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 to 3 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 the 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 $R_s$, of bovine serum albumin between pH 2 and 11 corresponded closely to changes in intrinsic viscosity (28) and sedimentation coefficient (29) of the protein, with no significant change in $R_s$ observed between pH 2 and 11.

The abbreviations used are: GdmCl, guanidinium chloride; $R_s$, radius of equivalent sphere (Stokes radius); STI, soybean trypsin inhibitor (Kunitz).

* Portions of this paper (including "Experimental Procedures," Figs. S1 to S3, and Tables I and II) are presented in miniprint at the end of this paper. Miniprint can be easily read with the aid of a standard magnifying glass. Full size photocopies are available from the Journal of Biological Chemistry, 9560 Rockville Pike, Bethesda, Md. 20014. Request Document No. 78M-951, cite author(s), and include a check or money order for $2.25 per set of photocopies.

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A. The corresponding (interpolated) apparent R₂ values obtained by gel filtration were 30.2 and 27.0 Å, respectively. At pH 7.8 D₂O, \( w = 7.80 \times 10^{-7} \text{ cm}^2/\text{s} \) (16), yielding \( R₂ = 27.5 \) Å. At pH 4.8 D₂O, \( w = 7.25 \times 10^{-7} \text{ cm}^2/\text{s} \) (12), yielding \( R₂ = 29.6 \) Å. Gel filtration may be compared with \( R₂ \) values calculated from diffusion coefficients measured at an ionic strength of 0.1 M. Two of the \( R₂ \) values of the basic protein obtained by gel filtration may be compared with \( R₂ \) values calculated from diffusion coefficients measured at an ionic strength of 0.1 M. At pH 4.8 \( D_{m, w} = 7.25 \times 10^{-7} \text{ cm}^2/\text{s} \) (12), yielding \( R₂ = 29.6 \) Å; at pH 7.8 \( D_{m, w} = 7.80 \times 10^{-7} \text{ cm}^2/\text{s} \) (16), yielding \( R₂ = 27.5 \) Å. The corresponding (interpolated) apparent \( R₂ \) values obtained by gel filtration were 30.2 and 27.0 Å, respectively.

Variation of Stokes Radius with Ionic Strength at pH 2.0—The results presented in Fig. 2 show that in the absence of salt (0.01 M HCl) myelin basic protein was highly expanded, with an apparent \( R₂ \) of about 34 Å. Under these conditions, serum albumin, myoglobin, and cytochrome c behaved in a similar fashion, whereas ovalbumin, STI, and RNase remained fairly compact. The results obtained for the standard proteins under these conditions are in complete accord with results obtained by the examination of other physical parameters (25-28, 31-34). Myoglobin, a molecule without disulfide bridges and with a molecular weight approximately ninetenths that of the basic protein, was expanded to nearly the same degree (apparent \( R₂ = 33 \) Å) as the latter, in agreement with earlier studies (35). At ionic strengths greater than about 0.25 M, serum albumin, myoglobin, cytochrome c, and myelin basic protein showed a marked inhibition of expansion. A further increase in ionic strength to 0.5 M resulted in little additional change. It would appear from the slopes of the curves that electrostatic shielding was essentially complete at ionic strengths between 0.25 and 0.5 M and that at higher ionic strengths the expansion of serum albumin, myoglobin, and the basic protein was inhibited by a NaCl-induced enhancement of intramolecular hydrophobic interactions. The hydrophobic bond-promoting effect of NaCl was demonstrated in the present study by the salting-out of denatured serum albumin and myoglobin in 0.01 M HCl by addition of the salt to 1 M and that of the basic protein by addition of the salt to 2 M.

