The thermodynamically stable state of myelin basic protein in aqueous solution is a flexible coil

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Conformational studies of myelin basic protein (MBP) in solution generally have used protein purified in organic solvents and acid. The use of such conditions raises the possibility that the secondary structure reported for the basic protein represents a denatured state. Therefore we have purified this protein by using a procedure that avoids denaturants. Bovine myelin was extracted with 0.2 M-CaCl₂ and the protein was purified from the supernatant by chromatography on Sephadex G-75. The conformation of the basic protein was characterized by using c.d. and ¹H-n.m.r. spectroscopy. In solution, it appeared to be predominantly randomly coiled, with only small segments of persistent structure. However, in the presence of myristoyl lysophosphatidylcholine the secondary structure of MBP became more ordered, and sedimentation-velocity experiments showed that MBP aggregated. Comparison of our results with published data indicates that Ca²⁺-extracted basic protein behaves similarly to the protein purified by traditional methods with respect to its ordered conformation in solution in the absence and in the presence of lipid and with respect to its self-association. Thus its thermodynamically stable structure in aqueous solution appears to be a highly flexible coil.

INTRODUCTION

Myelin basic protein (MBP) is an extrinsic membrane protein that resides on the cytoplasmic surfaces of central-nervous-system myelin and is thought to play a major role in compacting and stabilizing this multilamellar membrane [1,2].

The structure of MBP in solution has been studied extensively and two models for its conformation have been proposed. The first is a compact prolate ellipsoid of revolution with an axial ratio of approx. 1:10 [3], whereas the second model describes MBP as a randomly coiled protein with little persistent ordered structure (for example see refs. [4–10]). The latter model has gained general acceptance [11].

Lee *et al.* [12] have observed that in solution proteins up to about 100 amino acid residues in length, without cysteine residues, are generally unstructured. Proteins the size of MBP, which contains about 170 residues, generally have compact ordered structures. One explanation for the apparent lack of definite secondary structure in MBP is that the commonly used purification procedures may denature this protein.

Structural studies on MBP have generally employed protein purified with the use of chloroform/methanol to remove lipid, followed by aqueous extraction of the residue at either pH 3 or pH 1.6–1.7 (described here as CM-MBP). The use of such conditions is not conducive to the retention of ordered structure. To avoid potentially denaturing conditions, we have purified MBP by using CaCl₂ extractions of the myelin membrane; protein prepared in this manner is described here as Ca-MBP. From the data available [13,14] calcium salts do not appear to cause gross conformational changes in proteins

except at much higher concentrations than that used in the current study.

By comparing the results for CM-MBP and Ca-MBP from c.d., n.m.r. and sedimentation-velocity experiments, we conclude that the method of preparation has little influence on the final conformation or self-association of the protein in aqueous solution.

EXPERIMENTAL

Materials

Egg-yolk phosphatidylcholine (egg PC), myristoyl lysophosphatidylcholine (lysoMPC) and *M_r* markers (*M_r* 14000–70000) for SDS/polyacrylamide-gel electrophoresis were obtained from Sigma Chemical Co. (St. Louis, U.S.A.). Myelin was purified from bovine white matter by a modification [15] of the procedure of Autilio *et al.* [16]. All other chemicals used were of analytical grade.

Purification of MBP

Crude CM-MBP was prepared by the method of Oshiro & Eylar [17]. Bovine white matter was homogenized for 5 min in chloroform/methanol (2:1, v/v) at –70 °C, and the residue obtained after centrifugation was homogenized for 5 min in acetone, also at –70 °C. The residue obtained by centrifugation was homogenized for 30 s in 0.01 M-HCl, adjusted to pH 1.6–1.7, and then stirred overnight at 4 °C. After centrifugation, the supernatant was adjusted to pH 7, then left at 4 °C for 60 min to allow a precipitate to form. This precipitate was removed by centrifugation.

Chromatography on CM-cellulose (CM-32; What-

Abbreviations used: MBP, myelin basic protein; lysoMPC, myristoyl lysophosphatidylcholine; egg PC, egg-yolk phosphatidylcholine; C/A/M/Ac/W, chloroform/acetone/methanol/acetic acid/water (8:4:2:2:1, by vol.).

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man, Maidstone, Kent, U.K.), as described by Deibler & Martenson [18], was used as the final purification step. CM-MBP was eluted in three major peaks; the final peak to emerge from the column was used for subsequent experiments. This component is not post-translationally modified.

