The Use of Gel Filtration to Follow Conformational Changes in Proteins

CONFORMATIONAL FLEXIBILITY OF BOVINE MYELIN BASIC PROTEIN

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The hydrodynamic behavior of bovine myelin basic protein was studied by gel filtration through Sephadex G-100 under conditions which included variations in pH from 2 to 12, variations in ionic strength from 0.01 to 1.5 M at pH 2 and from 0.1 to 2 M at pH 7, and variations in guanidinium chloride concentration from 0 to 6 M. A number of well characterized compact globular proteins were subjected to the same conditions for comparison. Compact globular proteins showed major conformational transitions due to acid, alkali, and guanidinium chloride denaturation and, possibly, minor transitions as well. Myelin basic protein behaved like a flexible linear polyelectrolyte, expanding continuously between pH 11 and pH 2 at ionic strength 0.1 M and contracting continuously with increase in ionic strength at pH 2 and at pH 7 to the point of salting-out. Relatively low concentrations of guanidinium chloride (~0.5 M) were sufficient to cause the basic protein to expand. With increasing concentration of the denaturant the molecule continued to expand, but in a noncooperative manner. These results demonstrated the lack of significant intramolecular stabilization in the protein.

Since its initial isolation (1) and subcellular localization (2), myelin basic protein of the central nervous system has been studied extensively (3). The amino acid sequences of the bovine (4, 5), human (6), and smaller of the two rat (7) basic proteins have been determined. Hydrodynamic (8-12) and low angle x-ray scattering (11, 13) studies have shown that the molecule cannot be compact and globular. NMR studies of the protein in aqueous solution (12, 14, 15) have indicated that most of its residues are mobile, and studies of ORD (10, 16-18) and CD (11, 12, 14, 18-21) have shown little or no α helical or β sheet structures in the molecule under these conditions. Studies of the intrinsic fluorescence of the protein have indicated that in aqueous solution the single tryptophanyl residue is largely exposed to the solvent (22, 23) and sufficiently far from the 4 tyrosine residues to preclude resonance energy transfer from tyrosine to tryptophan (22). Moreover, the tyrosyl residues titrate normally (8) and appear to be exposed (22). While these studies indicate that the basic protein is largely unfolded in aqueous solution, the conformation of the protein does not appear to be random. The intrinsic viscosity of the molecule is increased significantly in the presence of 6 M GdmCl (10, 11), its CD spectrum is altered slightly (11), and the peaks in natural abundance 13C NMR spectra due to aromatic and aliphatic residues are sharpened (24).

The extent to which the conformation of the basic protein can be altered by changes in pH is not clear. This is largely because the ORD, CD, and NMR spectra of the protein vary only slightly over a wide pH range (11, 12, 15, 17, 19, 20). The intrinsic viscosity of the protein was reported to be the same at pH 2.7 and at pH 8.0 (ionic strength, 0.1 M) by Epand et al. (11), suggesting a relatively rigid conformation. In contrast, Chao and Einstein (10) observed a marked dependence of the reduced viscosity of the protein on the ionic strength at pH 4.6, behavior typical of a linear flexible polyelectrolyte and altogether at variance with the view that the molecule is rigid.

In the present study the extent to which bovine myelin basic protein is flexible was examined by gel filtration of the protein through Sephadex G-100 under a variety of conditions of pH and ionic strength. In addition, gel filtration of the protein in the presence of increasing concentrations of GdmCl was carried out to determine if a transition characteristic of a denaturation occurs. To validate these studies the same procedure was carried out with a number of well investigated proteins. The results demonstrate that gel filtration is a useful and sensitive method for following changes in the hydrodynamic properties of proteins. The results show that the myelin basic protein is a flexible molecule, whose conformation is stabilized primarily by interactions with the solvent.

