunits will involve extensive computational resources. Analysis of data such as illustrated in Fig. 2 and 3 will be dependent upon computer simulation to fit four rate constants if the most simple model representation is useful. The multiple protein interactions will be emulated numerically by cal-

culating changes in the concentrations of discrete oligmer species during time intervals on the order of 0.001 s or smaller. Simulated data in Figure 5 schematically illustrate the dependence of elution profile on the magnitude of the affinity governing the polymerization reaction. However, because the MicroVax system used to generate this data does not have the speed necessary to complete a full-scale simulation, the chromatograms shown in Figure 5 are intended only to illustrate the sensitivity of elution profiles to the polymerization affinity; the transition from predominantly high-order molecular weight to minimal molecular weight occurs within a range of only two orders of magnitude. This corresponds to the free energy change that can be accomplished by substitutions of one to two amino acids at the appropriate locations.

Working simulations of the chromatographic behavior of interacting light chains will take advantage of the fact that the molecular events occuring simultaneously at two separated positions in the chromatography column are independent and therefore can be numerically mimicked simultaneously. This allows us to use the developing technology of massively parallel (concurrent) supercomputers such as the Intel Touchstone Delta machine, designed to provide 30 billion floating point operations per second distributed among more than 500 processors. Computers capable of more than one trillion floating point operations, and even faster descendents, will likely provide the means to dynamically model protein-protein interactions and to predict the consequences of amino acid substitution that can be experimentally measured by chromatography and other means.

4.0 CONCLUSION

The multiple association mechanisms of antibody light chains have not yet been fully characterized. The formation of dimers by light chains is determined by interactions between β-sheets. Polymerization of light chain dimers is hypothesized to be controlled by interactions between the loops which connect β-strands. Additional interactions, which might contribute to the formation of amyloid fibrils, and may be analogous to interactions involved in the assembly of viral capsids, may also involve β-sheet interactions. Because these multiple interaction capabilities are sensitively linked to substitutions of specific amino acids, which remain to be determined, and because the affinity of these interactions can be changed by manipulation of solution conditions, it is clear that the diverse array of β-domain modules provided by antibody light chains represents a rich resource for study of the fundamental principles of controlled polypeptide assembly.

ACKNOWLEDGEMENTS

This work was supported by the United States Department of Energy, Office of Health and Environmental Research, under Contract W-31-109-ENG-38. We are grateful to T.J. O'Donnell and K.F. Koehler (Searle) for preparation of the molecular graphics images in Figures 1 and 4.

FIGURE LEGENDS

Figure 1. Backbone representation of a typical antibody light chain variable domain dimer. Coordinates of protein Rei (Epp *et al.* 1974,1975) were obtained from the Brookhaven Protein Data Bank.

Figure 2. Size-exclusion chromatograms illustrating solution-dependence of polymerization properties. Upper Panel: 50 mM $NaPO_4$, 100 mM NaCl, pH 7.2; approximate protein concentrations 4 mg/ml (———), 2 mg/ml (· · ·), 1 mg/ml (- - -), 0.5 mg/ml (— -). Lower Panel: Acetate buffer, pH 4.5; approximate protein concentrations 5 mg/ml (———), 1 mg/ml (· · ·), 0.5 mg/ml (- - -).

Figure 3. Size-exclusion chromatographic demonstration of high-order aggregation under neutral conditions (buffer as in upper panel of Figure 1). Protein concentrations 2.5 mg/ml (———), .25 mg/ml (· · ·), 0.02 mg/ml (- - -).

Figure 4. Hypothetical model of light chain polymerization. Adjacent light chain variable domain dimers are related by an approximate 90° rotation about the twofold symmetry axis which relates the two identical monomers in the dimer. Dual salt bridges contributed by suitably located amino acids of opposite charge present on many, but not all, light chains are highlighted. The constant domains, which do not contribute to the polymerization process in this model, are not shown.

Figure 5. Simulated elution profiles. Descending panels represent consecutive tenfold decreases in the affinity of polymerization between light chain dimers.

