Sedimentation Analysis of the Self-Association of Bovine Myelin Basic Protein†

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ABSTRACT: Myelin basic protein aggregates at pH values far from its isoelectric point. It has recently been proposed that this protein–protein interaction may assist in the maintenance of the structure of myelin, with dimers of basic protein providing noncovalent cross-links between opposed membrane surfaces. The self-association of bovine myelin basic protein has been studied to further examine this hypothesis. At pH 4.8 the sedimentation coefficient falls linearly with increasing protein concentration, but at pH 7.4 and 9.0 the coefficient increases as a function of concentration, indicating self-association. In the pH range 3–9 the sedimentation coefficient follows a curve similar to the expected titration curve for the imidazole groups of histidine, with an apparent pK at 6.2 ± 0.2. Sedimentation equilibrium experiments at pH 4.8 verified that the protein remained monomeric to 10 g L^{-1} and showed deviations from ideal solution behavior to be negligible at an ionic strength of 0.30. The equilibrium data at pH 7.4 are best described by the formation of dimers, with association constant $K_1 = 128 \pm 5$ M^{-1} (at 20 °C), which then undergo an indefinite isodesmic self-association with association constant $K_2 = (3.4 \pm 0.2) \times 10^4$ M^{-1}. At 5 °C these constants are 61 ± 2 and $(6.0 \pm 0.3) \times 10^4$ M^{-1}, respectively. The results are interpreted in view of recent observations that basic protein dimerizes in dodecyl sulfate solutions and is partially isolated as dimer following covalent cross-linking within myelin.

Basic protein self-associates and associates with lipids. It has not been established whether these interactions are mutually exclusive or utilize independent sites, though at high detergent concentrations the protein appears to bind two micelles of dodecyl sulfate and remain monomeric whereas at lower concentrations the protein–detergent complex contains two polypeptide chains (Smith & McDonald, 1979) which can be covalently cross-linked (Golds & Braun, 1978b). An answer to this question would clarify the mechanism of lipid bilayer cross-linking by basic protein (Smith, 1977a; Smith & McDonald, 1979; Braun, 1977; Rumsby, 1978). The existence of independent lipid and protein binding sites would favor cross-linking mediated by pairs of protein molecules. Competitive binding, on the other hand, would result in a thermodynamic equilibrium between protein–lipid and protein–protein contacts determined by the free energies associated with each type of interaction and could lead to cross-linking by single protein molecules. Information on the mechanism and extent of self-association has been sought to aid in assessment of these possibilities.

Self-association of myelin basic protein was initially inferred from measurements of light scattering above pH 6 (Liebes et al., 1975; Smith, 1977a). Above this pH there are increases in the line widths of the ^1H NMR peaks assigned to the side chains of aliphatic amino acids (Liebes et al., 1975; Block et al., 1973) and of the ^{13}C resonances of the aromatic amino acids and several aliphatic amino acids (Chapman & Moore, 1976) which have been interpreted as resulting from intermolecular protein contacts. These NMR results appear to implicate hydrophobic amino acids but provide no other information on the mechanism or extent of self-association. In this work sedimentation analyses have been employed to more completely analyze this protein aggregation.

Materials and Methods

Basic protein was prepared from fresh bovine white matter following standard techniques and its purity assessed by electrophoresis in both dodecyl sulfate solutions and pH 4.3 buffer as previously described (Smith, 1977a,b); in both systems it behaved as a single polypeptide. The homogeneity and molecular weight were also verified in the course of the ultracentrifugal analyses. Other reagents were of analytical grade. Protein concentrations (except in sedimentation equilibrium experiments) were determined spectrophotometrically by using $E_{1cm}^{1\%} = 5.44$ at 280 nm.

Basic protein undergoes limited covalent modification in vivo and in vitro. The unmodified form was isolated by ion-exchange chromatography on carboxymethylcellulose at pH 10.6 and 4 °C (Deibler & Martenson, 1973) and its integrity established as for the unfractionated protein.

The partial specific volume of the protein was derived from measurements of density obtained to within ±0.00001 g cm^{-3} on an Anton Paar (Graz, Austria) DMA 02C instrument calibrated with dry air and glass-distilled water. Protein solutions were dialyzed at 5 °C for 48 h against pH 4.8, 0.30 ionic strength buffer before reequilibration at room temperature for several hours and then measurement at 20.00 ± 0.01 °C. The temperature of the density meter was kept constant to within ±0.002 °C.