To obtain Ca-MBP, purified bovine myelin was suspended for 5 min in 5 mM-Tris/HCl buffer, pH 7.4, then centrifuged at 15000 g (r_{av} 7 cm) for 10 min at 4 °C, and the pellet was stirred at 37 °C overnight in sufficient 0.2 M-CaCl₂/5 mM-Tris/HCl buffer, pH 7.4, to give a protein concentration of 1 g/l [19]. The supernatant, containing approx. 50 mg of protein, from this extraction was concentrated to 2 ml by ultrafiltration with a YM-10 membrane, and dialysed either against water then 0.2 M-NaCl/0.1 M-Na₂HPO₄ buffer, pH 7.4 and 1.0.48 (phosphate/chloride buffer), or against 0.1 M-NaCl/0.01 M-Tes (Calbiochem-Behring, La Jolla, CA, U.S.A.)/NaOH buffer, pH 7.5 and 1.0.22 (Tes/chloride buffer), overnight at 4 °C in preparation for column chromatography. The proteins were then loaded on to a 2.2 cm diam. $\times$ 70 cm Sephadex G-75 (Pharmacia Fine Chemicals, Uppsala, Sweden) column equilibrated with the appropriate buffer (phosphate/chloride or Tes/chloride); the column was run at 40 ml/h. Two major peaks were eluted, the second being Ca-MBP. The amount of Ca²⁺ bound to the protein, measured by atomic absorption spectroscopy, was less than 7 mol % (from quadruplicate measurements on 0.5–1.0 g/l protein samples).

Protein assays

Protein concentrations were measured by the method of Lowry *et al.* [20], with purified MBP as standard. Protein concentrations of CM-MBP and Ca-MBP preparations were also determined spectrophotometrically assuming a specific absorption coefficient of 0.54 litre $\cdot$ g⁻¹ $\cdot$ cm⁻¹ at 280 nm and M_r 18400 [21].

Lipid analyses

Freeze-dried samples of CM-MBP (one preparation of 10 mg) and Ca-MBP (five preparations of between 1 and 2 mg) were extracted overnight at room temperature with 0.2–1.0 ml/mg of protein of chloroform/acetone/methanol/acetic acid/water (8:4:2:2:1, by vol.) (C/A/M/Ac/W). The clear supernatant obtained by centrifugation was concentrated by evaporation under N₂ at 50 °C and then loaded on to pre-activated 0.5 mm-thick silica gel 60 F₂₅₄ glass-backed t.l.c. plates (Merck, Darmstadt, West Germany). The plates were developed in C/A/M/Ac/W and, after drying, fluorescence was used to detect spots. Thereafter the plates were charred with 40 % (v/v) H₂SO₄ or sprayed with one of, or a combination of, three specific lipid stains [22]: α -naphthol for sugars, Zinzadze reagent for phosphodiester or ninhydrin for free amino groups.

The amounts of lipid extracted from CM-MBP and Ca-MBP preparations were estimated by comparing charred spots from myelin lipids on t.l.c. plates with charred spots from known amounts of an egg PC standard.

Secondary structure

C.d. spectra were obtained at 20 °C with a Jobin Yvon (Roussel-Jouan, France) Dichrographe III that had been calibrated at 304 nm with an epiandrosterone standard,

as recommended by the manufacturers. A 0.098 mm-path-length fused-silica demountable cell was used for all measurements. A 2 nm band pass was used with a time constant of 20 s and a scan speed of 0.01 nm/s. The protein solutions, containing 2.3–3.1 g of MBP/l, were dialysed overnight at 4 °C against phosphate/chloride buffer. To several protein solutions small volumes of lysoMPC dissolved in phosphate/chloride buffer were added from a concentrated stock to give an overall lipid/protein molar ratio of 6:1, and the spectra were then recorded. The c.d. spectrum of Ca-MBP in 0.2 M-CaCl₂/0.1 M-Tes/NaOH buffer, pH 7.5, was also obtained in the absence and in the presence of lysoMPC.