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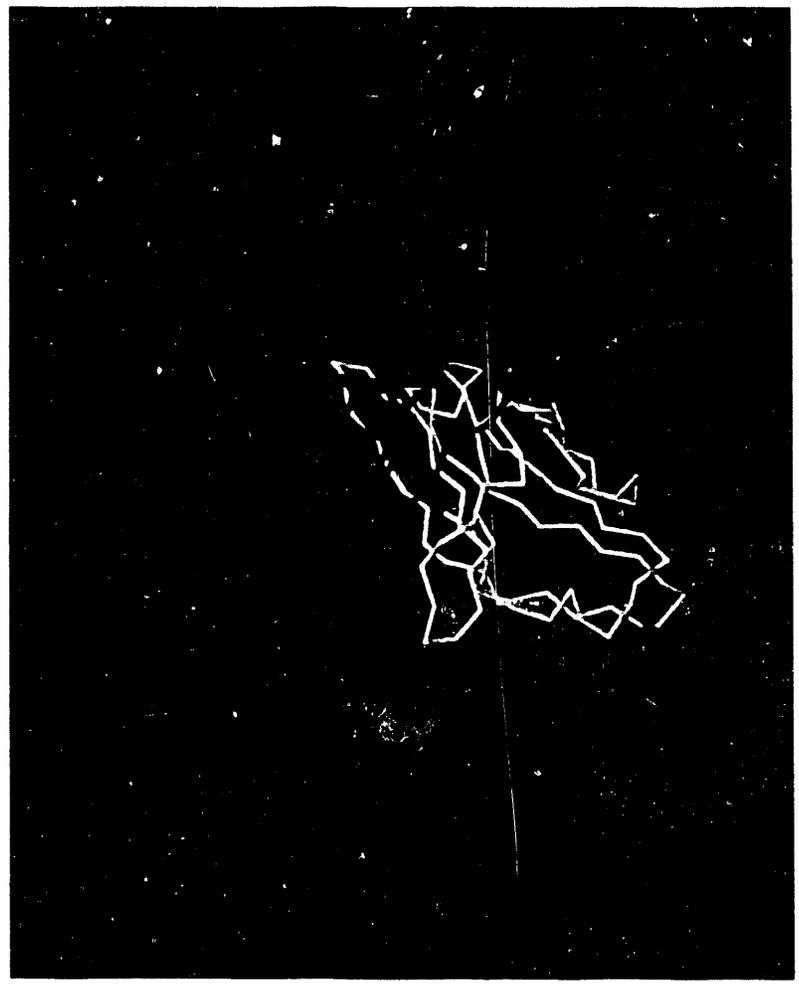