All sedimentation experiments were performed on a Beckman Model E ultracentrifuge equipped with interference and Schlieren optics. A double-sector synthetic boundary cell was used for sedimentation velocity experiments at rotation rates between 48 000 and 36 000 rpm; the use of the latter speed in a few experiments was dictated by instrumental limitations. All experiments were carried out in the range 17–24 °C in water ($s_{20,w}$). Particularly with basic protein samples at neutral and alkaline pH, the solutions were prepared within an hour of initiation of the centrifuge run. Following centrifugation the pH and protein concentration of the solution were reexamined. It was constantly found with many different preparations, including forms of basic protein isolated by ion-exchange chromatography (see above), that the protein was particularly susceptible to proteolysis at alkaline pH and was occasionally degraded at pH 4.8. The usual precautions, including addition of 2 mM sodium azide to all solutions, were taken to avoid the growth of microorganisms.

† From the School of Chemistry, University of Sydney, Sydney, N. S.W. 2006, Australia. *Received May 10, 1979.* This work was supported by grants from the Australian Research Grants Committee, the Sydney University Research Grants Committee, and the National Multiple Sclerosis Society of Australia.

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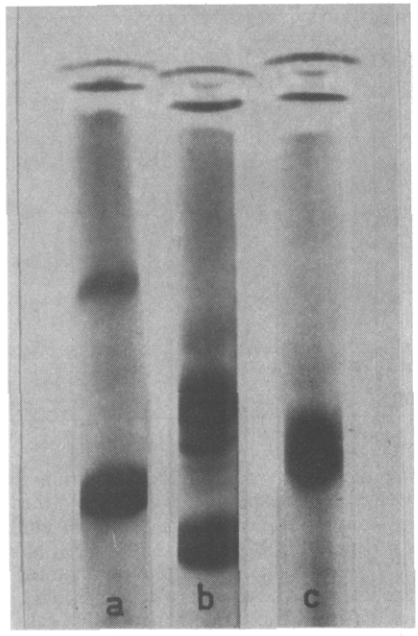

FIGURE 1: Dodecyl sulfate–polyacrylamide gel electrophoresis of (a) lysozyme, (b) the precipitate from myelin basic protein, and (c) basic protein. Approximately 50 μg of protein was applied to each of the gels, which were stained with Coomassie brilliant blue R250.

Sedimentation equilibrium experiments were used to define the extent of oligomerization. One hundred and thirty to one hundred and fifty microliters of solvent and of solution were used in a double-sector cell with a capillary-type synthetic boundary center piece and sapphire windows. Protein solutions were generally dialyzed at 4 °C for 24–48 h against the solvent prior to centrifugation. Fluorocarbon oil was not used in the cell as the protein appeared to partially dissolve in it. Photographs of the Rayleigh interference pattern were taken upon reaching full angular velocity (to check for cell window distortion) and after a time sufficient to give no detectible change in the interference pattern over 4–5 h (~24 h at 20 °C but times up to 40 h were used at 5 °C).

In experiments in which the meniscus was not depleted at equilibrium, the actual concentration in the cell was determined in a subsequent synthetic boundary experiment. After formation of the boundary, five photographs of the interference pattern were taken at 3–10-min intervals. The zero-time value of the fringe displacement across the boundary was obtained by linear extrapolation. Fringe and boundary displacements were measured to ±5 μm on a microcomparator (Chervenka, 1970).

Results

Aggregation of basic protein has been inferred from the observation of turbidity in solutions of purified protein above pH 7 (Liebes et al., 1975; Smith, 1977a). This precipitate is, however, a small fraction of the protein in solution. In a typical experiment a solution of 16.8 g L^{-1} basic protein at pH 3.5 was adjusted to pH 9.04 and centrifuged. The clear supernatant was readjusted to pH 3.5 and the concentration measured; the absorbance had fallen by 2.6%. The precipitate was hetergeneous in polypeptide composition with the major components differing in electrophoretic mobility from the unprecipitated protein (Figure 1). But it is shown below that the soluble protein does aggregate under conditions where turbidity is extant.

Sedimentation Velocity. As previously observed (Liebes et al., 1975; Eylar & Thompson, 1969), at pH 4.8 the sedimentation coefficient falls linearly with increasing protein concentration. The present data fit the equation $s_{20,w} = s^0_{20,w}(1 - kc)$, where $k = 0.021$ cm^3 g^{-1}, c is the concentration in g L^{-1}, and $s^0_{20,w} = 1.50 \pm 0.03$ S. The value at infinite dilution is close to those of Liebes et al. (1975) and Chao & Einstein (1970) (using $\bar{v} = 0.72$ cm^3 g^{-1}) but is substantially lower than that of Eylar & Thompson (1969). The protein used in the present study was intact as demonstrated by subsequent sedimentation equilibrium and electrophoresis experiments.