RESULTS

Variation of Stokes Radius with pH at Constant Ionic Strength—The results shown in Fig. 1 are in good agreement with results obtained by other methods. Buzzel and Tanford (25) found very little change in the intrinsic viscosity of RNase between pH 1 and 11, and according to the data of Hermans and Scheraga (26, 27), no significant unfolding of the molecule should occur at pH 2 under the conditions of the gel filtration. The pH-dependent changes in $R_e$ of bovine serum albumin between pH 2 and 11 corresponded closely to changes in intrinsic viscosity (28) and sedimentation coefficient (29) of
Gel Filtration and Protein Conformational Changes

FIG. 1 (left). Variation of R, with pH at constant ionic strength (0.1 M NaCl plus 0.01 M HCl buffer). Bp, myelin basic protein; OVAL, ovalbumin; BSA-1, bovine serum albumin monomer; BSA-2, bovine serum albumin dimer. Below pH 4 and above pH 9, bovine serum albumin dimer emerged too close to the void volume for accurate determination of R,. Roughly 25% of the STI at pH 12 had an apparent R, greater than 27.2 Å. For most of the larger proteins the apparent R, was 31.6 Å. Each point represents the average of results of at least two experiments.

FIG. 2 (center). Variation of R, with molar ionic strength at pH 2 (0.01 M HCl plus NaCl). Cyt c, cytochrome c; other abbreviations are given in Fig. 1. Each point represents the average of results of at least two experiments.

FIG. 3 (right). Variation of R, with molar ionic strength at pH 7 (0.01 M buffer plus NaCl). Abbreviations are given in Fig. 1. Each point represents the average of results of at least two experiments.

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shrinkage of the same magnitude was observed when the pH was reduced from 6 to 4 at an ionic strength of 0.1 M (see Fig. 1). Since electrostatic shielding was essentially complete at an ionic strength of 0.5 M, the continual contraction of the basic protein at higher ionic strengths probably arose from increased intramolecular hydrophobic interactions. In 1.5 M NaCl, the solubility of the protein was about 0.2 mg/ml, and in 2 M NaCl only a very dilute solution (0.04 mg/ml) of the protein could be examined. In contrast, all of the standard proteins except fibrinogen were soluble in 2 M NaCl at a concentration of 1 mg/ml. It is clear from these and the preceding experiments that the minimum apparent concentration of 1 mg/ml. It is clear from these and the preceding experiments that the minimum apparent concentration of 1 mg/ml. It is clear from these and the preceding experiments that the minimum apparent concentration of 1 mg/ml. It is clear from these and the preceding experiments that the minimum apparent concentration of 1 mg/ml. It is clear from these and the preceding experiments that the minimum apparent concentration of 1 mg/ml. It is clear from these and the preceding experiments that the minimum apparent concentration of 1 mg/ml.
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The present studies have demonstrated that gel filtration is a useful and sensitive method for following changes in the hydrodynamic properties of proteins. As pointed out by Leach (37), the procedure is particularly suited for denaturation studies involving proteins that display a "random coil" type of CD spectrum in the native state. Gel filtration has been used by Cohn et al. (48) to demonstrate pH-dependent conformational changes in parathyroid and parathyroid hormones. Like myelin basic protein, the hormone and its precursor show a steady decrease in Rs, the radius of gyration, between pH 2.5 and 11, indicating an essentially randomly coiled form of the protein at low pH that becomes more folded and symmetric at higher pH. More recently, Lizarraga et al. (49) have used gel filtration to follow the increase in Rs with pH closely resembles the titration curve for which the intrinsic viscosities are low and difficult to measure precisely; 2) it can detect the presence of aggregates, and, if they are stable, can reveal their size; 3) several proteins can frequently be studied simultaneously; and 4) very little protein is required. Since it is possible to apply relatively dilute solutions of protein (<0.2 mg/ml) to the chromatographic column, studies can be carried out under conditions where the solubility of the protein is relatively low. In the present study, myelin basic protein has been examined under conditions where it is nearly electrically neutral, yet monodisperse. It should be pointed out that the use of relatively dilute solutions of proteins is not only desirable but is essential to ensure an approach to thermodynamic ideality (50). Loading concentrations of 0.5 to 1 mg/ml, as used in the present study, would appear to be satisfactory in this regard.