For ¹H-n.m.r. experiments CM-MBP and Ca-MBP were dialysed against 5 mM-Na₂HPO₄ buffer, pH 7.4, for several hours at 4 °C. Water was exchanged for 99.75 % (v/v) ²H₂O (Australian Atomic Energy Commission, Sydney, N.S.W., Australia) in two freeze-dry cycles. The proteins were then reconstituted in 99.75 % ²H₂O to give a final concentration of 5 g/l in buffer of the original composition. Some samples of Ca-MBP showed minor precipitation on reconstitution in ²H₂O; this precipitate was removed by the routine centrifugation of the samples before collection of the spectrum. Although these samples were optically clear after centrifugation, it is likely that soluble aggregates were still present.

¹H-n.m.r. spectra were obtained at 23 °C with a JEOL GX-400 spectrometer operating at 399.78 MHz. In all 500–1500 scans were collected, over 16 k data points with a 90° radio frequency pulse of 14.0 μ s, a spectral width of 5 kHz, a line-broadening of 0.1 Hz, an acquisition time of 0.89 s and a recycle time of 1.5 s. The chemical shifts were measured relative to an internal dioxan standard, which was added after initial acquisition of the spectrum.

Self-association

Sedimentation-velocity measurements were carried out at 20 °C using a Beckman model E ultracentrifuge fitted with electronic speed control, as previously described [23]. CM-MBP and Ca-MBP were dialysed at 20 °C for 24 h against phosphate/chloride buffer; the protein concentration was 3.5 $\pm$ 0.2 g/l for all measurements. LysoMPC, in negligibly small volumes of phosphate/chloride buffer, was added to the protein solutions to increase the lipid/protein ratio in successive experiments.

RESULTS

Protein purity

The purities of CM-MBP and Ca-MBP were determined by using SDS/polyacrylamide-gel electrophoresis [24], with M_r markers and purified myelin as standards (Fig. 1). Both proteins ran as single major bands, aligned with MBP from purified myelin, with M_r = 20000 $\pm$ 1000. This value is consistent with previously published estimates from gels [25]. A very small amount of a slightly lower- M_r component is visible on the gel (lanes c and d); on the basis of past observations this is probably a breakdown product from MBP.

Myelin lipids

MBP purified by several different methods has been shown to be associated with lipids [26–28], which may influence its properties. We have also found purified CM-MBP to contain lipids from several major classes, in accord with the published results. An upper limit of

Fig. 1. Purity of CM-MBP and Ca-MBP

SDS/polyacrylamide gel (15%) stained with Coomassie Brilliant Blue R250. Lanes a and e, M_r markers (bovine albumin, egg albumin, glyceraldehyde-3-phosphate dehydrogenase, carbonic anhydrase, trypsinogen, trypsin inhibitor and α -lactalbumin); lane b, purified whole myelin (approx. 30 μ g of protein); lane c, purified CM-MBP (5 μ g); lane d, purified Ca-MBP (5 μ g).

10–15 μ g of lipid/mg of CM-MBP was estimated for CM-MBP by comparing lipid spot sizes with an egg PC standard. As a measure of the efficiency of the extraction of lipid from CM-MBP, 0.5–3 mg samples of C/A/M/Ac/W-extracted CM-MBP, in Tes/chloride buffer, were assayed for phosphorus [29]. The phosphorus/protein ratio was estimated to be 0.03 ± 0.01 mol/mol.

T.l.c. of the residue from the organic-solvent extractions of Ca-MBP consistently revealed one compound, which was detectable by fluorescence, with R_f 0.25. This compound did not react with any of the specific lipid stains used for sugars, free amino groups or phosphorus, but was visible as a negative spot upon charring. The amounts of this compound could not be

accurately measured because of the lack of a suitable standard.

Phospholipids were not detected on t.l.c. of organic-solvent extracts of Ca-MBP. However, this does not preclude the association of lipids with the protein, as the Zinzadze stain for phosphodiesterases does not react well with some lipids such as phosphatidic acid (a phosphomonoester). For this reason we determined the phosphorus content of Ca-MBP as another measure of the amount of phospholipid associated with the protein. Ca-MBP was found to contain 0.23 ± 0.09 mol of phosphorus/mol of protein (mean $\pm$ s.d. for four preparations). This phosphorus content may be accounted for by post-translational phosphorylation of Ser-65 and Thr-98 residues, which has been estimated at 0.2 mol/mol in bovine MBP [30]. Therefore the amount of phospholipid co-purifying with the protein must be negligible.

Secondary structure

A well-defined characteristic of MBP in aqueous solution is its lack of persistent ordered secondary structure in the absence of lipids. We have used c.d. to compare the secondary structures of CM-MBP and Ca-MBP.