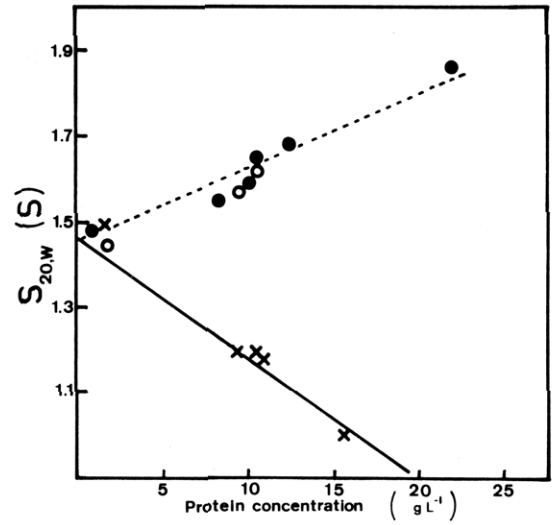

FIGURE 2: Sedimentation coefficient, $s_{20,w}$ (in svedbergs), as a function of protein concentration at pH 4.8 (×), 7.4 (●), and 9.0 (○) at an ionic strength of 0.30. The solid line represents the results of Liebes et al. (1975) at pH 4.8, 0.35 ionic strength.

At pH 7.4 the sedimentation coefficient increases with concentration as the result of self-association (Figure 2). Only a single Schlieren peak close to the expected Gaussian form was observed at all concentrations; the monomer and oligomers must be in equilibrium on a time scale short in comparison with that of the sedimentation experiment. The limiting sedimentation coefficient, $s^0_{20,w}$, is again 1.50 ± 0.03 S, indicating no gross conformation change from pH 4.8 to 7.4. This value may be compared with the value expected for an unsolvated spherical protein, 2.61 S.

Figure 3 shows the variation in $s_{20,w}$, at a fixed protein concentration, with pH. Below pH 5 the protein appears to be monomeric (see below), but self-association increases above this pH, becoming constant above pH 7. Comparison of Figure 3 with the titration data for other proteins (Tanford, 1961) suggests that self-association is dependent on the ionization of one or more histidine residues, groups which become weakly hydrophobic at pH values above their intrinsic pK at ~6.5 (Nozaki & Tanford, 1971). A less specific dissociation attendant upon increased intermolecular electrostatic repulsion at low pH appears unlikely. Although basic protein is close to randomly coiled, it does display some ^{1}H NMR chemical-shift heterogeneity that has been attributed to small elements of local structure (Littlemore, 1978). Two of the histidine residues have significant chemical shifts from the resonance positions in the free amino acid and small peptides.

Sedimentation Equilibrium. The interpretation of sedimentation equilibrium data is markedly influenced by the value adopted for the protein partial specific volume. A value of 0.723 ± 0.003 cm^3 g^{-1} was obtained from density measurements at protein concentrations from 7.8 to 15.7 g L^{-1} at pH 4.8, in agreement with the value of 0.72 cm^3 g^{-1} calculated following Cohn & Edsall (1943). No concentration dependence was evident.

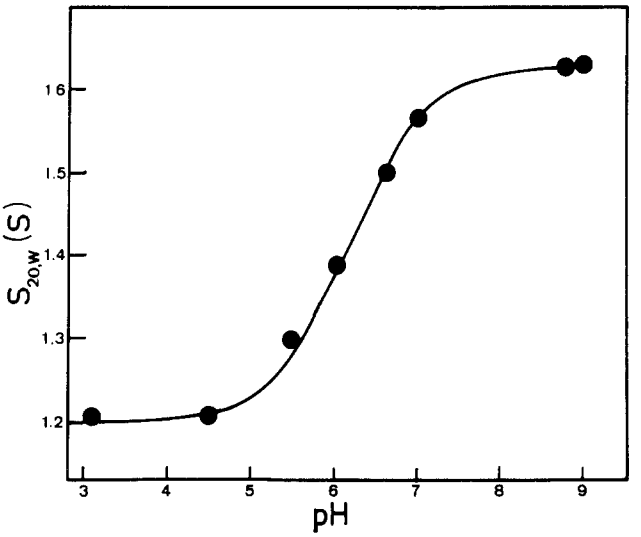

FIGURE 3: pH dependence of the sedimentation coefficient of basic protein at an ionic strength of 0.30. All measurements were made at a concentration of 10.9 g L^{-1} on the same solution. The pH was adjusted by addition of small volumes of concentrated acid or base. To ensure that the experiments at high pH had caused negligible protein degradation, the value at pH 3.0 was measured last. The line was calculated by assuming that aggregation was solely dependent on the ionization of histidine groups in the protein (with an assumed intrinsic pK of 6.2) using an equation of the form of the Henderson–Hasselbalch equation.