The major disadvantage of gel filtration is the distinct possibility of a more or less specific interaction between the protein and the gel matrix, particularly if the protein is unfolded like myelin basic protein. With myelin basic protein on Sephadex G-100 there was no evidence of anomalous behavior. In columns containing Sephadex G-100 that had been pre-washed with 1 M pyridine, no retardation of the protein was observed at pH 7 at ionic strengths as low as 0.06 M. The protein could not be examined satisfactorily by gel filtration at pH 7 in columns of polyacrylamide (Bio-Gel P-100) or agarose (Bio-Gel A-0.5 M), however. It was adsorbed to polyacrylamide at ionic strengths up to 0.5 M and to agarose at an ionic strength of 0.1 M. On agarose at ionic strengths of 0.5 and 1.5 M the protein was eluted in a normal-appearing, symmetrical peak, but at the position of myoglobin.

In the present study, the tacit assumption was made that the Rs values of randomly coiled or asymmetric proteins could be estimated accurately from a calibration curve obtained with compact globular proteins. This assumption would seem to be justified insofar as the results obtained with the basic protein at pH 4.8 and 7.8 and with the basic protein, myoglobin, and cytochrome c in 6 M GdmCl are concerned. Further studies will be required to establish the limits of accuracy of the procedure.

The Rs of myelin basic protein has been shown to vary markedly with pH. This variation may be compared with the theoretical titration curve of the protein, whose equation is:

$$Z_H = Z_{H_{\text{max}}} - \sum_i n_i \alpha_i$$

where $Z_H$ is the average net charge at any pH, $Z_{H_{\text{max}}}$ is the maximum value (+41) of this charge attained at low pH where all titrating groups are protonated, $n_i$ is the number of groups of type $i$ at a given pH, and $\alpha_i$ is defined by the approximate relationship

$$\log \left( \frac{\alpha_i}{1 - \alpha_i} \right) = pH - pK^*_i$$

where pK*_i is the intrinsic pK of group $i$. This approximation neglects the effects of electrostatic interactions. These are probably minimal, however, since the ionic strength is reasonably high and the electrostatic free energy is minimized by the continual expansion of the protein as the net charge increases. In any case, an approximate treatment is adequate for the comparison. The comparison (Fig. 5A) shows that the variation in Rs with pH closely resembles the titration curve throughout the entire pH range. The absence of any transitions in the curve $R_s$ versus $Z_H$ (Fig. 5B) demonstrates that the protein behaves like a linear flexible polyelectrolyte and cannot be stabilized to any significant degree by intramolecular interactions.

From the studies carried out at acid pH, it is obvious that the basic protein under these conditions behaves like dena-
tured serum albumin and myoglobin. Like the latter two proteins, the basic protein at acid pH shows a marked inhibition of expansion with increasing NaCl concentration as electrostatic shielding and intramolecular hydrophobic interactions are increased. Furthermore, like the acid-denatured serum albumin and myoglobin molecules, the basic protein molecules experience intermolecular hydrophobic interactions at relatively low NaCl concentrations, and the protein is readily salted-out.

At neutral pH where the standard proteins exist in their native, more or less compact, conformations, an increase in NaCl concentration of the medium should have no appreciable effect on their $R_e$ values, as was indeed observed experimentally. Quite the opposite was true for myelin basic protein, however; an increase in the NaCl concentrations from 0.1 to 2 M caused a reduction in its apparent $R_e$ from 28 to 24 A. Furthermore, unlike the compact proteins, the basic protein is salted-out at relatively low NaCl concentrations at pH 7, indicating that a considerable number of hydrophobic side chains must lie on the surface of the molecule and thus be unavailable for intrachain stabilization and compaction of the molecule.

The behavior of the basic protein in the presence of increasing amounts of GdmCl demonstrates that in this protein the usual forces that stabilize native proteins in solution, particularly intramolecular hydrophobic interactions and hydrogen bonds, must be largely lacking or very weak. Even under conditions where the net charge on the protein is reduced to nearly zero (pH 11) and the protein is partially collapsed, intramolecular interactions are still too weak to stabilize the molecule even against concentrations of GdmCl as low as 0.25 M. The most stable conformation of the protein in aqueous solution appears to be one in which interactions between the polypeptide chain and the solvent are maximized.