In the absence of detergent (Fig. 2a), at protein concentrations of 2.3–2.9 g/l, spectra for CM-MBP and Ca-MBP are characteristic of randomly coiled proteins. These spectra are similar to those obtained for C/A/M/Ac/W-extracted CM-MBP at 2.8 g/l (results not shown), CM-MBP at concentrations below 1 g/l (results not shown) and published spectra for CM-MBP [4,5,31,32]. Since the spectrum of C/A/M/Ac/W-extracted CM-MBP did not differ significantly from that for CM-MBP or Ca-MBP, the presence of traces of myelin lipids with CM-MBP and the unidentified compound with Ca-MBP preparations appeared to have little influence on the protein structure.

After the addition of a 6-fold molar excess of lysoMPC,

Fig. 2. C.d. spectra of CM-MBP and Ca-MBP

(a) CM-MBP ($\square$) (2.3 g/l) and Ca-MBP ($\triangle$) (2.2–2.9 g/l; average of three preparations). (b) CM-MBP ($\square$) (3.1 g/l) and Ca-MBP ($\triangle$) (2.9 g/l) in the presence of a 6-fold molar excess of lysoMPC. (c) Ca-MBP (3.0 g/l) + 0.2 M CaCl_2 in the absence ($\circ$) and in the presence ($\diamond$) of lysoMPC (again 6:1).

the spectra of both CM-MBP and Ca-MBP were altered to similar extents; the inflexion near 220 nm was deepened approx. 2-fold and the minimum near 200 nm, although not significantly different in magnitude, was red-shifted.

The c.d. spectrum of Ca-MBP was also recorded in the presence of 0.2 M-CaCl₂ to determine the effect of this salt on the protein and on its self-association in the presence of lipids. The spectra in Fig. 2(c) are experimentally indistinguishable from the corresponding spectra in Figs. 2(a) and 2(b), which were obtained in the absence of CaCl₂, indicating that at this concentration it does not influence the conformation of the protein.

The n.m.r. spectrum of MBP is distinctive in that the resonances are unusually narrow for a protein of this size, indicating that most of the polypeptide has substantial conformational flexibility, with only small amounts of persistent ordered structure [5,7,8]. Despite broadening of several resonances in the Ca-MBP spectrum compared with that of CM-MBP, overall the spectra shown in Fig. 3 are similar to each other and to previously published spectra obtained at pD 7.7 [33], and we conclude that Ca-MBP does not differ significantly in structure from CM-MBP.

However, comparison of the two spectra in Fig. 3 does reveal several minor differences. Peaks at 7.70 and 6.97 p.p.m. belonging to the C-2 and C-4 resonances of histidine respectively and two unassigned resonances just upfield from the histidine C-4 resonances (6.89 p.p.m.) were significantly broadened. These changes were greater in Ca-MBP preparations that showed signs of aggregation, consistent with previous reports that MBP self-

Fig. 3. ¹H-n.m.r. spectra of CM-MBP and Ca-MBP

The upper spectrum was obtained from CM-MBP (1500 scans) and the lower spectrum from Ca-MBP (500 scans), each at 5 g/l in 5 mM-Na₂HPO₄ at pD 7.7 (uncorrected for the deuterium isotope effect). Asterisks mark the positions of unassigned resonances that differ between the two spectra.

Fig. 4. Sedimentation coefficient of CM-MBP and Ca-MBP as a function of lipid/protein ratio

The data for CM-MBP (□) were obtained from ref. [23]. Ca-MBP data (△) (3.5 ± 0.2 g/l; from three preparations) with the indicated amounts of lysoMPC added were obtained during the present study.

association is mediated through the histidine residues [21].

Several differences were also evident further upfield. The most prominent is a narrow resonance near 3.75 p.p.m., which is present only in the Ca-MBP spectrum in Fig. 3; this peak has been seen in some preparations of both CM-MBP and Ca-MBP and we therefore assume that it was derived from a contaminant. Minor differences in other regions of the spectrum have been marked by asterisks in Fig. 3; additional studies will be required to define the origin of these changes.

Self-association

A second well-studied characteristic of MBP is its self-association and the enhancement of this association by lipids. We have examined the self-association of Ca-MBP in the presence of lysoMPC, by using sedimentation-velocity measurements, and have compared the results with published data for CM-MBP [23].