Estimates of deviation from ideal solution behavior were sought by examining the sedimentation equilibrium protein distribution at pH 4.8, 0.30 ionic strength. The usual plot of ln (concentration) against the square of the radial distance was strictly linear up to 10 g L^{-1}. Deviations from ideality are therefore insignificant or fortuitously balanced by minor aggregation at pH 4.8. The second virial coefficient, B, has been measured by light scattering (Liebes et al., 1975) and low-angle X-ray scattering (Krigbaum & Hsu, 1975), but the reported values are discrepant. B must fall between the figure calculated from the excluded volume of an unsolvated compact sphere of molecular weight 18 400, 1.5×10^{-4} cm^3 mol g^{-2}, and that measured for a randomly coiled protein of this molecular weight, 1.1×10^{-3} cm^3 mol g^{-2} (Lapanje & Tanford, 1967). If a value of 2.2×10^{-4} cm^3 mol g^{-2} is assumed [derived from Krigbaum & Hsu (1975)], this yields an apparent molecular weight of 17 550 at a concentration of 6 g L^{-1}, the highest monomer concentration encountered at pH 7.4. This is 4.5% below the actual figure; such deviations would not have a substantial effect on the calculations below. Thus, in this analysis it has been assumed that the activity coefficients for the monomer and for each oligomer are unity; the concentrations of aggregated forms in all experiments are considerably lower than that of the monomer.

For a self-associating system we introduce the $\Omega(r)$ function (Milthorpe et al., 1975), which makes direct use of the equilibrium protein distribution to calculate the activity of monomer at each point in the centrifuge cell

$$\Omega(r) = [\bar{c}(r)/\bar{c}(r_F)] \exp[\phi_1 M_1(r_F^2 - r^2)] \quad (1)$$

where $\bar{c}(r)$ and $\bar{c}(r_F)$ are the total weight concentrations of protein at distances r and r_F from the center of rotation, M_1 is the monomer molecular weight (18 400), and

$$\phi_1 = (1 - \bar{v}_1\rho)\omega^2/(2RT) \quad (2)$$

$\bar{v}_1$ is the monomer partial specific volume, ρ is the solution density, ω is the angular velocity, R is the gas constant, and T is the absolute temperature.

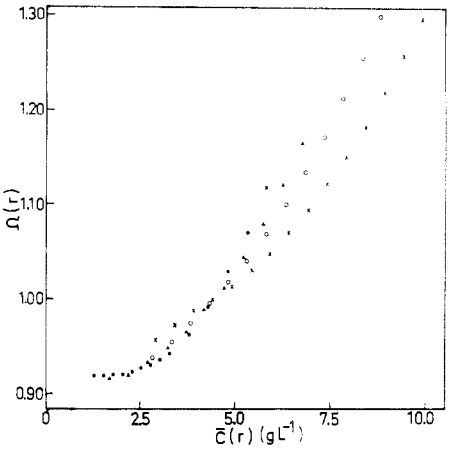

FIGURE 4: Representative sedimentation equilibrium data at pH 7.4, 0.30 ionic strength. The experiments were performed with the following initial concentrations (grams per liter) and rotor speeds (revolutions per minute): 2.53, 16 022 (●); 3.52, 14 014 (▲); 5.12, 12 009 (○); 5.27, 12 009 (×). All data were obtained at 20 ± 1 °C except for the last, which were obtained at 5.1 °C. In each the reference concentration was 17.64 fringes (4.48 g L^{-1}).

$\Omega(r)$ is a measure of the degree of self-association at r relative to that at r_F. Assuming no deviation from ideal solution behavior

$$\Omega(r) = \frac{\bar{c}(r)/c_1(r)}{\bar{c}(r_F)/c_1(r_F)} \quad (3)$$

As $\bar{c}(r)$ and $\bar{c}(r_F)$ are obtained experimentally, eq 3, coupled with the fact that $\bar{c}(r) \rightarrow c_1(r)$ as $\bar{c}(r) \rightarrow 0$, can be used with $\Omega(r)$ from eq 1 to determine c_1 at any radial distance.