At pH 2, ionic strength 0.5 M, the basic protein had nearly the same apparent \( R₂ \) (28 Å) as did at pH 7, ionic strength 0.1 M; at pH 2, ionic strength 1.5 M, the apparent \( R₂ \) (24.5 Å) was equivalent to that at pH 10, ionic strength 0.1 M (see Fig. 1). Thus, even in acid solution, the basic protein could be made to contract to nearly its isoelectric size by the addition of sufficient NaCl. The same appeared to be true for serum albumin (apparent \( R₂ = 34 \) Å), but not for myoglobin. Whereas native myoglobin has a Stokes radius of 19 Å, the acid-denatured protein salted-out after its apparent \( R₂ \) had decreased to 23 Å. Considering the similarity in molecular weights of the basic protein and myoglobin, it is likely that the basic protein (apparent \( R₂ = 24.5 \) Å) is about as compact as acid-denatured myoglobin (apparent \( R₂ = 23 \) Å) just before the two proteins are salted out.

Variation of Stokes Radius with Ionic Strength at pH 7.0—As the ionic strength was increased from 0.1 to 2 M at pH 7, the apparent \( R₂ \) of myelin basic protein progressively decreased from about 28 to 24 Å (Fig. 3). The behavior of the basic protein differed considerably from that of the other proteins, which, not unexpectedly, underwent very little, if any, change in molecular size. Of the compact globular proteins only STI showed a significant change in \( R₂ \), limited to a slight contraction at ionic strengths between 0.1 and 0.5 M. A
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.

Variation of Stokes Radius with GdmCl Concentration—The effects of increasing concentrations of GdmCl on the \( R_e \) of myelin basic protein and three other proteins at pH 7 are shown in Fig. 4A. Two proteins (horse myoglobin and cytochrome c) undergo cooperative unfolding from the native to the random-coiled state, whereas the third (ST1) is extremely resistant to such denaturation (36, 37). The transition curve of horse myoglobin had a midpoint at approximately 1.9 M GdmCl, while the midpoint of the cytochrome c transition curve was close to 2.6 M GdmCl. These results, which were obtained at pH 7.0 and 20°C, are comparable to those obtained from other physical parameters at slightly different pH values and at 25°C (38-40). The curves for myoglobin and cytochrome c show that once complete unfolding of the protein occurred, a gradual expansion followed as the concentration of GdmCl was further increased. Such an expansion has been observed in viscosity studies (41); it apparently results from a progressive increase in the solvation of the unfolded molecule.

Exposure of ST1 to low concentrations of GdmCl resulted in a slight contraction of the molecule. This was probably an ionic strength effect and is reminiscent of the slight contraction shown for this protein with increase in NaCl concentration or a lowering of pH. With increasing GdmCl concentrations, the apparent \( R_e \) increased gradually to a final value of about 24 Å in 6 M GdmCl. This value is the same as that found by Leach and Fish (37) for ST1 in 6 M GdmCl. These workers ascribed the slight increase in \( R_e \) from 21 to 24 Å to the small fraction of native ST1 molecules that unfolded during gel filtration. It could also be ascribed to a slight swelling of the molecule in the absence of significant polypeptide chain unfolding. Complete unfolding to a disulfide-linked random coil yields \( R_e = 38 \) Å (37).

When the curve of myelin basic protein is compared with those of the standard proteins, it is apparent that the former did not undergo a cooperative unfolding since no sigmoidal curve was observed over any GdmCl concentrations. At low GdmCl concentrations, the increase in \( R_e \) with GdmCl concentration was fairly rapid, but above 1 M, the basic protein expanded in exactly the same fashion as did the completely unfolded myoglobin. Similar results based on CD spectral changes have been obtained by Thomas et al. (20) for the basic protein in urea solutions.

It should be pointed out that myelin basic protein in 6 M GdmCl behaves as a random coil (10) on the basis of the relationship between its intrinsic viscosity and molecular weight or the number of amino acid residues in its polypeptide chain (42). It also behaves as a random coil (9) during gel filtration in concentrated GdmCl (43, 44). Thus, although the curve for the basic protein in Fig. 4A does not show a transition characteristic of a denaturation, the final product is, nevertheless, a random coil.