From low angle x-ray scattering studies of the basic protein in aqueous solution at pH 5.2 (ionic strength 0.07 M), Krigbaum and Hsu (13) determined that the molecule is asymmetric and has a hydrodynamic volume of 41,300 A$^3$. They concluded that the protein could best be represented by a worm-like (stiff) chain having definite coil- and rod-like regions. Calculation of the volume occupied by the anhydrous protein from its mass (18,400 g/mol) and partial specific volume (0.72 cm$^3$/g) (12) yields a value of only 22,000 A$^3$, indicating that the solvent occupies approximately half the volume of the hydrodynamic particle under these conditions. Consequently, the reduction in $R_e$ of the protein observed at high pH or in the presence of NaCl is understandable in terms of a decrease in hydration, although it is also possible that the asymmetry of the molecule decreases as well.

The asymmetry, open conformation, and flexibility of the basic protein molecule apparently provides a relatively large number of hydrophobic contact surfaces capable of interactions leading to aggregation once the net charge on the molecules is reduced sufficiently or the surface tension of the solvent is increased sufficiently by the addition of an appropriate neutral salt (52). The extensive exposure of nonpolar, as well as polar and charged, residues to the external milieu undoubtedly plays a key role in the ability of the protein to penetrate or deform lipid monolayers (53, 54); increase the permeability (54, 55), alter the thermotropic properties (54, 56), and promote the fusion and aggregation (57) of liposomes; and, according to current views (58), maintain the structure of myelin by holding the cytoplasmic surfaces of the oligodendroglial plasma membrane in apposition.

REFERENCES

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58. Rumsey, M. G. (1978) Biochem. Soc. Trans. 6, 448-452

Additional references are found on p. 8960.

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**Figure 5d.** Dependence of some properties of carrier protein A and B on the ionic strength of the buffer. (A) protein standards listed in Table I. (B) Various proteins and protein standards. (C) Various proteins and protein standards. (D) Various proteins and protein standards.

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**Figure 5e.** Relationship between the ratio of the Stokes radii of proteins and polypeptides at pH 7.0. The data are fitted to the following function:

\[ \frac{r}{R} = \frac{1}{1 + (k_R - 1)^2} \]

where \( R \) is the radius of the protein, \( r \) is the radius of the polypeptide, and \( k_R \) is the protein radius factor. The data are shown in Table II. Each point represents the average of at least two experiments.

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**Table I.** Fractional Stokes radius of protein standards at pH 7.0, 20°C, Ionic strength 0.1 M

| Protein | \( r^2 \times 10^{-6} \) | \( \log r^6/2 \) | Reference | \( r_A^2 \) | \( \log r_A^6/2 \) | \( r_B^2 \) | \( \log r_B^6/2 \)
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<tbody>
<tr>
<td>Albumin</td>
<td>1.333</td>
<td>40</td>
<td>1.33</td>
<td>20.0</td>
<td>17.9</td>
<td>17.0</td>
<td>19.0</td>
</tr>
<tr>
<td>Lysozyme</td>
<td>1.125</td>
<td>32</td>
<td>1.12</td>
<td>24.8</td>
<td>24.8</td>
<td>26.1</td>
<td>26.1</td>
</tr>
<tr>
<td>Ribonuclease</td>
<td>1.125</td>
<td>32</td>
<td>1.12</td>
<td>24.8</td>
<td>24.8</td>
<td>26.1</td>
<td>26.1</td>
</tr>
<tr>
<td>Ovalbumin</td>
<td>1.125</td>
<td>32</td>
<td>1.12</td>
<td>24.8</td>
<td>24.8</td>
<td>26.1</td>
<td>26.1</td>
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<tr>
<td>Bovine serum albumin</td>
<td>1.125</td>
<td>32</td>
<td>1.12</td>
<td>24.8</td>
<td>24.8</td>
<td>26.1</td>
<td>26.1</td>
</tr>
</tbody>
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**Table II.** Viscosity Stokes radius of protein standards at pH 7.0, 20°C, Ionic strength 0.1 M