The experimental data are represented in the curves for $\Omega(r)$ in Figure 4. The results in each concentration range were closely reproducible and were the same for the whole basic protein and for the unmodified form (which is the major component) isolated by ion-exchange chromatography. Extrapolation of the data obtained near 20 °C is straightforward and yields for the chosen $\bar{c}(r_F)$ (17.64 fringes or 4.48 g L^{-1}) the limit as $\bar{c}(r)$ tends to zero of $\Omega(r) = 0.920 \pm 0.005 = c_1(r_F)/\bar{c}(r_F)$. The corresponding value at 5.1 °C is 0.945 ± 0.010.

The data from experiments spanning different concentration ranges and at different angular velocities do not overlap, showing that oligomerization is accompanied by a decrease in partial specific volume. Comparison of results obtained at high initial concentration and the two lowest speeds, 8986 and 12 009 rpm, showed little variation at these centrifugal fields (Figure 5), and the data could be used directly to analyze the self-association mechanism. This avoids mathematical correction for the effects of volume changes which are difficult unless only a single oligomer is in equilibrium with the monomer. Calculations performed for a pressure-dependent monomer–dimer equilibrium (Howlett et al., 1972) failed to provide a satisfactory fit to the experimental results.

As has often been noted, synthetic boundary concentration measurements are poorly reproducible. This was observed occasionally in the present work, but the results presented are from solutions whose concentrations were derived from many measurements which were generally self-consistent and consistent with the protein concentrations deduced from absorption measurements. Residual errors are reflected in the calculated meniscus concentrations. The correctness of these values can be judged by the molecular weights estimated from the data

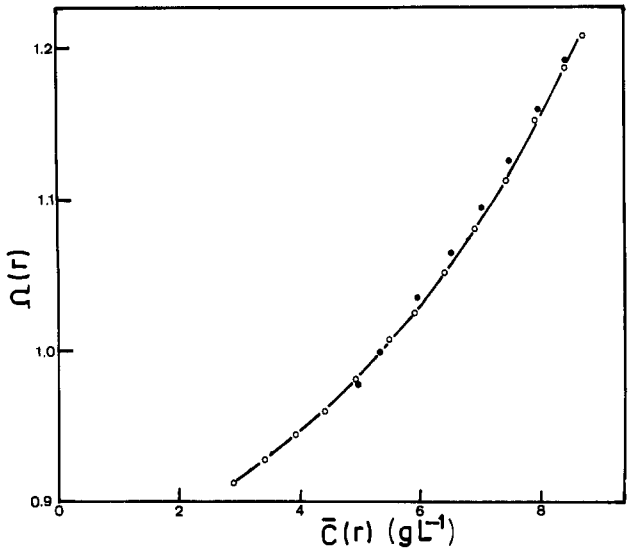

FIGURE 5: Sedimentation data obtained at low angular velocity: at 12 009 rpm with initial concentration of 5.27 g L^{-1} (○) and at 8986 rpm at 6.92 g L^{-1} (●). Both experiments were performed at 20 °C, ionic strength 0.30. The reference concentration $[\bar{c}(r_F)]$ was 21.41 fringes.

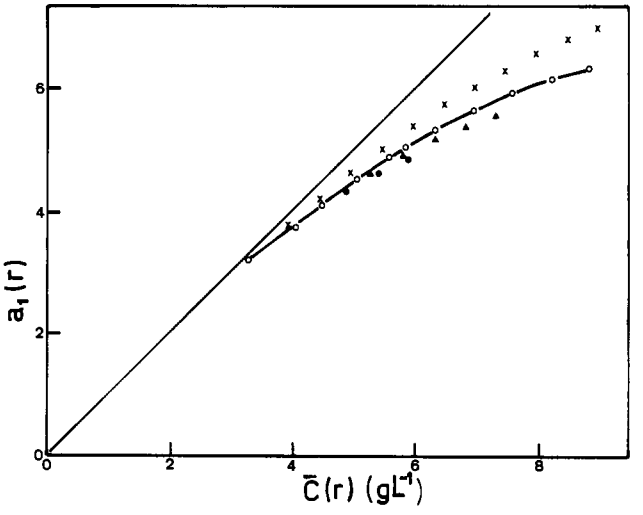

FIGURE 6: Activity of monomer $[a_1(r)]$ as a function of total concentration $[\bar{c}(r)]$. These curves were constructed from the data in Figure 4, and the same symbols have been used to identify experiments. Below 3.5 g L^{-1} the data are coincident with the solid straight line, which shows the results expected in the absence of association.

at low protein concentrations; in the absence of demonstrable proteolysis, they were within 3 to 4% of 18 400.