In order to determine if the basic protein in a more compact conformation is more resistant to the effects of GdmCl, the protein was subjected to low concentrations of the denaturant at pH 11. As shown in Fig. 4B, no increase in the stability of the protein occurred at a pH close to its isoelectric point since even very low concentrations of GdmCl were sufficient to cause an increase in \( R_e \). Fig. 4B also shows that addition of \( \text{SO}_4^{2-} \) ion counteracted the action of GdmCl, as anticipated from the ion's hydrophobic bond-promoting and protein-stabilizing properties (45, 46).

By gel filtration in 6 M GdmCl, the random coils of myelin basic protein, myoglobin, and cytochrome c were estimated to have apparent \( R_e \) values of 37 Å, 35 to 36 Å, and 29 to 30 Å, respectively. These values can be compared with viscosity Stokes radii calculated from the intrinsic viscosities of these proteins determined in 6 M GdmCl. The calculated values are 39.3 and 40.8 Å for the basic protein (10, 11), 38.5 and 38.0 Å for myoglobin (42, 47), and 30.6 Å for cytochrome c (39). It can be seen that the agreement is reasonably close.
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-dependencies of type I at a given pH, and $\bar{\alpha}_i$ is defined by the approximate relationship

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

where pK$^\text{iso}$ 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 $R_e$ with pH closely resembles the titration curve throughout the entire pH range. The absence of any transitions in the curve $R_e$ versus $\bar{Z}_H$ (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 Rg 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 Rg from 28 to 24 Å. 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 Å3. 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 cm3/g) (12) yields a value of only 22,000 Å3, 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.

REFERENCES


Additional references are found on p. 8893.

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**Figure 26.** Relationship between $V_e/V_0$ of protein standards and solar ionic strength at pH 7.0, 20°C: Tyr, tyrosine; other abbreviations are given in Fig. 15. Each point represents the average of results of at least two experiments.

**Figure 27.** Relationship between $V_e/V_0$ of protein standards and solar ionic strength at pH 7.0, 20°C. The points show the values determined with blue dextran and tyrosine, respectively. Column dimensions, 3.5 x 50 cm.

**Table I**

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Gel Filtration and Protein Conformational Changes

SUSAN G. HACKETT

EXPERIMENTAL PROCEDURES

MATERIALS—Bovin basic protein was obtained from bovine brain. The pro-

tein—purified by ion-exchange chromatography on a column of carboxy-

ymethylcellulose—(CM). The most basic of the species, the protein, com-

ponent I (I), was used. Other proteins were purchased com-

pletely from Sigma Chemical Co. Bovine serum albumin, casein, foraminiferous 

hydroxide (alizarin pure) was a product of Schwarz/Mann.

General Procedure—Endochromatography was performed for 3 h at 22°C. 

The proteins were applied to, and eluted from, a column of carboxy-

ymethylcellulose (CM). The most basic species of the protein, component I 

(1), was used. Other proteins were purchased com-

pletely from Sigma Chemical Co. Bovine serum albumin, casein, foraminiferous 

hydroxide (alizarin pure) was a product of Schwarz/Mann.

Gel filtration was performed as described in detail elsewhere.1 The sol-

vents used were MeOH, H2O, and acetone. The column was made from a 

300 x 0.8 cm column of Sephadex G-25. The elution buffer was 

acetone, and the columns were equilibrated with a solution of 

acetone:MeOH (9:1) at 22°C. The column was calibrated with 

polypeptides with known molecular weights.

The gel filtration method was performed as described in detail elsewhere.1 The 

solvents used were MeOH, H2O, and acetone. The column was made from a 

300 x 0.8 cm column of Sephadex G-25. The elution buffer was 

acetone, and the columns were equilibrated with a solution of 

acetone:MeOH (9:1) at 22°C. The column was calibrated with 

polypeptides with known molecular weights.

The presence of proteins in the fractions was determined by the method of 

Lowry et al.2 The protein in each fraction was precipitated with 

1 Abbreviations used in this paper are the same as those used in the 

literature. Gel Filtration is abbreviated GFC. Protein Conformational Changes is abbreviated PCC.

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literature. Gel Filtration is abbreviated GFC. Protein Conformational Changes is abbreviated PCC.

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