| Protein | \( \eta \times 10^{-3} \) | \( \log \eta^{1/2} \) | Reference | \( R_A \) | \( R_B \) | Column A
<table>
<thead>
<tr>
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<tbody>
<tr>
<td>Albumin</td>
<td>23.760</td>
<td>3.30</td>
<td>11</td>
<td>19.3</td>
<td>19.5</td>
<td>19.5</td>
</tr>
<tr>
<td>Lysozyme</td>
<td>21.080</td>
<td>3.23</td>
<td>11</td>
<td>20.6</td>
<td>20.6</td>
<td>20.6</td>
</tr>
<tr>
<td>Ovalbumin</td>
<td>23.760</td>
<td>3.30</td>
<td>11</td>
<td>20.6</td>
<td>20.6</td>
<td>20.6</td>
</tr>
<tr>
<td>Bovine serum albumin</td>
<td>27.080</td>
<td>3.47</td>
<td>14</td>
<td>28.0</td>
<td>28.0</td>
<td>28.0</td>
</tr>
</tbody>
</table>

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**Figure 6.** Relationship between the ratio of the Stokes radii of protein standards and polypeptides at pH 7.0. The data are fitted to the following function:

\[ \frac{r}{R} = \frac{1}{1 + (k_R - 1)^2} \]

where \( R \) is the radius of the protein, \( r \) is the radius of the polypeptide, and \( k_R \) is the protein radius factor. The data are shown in Table II. Each point represents the average of at least two experiments.
Gel Filtration and Protein Conformational Changes

SUPPLEMENTAL MATERIAL

THE USE OF GEL FILTRATION TO STUDY CONFORMATIONAL CHANGES IN PROTEINS. CONFORMATIONAL FLEXIBILITY OF BOVINE MILK BASIC PROTEIN

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EXPERIMENTAL PROCEDURES

MATERIALS—Water basic protein was obtained from bovine brain. The protein hydrolyzate was prepared in 1 M formic acid and 1.0 M 2-mercaptoethanol. 1.0 M sodium acetate buffer (pH 5.0) and the enzyme was stored at -20°C.

General Procedure—The material was dissolved in 40 ml of 1 M sodium pyrophosphate buffer (pH 6.0) containing 6 M guanidine hydrochloride (final concentration) and 6 M guanidine hydrochloride (final concentration) was added to the solution until the final concentration was 6 M. The mixture was then heated to 100°C for 10 min and allowed to cool to room temperature before being used.

Gel filtration was carried out using a Sephadex G-100 column (2.6 x 70 cm) eluted with 0.1 M sodium phosphate buffer (pH 7.0). The effluent was monitored at 280 nm using a spectrophotometer.

To study conformational changes, the protein was dialyzed against 0.1 M sodium phosphate buffer (pH 7.0) containing 0.1 M sodium chloride for 24 h at 4°C. The dialyzed solution was then applied to a Sephadex G-100 column and eluted with the same buffer. The eluted fractions were monitored at 280 nm using a spectrophotometer.

RESULTS—Figure 1A shows the elution pattern of the protein as a function of column size. The peak eluted at the void volume is consistent with the native conformation of the protein. The peak eluted at the void volume is consistent with the native conformation of the protein.

Figure 1B shows the elution pattern of the protein as a function of column size. The peak eluted at the void volume is consistent with the native conformation of the protein. The peak eluted at the void volume is consistent with the native conformation of the protein.

Figure 1C shows the elution pattern of the protein as a function of column size. The peak eluted at the void volume is consistent with the native conformation of the protein. The peak eluted at the void volume is consistent with the native conformation of the protein.

DISCUSSION—The results of the experiments indicate that the protein is capable of undergoing conformational changes upon dialysis. The changes in the elution pattern of the protein suggest that the protein is undergoing a transition from a more compact to a more extended structure. The transition is likely to be associated with changes in the secondary and tertiary structure of the protein.

In summary, the gel filtration results presented in this study demonstrate that the protein is capable of undergoing conformational changes upon dialysis. The changes in the elution pattern of the protein suggest that the protein is undergoing a transition from a more compact to a more extended structure. The transition is likely to be associated with changes in the secondary and tertiary structure of the protein.

1 Abbreviations used: HPLC, high-performance liquid chromatography; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; M, molecular weight.
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