From the results in Figure 4 plots of monomer activity, $a_1(r) = c_1(r)$, against $\bar{c}(r)$ were constructed (Figure 6) which provided the basis for analysis of the self-association. Many simple limited association models were tested including monomer–dimer which is of particular interest because of its possible implication in the maintenance of the structure of myelin. However, the results were inconsistent with such an equilibrium even if allowance was made for nonideality by introduction of a concentration-independent second virial coefficient for the monomer. Unsatisfactory fits were also obtained by assuming ideal or nonideal indefinite isodesmic association. Of the models tested only two were at all satisfactory (Figure 7). A monomer–tetramer equilibrium could be made to fit the data at low concentrations but predicted less than the observed association at high concentrations. An excellent correspondence was provided by a mechanism in-

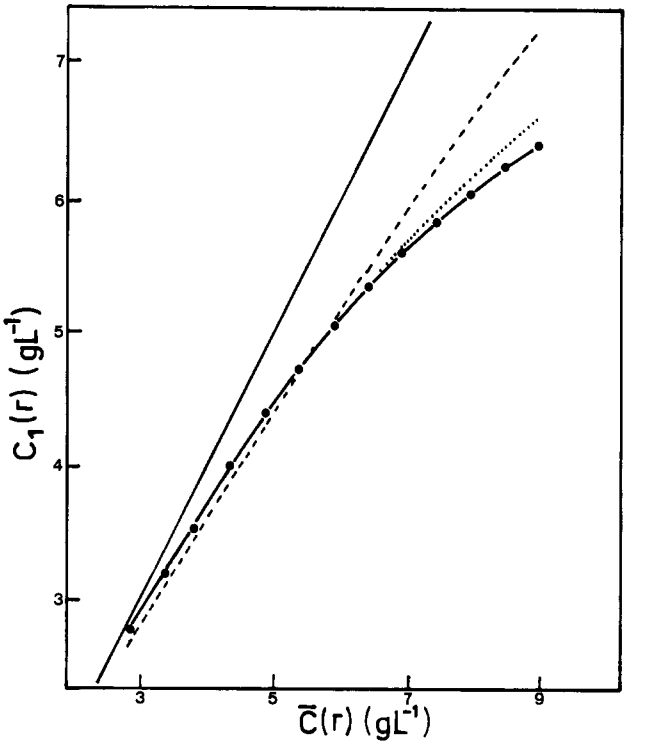

FIGURE 7: Comparison of the experimental (●) and predicted monomer concentrations assuming the following models: (a) dimer formation followed by ideal indefinite isodesmic association of dimers with $K_1 = 128\ M^{-1}$ and $K_2 = 3.4 \times 10^4\ M^{-1}$ (—); (b) an indefinite isodesmic ideal self-association with an association constant of 250 M^{-1} (---); (c) a monomer–tetramer equilibrium with an association constant of $12.4 \times 10^{-4}\ L^3\ g^{-3}$ (···). Below 6 g L^{-1} the calculated values fall on the solid line. The errors in the experimental data do not exceed the size of the points on the graph. The diagonal line at the left represents $\bar{c}(r) = c_1(r)$.

volving formation of dimers with subsequent indefinite isodesmic association of the dimers. The experimental data were fitted to the equation (Jeffrey et al., 1976)

$$\bar{c}(r) = \frac{M_1 m_1(r)[[1 - K_1K_2[m_1(r)]^2]^2 + 2K_1 m_1(r)]}{[1 - K_1K_2[m_1(r)]^2]^2} \quad (4)$$

where $K_1 = m_2(r)/[m_1(r)]^2$, $K_2 = m_{i+2}(r)/[m_2(r)m_i(r)]$, and m_i is the molarity of the ith oligomer.

An approximate value for K_1 was obtained by using the data at low concentrations and assuming $K_2 = 0$. This value was then refined by taking all of the data to calculate K_2 at each point (~30 points/run), accepting the K_1 leading to a constant value for K_2. At 20 ± 1 °C, $K_1 = 128 \pm 5\ M^{-1}$ and $K_2 = (3.4 \pm 0.2) \times 10^4\ M^{-1}$. By use of these constants, the solid line in Figure 7 was generated for comparison with the experimental results and typical curves predicted by several other association schemes.