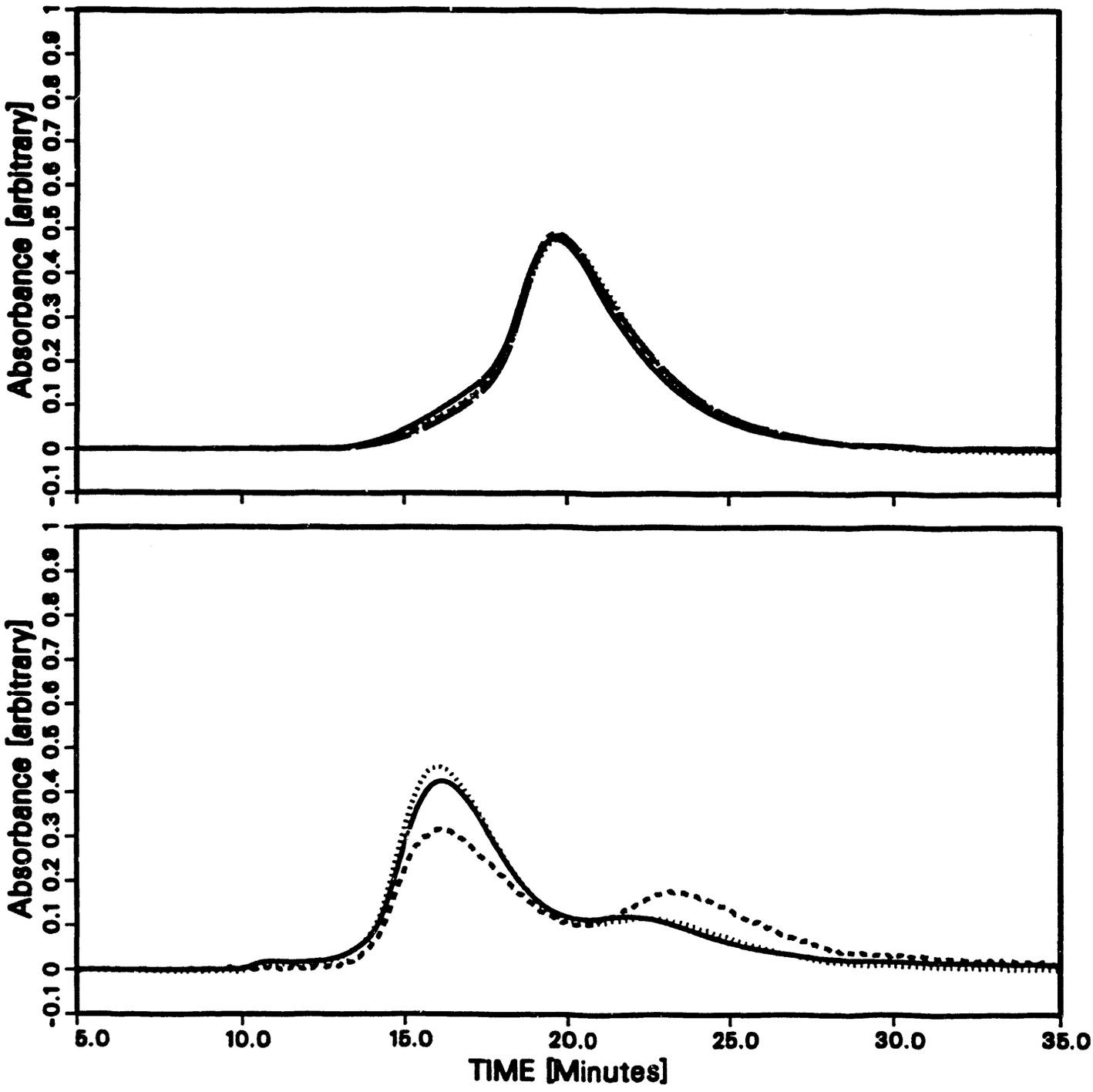
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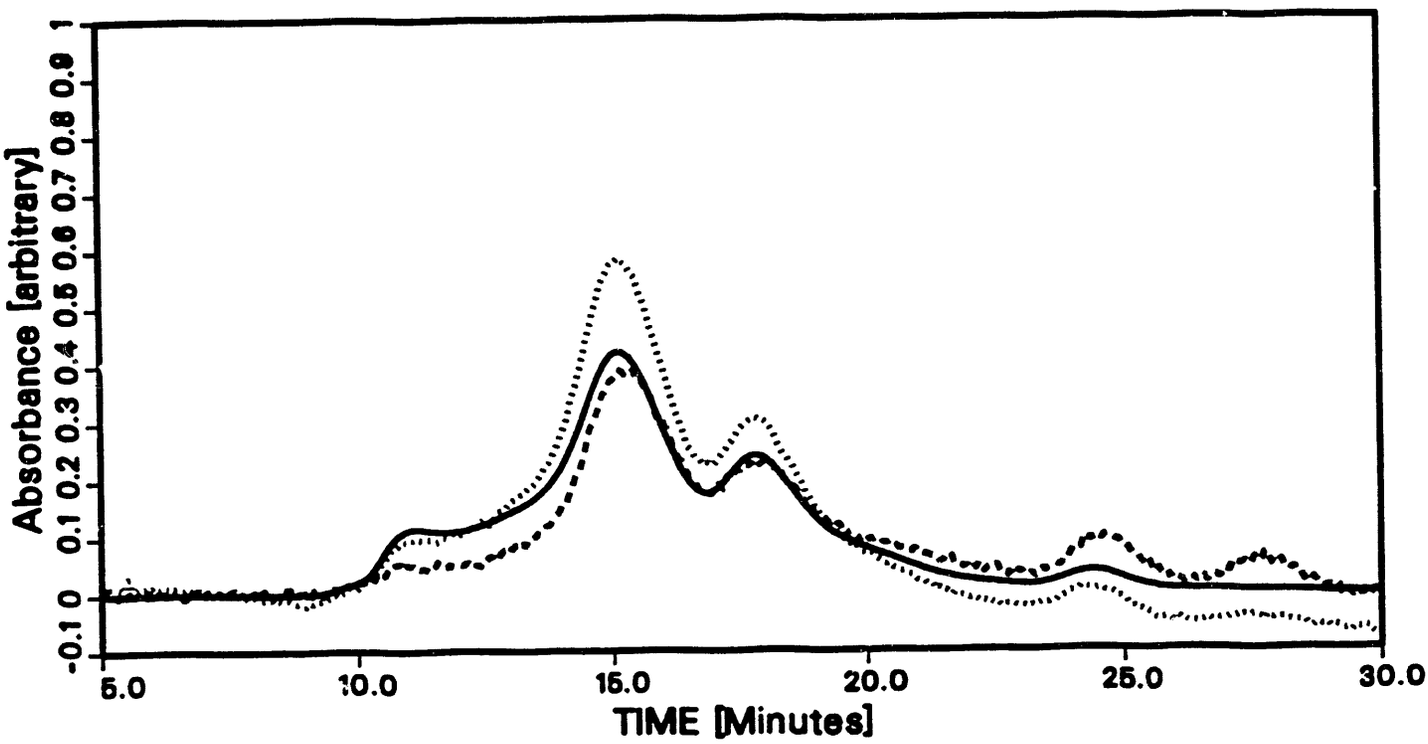
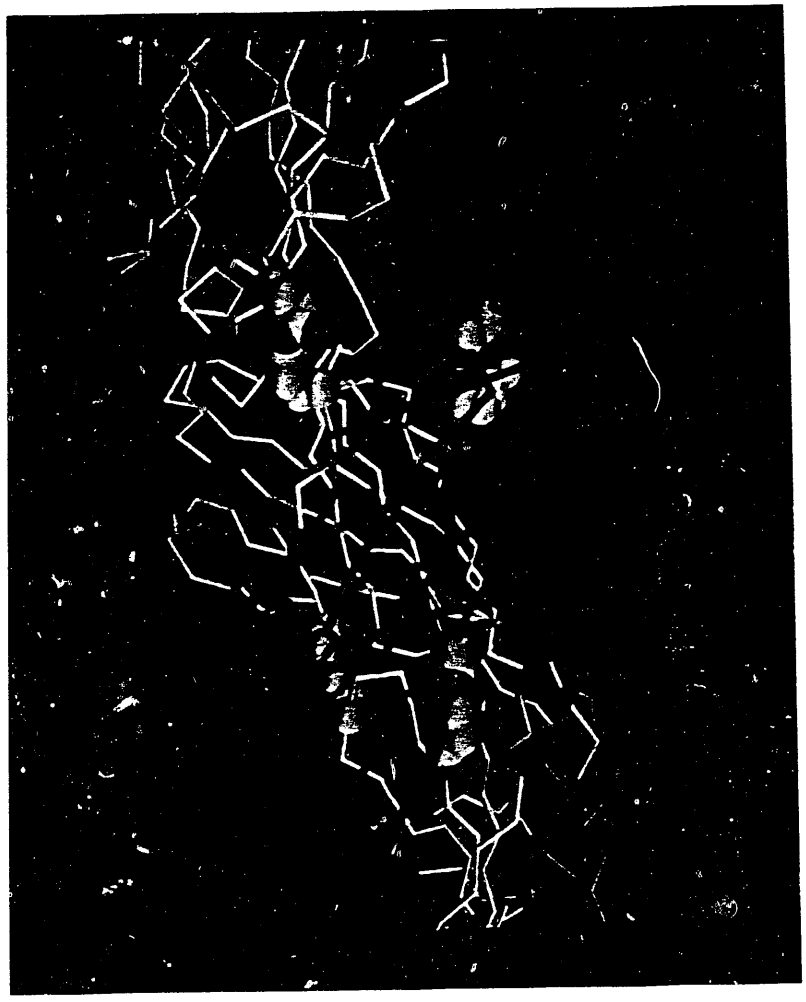
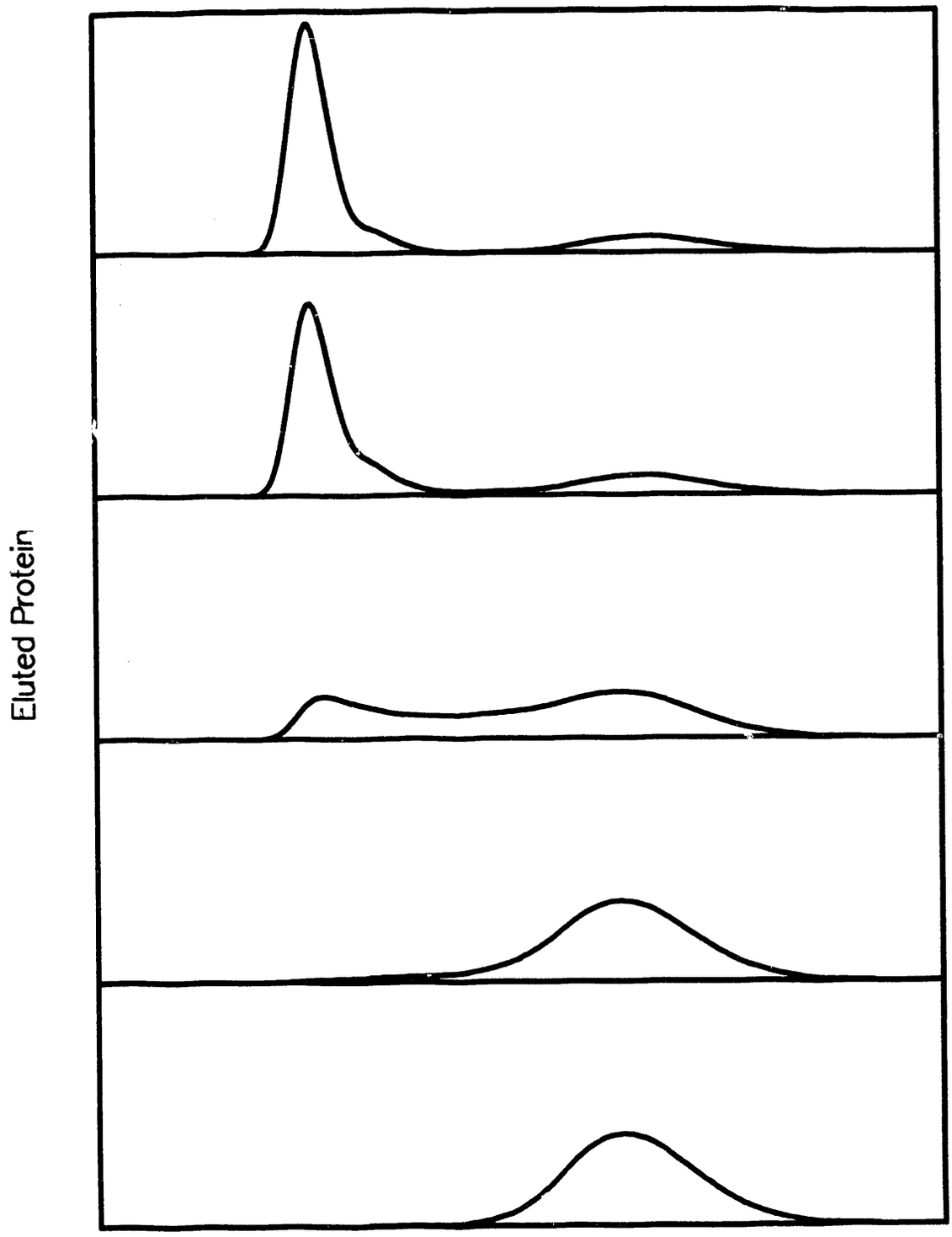
-0.1 0 0.1 0.2 0.3 0.4 0.5 0.6 0.7 0.8 0.9 1
Absorbance [arbitrary]
-0.1 0 0.1 0.2 0.3 0.4 0.5 0.6 0.7 0.8 0.9 1
5.0
10.0
15.0
20.0
25.0
30.0
35.0
TIME [Minutes]


Absorbance [arbitrary]
-0.1 0 0.1 0.2 0.3 0.4 0.5 0.6 0.7 0.8 0.9 1
5.0
10.0
15.0
20.0
25.0
30.0
TIME [Minutes]




Eluted Protein
Elution Time

END

DATE
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01/23/92