Fig. 4 indicates that both CM-MBP and Ca-MBP self-associate to similar extents. Values for the $s_{20,w}$ of CM-MBP (data from ref. [23]) between the lipid/protein ratios of 2:1 and 4:1 represent averaged data. In the schlieren photographs, two peaks from the migrating protein monomer and hexamer were present but not resolved. However, these peaks were well-resolved in the Ca-MBP experiments. Even at a detergent/protein ratio of 1:1 two discrete peaks were visible in the schlieren photographs, migrating with $s_{20,w}$ 1.4 S and 4.2 S. The distribution of Ca-MBP in the peaks changed as a function of increasing lipid/protein ratio; at ratios less than 3:1 most of the Ca-MBP migrated in the slower schlieren peak, but at ratios greater than 3:1 protein travelling in the fast peak dominated the schlieren pattern. At ratios greater than 4:1 Ca-MBP migrated in a single symmetrical peak with $s_{20,w}$ 4.2 S.

These results provide additional evidence for the equivalence of the protein prepared by the two methods.

DISCUSSION

There is a large body of evidence supporting the notion that purified MBP in aqueous solution behaves as

a randomly coiled protein with only a small amount of ordered structure. However, these studies have used protein purified with organic solvent and acid extractions, conditions that are known to cause protein denaturation [13,14,34]. In particular, the high content and even distribution of basic residues may make MBP unusually susceptible to denaturation at low pH.

We have purified MBP without the use of the denaturants mentioned above to determine whether the conformation of this protein is modified by the procedures commonly used to isolate it, or whether the ordered structure of MBP is inherently unstable outside its native membrane environment. CaCl_2 has been shown to alter the gross conformation of some proteins [13,14,34], but only at concentrations an order of magnitude higher than that used in the present study. This evidence suggests that 0.2 M- CaCl_2 should not directly modify the structure of MBP.

MBP has previously been purified with the use of detergents which are regarded as non-denaturing for membrane proteins. Riccio *et al.* [28] purified MBP from bovine myelin using the non-ionic detergent octyl polyoxyethylene. The protein was found to remain associated with a 20-fold molar excess of myelin lipids and under these conditions retained a significant amount of ordered structure. MBP has also been purified from crude bovine myelin by washing the membrane with deoxycholate at pH 9.2 [15]. The c.d. spectrum for this protein was found to be similar to the spectrum for CM-MBP in the presence of 1% deoxycholate at pH 9.2 and to the spectra of CM-MBP and Ca-MBP in the presence of lysoMPC, shown in Fig. 2(b). These results suggest that CM-MBP in solution can refold in the presence of detergents to a conformation that resembles the structure of detergent-extracted MBP.

There is evidence from other studies that single-chain proteins lacking disulphide bonds are able to refold spontaneously to their thermodynamically stable state. Proteins studied include staphylococcal nuclease, bacteriophage T₄ lysozyme and *Bacillus subtilis* α -amylase [14]; all possess both α -helix and β -sheet conformations in solution. Denaturation of these proteins by pH, guanidinium chloride, urea or heat is completely reversible, indicating that the information necessary for correct folding of the protein is inherent in the polypeptide chain [35,36]. From these studies one might therefore expect that MBP would adopt its most stable structure in solution even after exposure to denaturants; the equivalence of the structures observed for CM-MBP and Ca-MBP indicates that this is the case.

A number of other proteins, including glucagon [36], atriopeptin III [37] and β -endorphin [12], also exist in solution as relatively unstructured molecules. However, all of these molecules adopt a more ordered secondary structure on self-association or on binding to lipids. For MBP, the change on self-association is minor, but the protein has a substantially more ordered structure when bound to vesicles of acidic lipids. (Earlier indications that the secondary structure changed appreciably on hexamerization [32] have not been confirmed by experiments on more recent MBP preparations; the reported formation of β -sheet may have been caused by the presence of other non-covalently bound molecules.)

Blundell & Wood [36] have proposed that the high flexibility of small polypeptide hormones may be

important for rapid recognition when binding to receptors. In common with these proteins, a high degree of conformational flexibility may be advantageous for MBP in binding to the myelin membrane after its synthesis on free ribosomes.

In summary, the results from the present study indicate that MBP is not adversely affected, with respect to its secondary structure, interaction with lysoMPC and hexamerization, by the methods usually used to purify it. We conclude that MBP has as its thermodynamically preferred state in solution a randomly coiled conformation, with only local areas of ordered structure.

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