At 5 °C the constants derived are $K_1 = 61 \pm 2\ M^{-1}$ and $K_2 = (6.0 \pm 0.3) \times 10^4\ M^{-1}$. The limiting value of $\Omega(r)$ is less certain at this temperature, but allowing for generous error in this figure K_1 falls in the range 58–70 M^{-1} and $K_2 = (5.2–6.1) \times 10^4\ M^{-1}$. At lower temperatures the dimerization appears to be reduced but the subsequent oligomerization becomes energetically more favorable; the observed temperature dependence is consistent with hydrophobic bonding within dimers.

Discussion

Two models have been proposed for the structure of basic protein: a compact prolate ellipsoid of revolution (Epand et

al., 1974) and an expanded flexible coil (Krigbaum & Hsu, 1975). Both are compatbile with the available hydrodynamic data, but the latter is additionally supported by low-angle X-ray scattering results (Krigbaum & Hsu, 1975) and by recent NMR experiments. The majority of the atoms have spin–spin and spin–lattice relaxation rates, nuclear Overhauser effects, and chemical shifts that are consistent with a close-to-random-coiled conformation (Chapman & Moore, 1978). The sedimentation velocity data show that $s^0_{20,w}$ is invariant from pH 9.0 to 4.8; no expansion accompanies the increased intramolecular repulsions expected on protonation of the histidine residues and partial protonation of the carboxyl groups.

In earlier work from this laboratory (Smith, 1977a) a parallel was noted between basic protein-induced aggregation of diacylphosphatidylcholine vesicles and precipitation of basic protein. This led to the proposal that basic protein noncovalently cross-linked the vesicles, possibly (though not necessarily) through protein–protein interaction between molecules attached to different vesicles. Although we have now shown that the precipitate is not unmodified basic protein, these conclusions remain valid as the protein *does* aggregate over much the same pH range as the lipid–protein complex. It was of particular interest to determine whether the protein readily forms specific protein–protein contacts. The sedimentation analyses have clearly demonstrated self-association under conditions where the molecules bear a substantial net positive charge. Among the models tested, dimerization followed by an indefinite isodesmic association of dimers provides a uniquely good fit to the data. Separate molecular mechanisms for dimer and oligomer formation are also indicated by the observed temperature dependence of each type of protein interaction.

Golds & Braun (1978a,b) have recently found that following covalent cross-linking of basic protein in dodecyl sulfate solutions and in myelin the protein is isolated as monomer and dimer with negligible formation of higher oligomers. We have shown that at high detergent concentrations the protein is monomeric but at free dodecyl sulfate concentrations near 0.25 mM the protein appears to be completely dimeric, even at low concentrations (Smith & McDonald, 1979). This observation appears particularly relevant to the proposed role within myelin (Braun, 1977; Smith, 1977a) for, as is supported by the present work, it suggests that under appropriate conditions oligomerization is terminated before tetramer formation and that in some systems dimerization is considerably more favorable than it appears to be in aqueous solution. If the increased tendency to dimerize in dodecyl sulfate solutions results from decreased intermolecular electrostatic repulsions consequent on association with negatively charged detergent, a similar enhancement of self-association in myelin may be brought about by preferential interaction with negatively charged lipid (Boggs et al., 1977) or by covalent modifications, such as phosphorylation, which lower the charge on the protein.

Covalent cross-linking at pH 6.8 in aqueous solution led to the apparent formation of dimer, trimer, and smaller amounts of higher oligomers (Golds & Braun, 1978b). The authors noted that the oligomers could have formed by cross-linking of molecules during random collisions. If the products of such a covalent reaction are representative of species extant in solution, the appearance of dimer, tetramer, hexamer, and so on would have been expected from the sedimentation analyses; an indefinite isodesmic association of the monomer is not compatible with the sedimentation results.

The secondary structure of the monomer does not vary markedly over a wide pH range (Moscarello et al., 1974; Liebes et al., 1975), but the structure of the monomer within oligomers may differ. Circular dichroism spectra are generally recorded under conditions where the aggregate concentrations are low and the monomer spectrum would be dominant. Binding to lipid vesicles and micelles induces a transition in the secondary structure of the protein that might also modify the self-association (Keniry & Smith, 1979). The presence of several species of oligomer at most concentrations precludes drawing firm conclusions from the sedimentation velocity data about the structure of the dimer and higher aggregates in aqueous solution.

Although detergent binding was originally interpreted as favoring a unimolecular bilayer bridging (Smith & McDonald, 1979), the self-association of the protein in aqueous solution, in dodecyl sulfate solutions at low concentrations, and in myelin is consistent with a bimolecular mechanism. Formation of monomer at high dodecyl sulfate concentrations may be caused by detergent association not only with lipid binding sites on each molecule but also with the putative self-association site. If dimerization does utilize different sites from those causing subsequent indefinite association in aqueous solution, it would be of interest to know the correspondence between these protein sites and those binding lipids and other protein molecules in the detergent complexes and in myelin. The sites causing the initial dimerization, which must be at least partially responsible for the pH dependence seen in Figure 3, cannot a priori be taken as those involved in protein–protein interaction in the other systems.

Acknowledgments

The author acknowledges with gratitude the generous assistance of Professor L. W. Nichol, Australian National University.

References

Block, R. E., Brady, A. H., & Joffe, S. (1973) *Biochem. Biophys. Res. Commun. 54*, 1595–1602.

Boggs, J. M., Moscarello, M. A., & Papahadjopoulos, D. (1977) *Biochemistry 16*, 5420–5426.

Braun, P. E. (1977) in *Myelin* (Morell, P., Ed.) pp 91–115, Plenum Press, New York.

Chao, L.-P., & Einstein, E. R. (1970) *J. Neurochem. 17*, 1121–1132.

Chapman, B. E., & Moore, W. J. (1976) *Biochem. Biophys. Res. Commun. 73*, 758–766.

Chapman, B. E., & Moore, W. J. (1978) *Aust. J. Chem. 31*, 2367–2385.

Chervenka, C. H. (1970) in *A Manual of Methods for the Analytical Ultracentrifuge*, pp 6–12, Beckman Instruments Inc., Palo Alto, CA.

Cohn, E. J., & Esall, J. T. (1943) in *Proteins, Amino Acids and Peptides*, pp 370–381, Reinhold, New York.

Diebler, G. E., & Martenson, R. E. (1973) *J. Biol. Chem. 248*, 2392–2396.

Epand, R. M., Moscarello, M. A., Zierenberg, B., & Vail, W. J. (1974) *Biochemistry 13*, 1264–1267.

Eylar, E. H., & Thompson, M. (1969) *Arch. Biochem. Biophys. 138*, 606–613.

Golds, E. E., & Braun, P. E. (1978a) *J. Biol. Chem. 253*, 8162–8170.

Golds, E. E., & Braun, P. E. (1978b) *J. Biol. Chem. 253*, 8171–8177.

Howlett, G. J., Jeffrey, P. D., & Nichol, L. W. (1972) *J. Phys. Chem. 76*, 777–783.

Jeffrey, P. D., Milthorpe, B. K., & Nichol, L. W. (1976) *Biochemistry 15*, 4660–4665.
Keniry, M. A., & Smith, R. (1979) *Biochim. Biophys. Acta 578*, 381–391.
Krigbaum, W. R., & Hsu, T. S. (1975) *Biochemistry 14*, 2542–2546.
Lapanje, S., & Tanford, C. (1967) *J. Am. Chem. Soc. 89*, 5030–5033.
Liebes, L. F., Zand, R., & Phillips, W. D. (1975) *Biochim. Biophys. Acta 405*, 27–39.
Littlemore, L. A. T. (1978) *Aust. J. Chem. 31*, 2387–2398.
Milthorpe, B. K., Jeffrey, P. D., & Nichol, L. W. (1975) *Biophys. Chem. 3*, 169–176.
Moscarello, M. A., Katona, E., Neumann, A. W., & Epand, R. M. (1974) *Biophys. Chem. 2*, 290–295.
Nozaki, Y., & Tanford, C. (1971) *J. Biol. Chem. 246*, 2211–2217.
Rumsby, M. G. (1978) *Biochem. Soc. Trans. 6*, 448–462.
Smith, R. (1977a) *Biochim. Biophys. Acta 470*, 170–184.
Smith, R. (1977b) *Biochim. Biophys. Acta 491*, 581–590.
Smith, R., & McDonald, B. J. (1979) *Biochim. Biophys. Acta 554*, 133–147.

High Levels of a Heat-Labile Calmodulin-Binding Protein ($CaM\text{-}BP_{80}$) in Bovine Neostriatum†

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ABSTRACT: Bovine brain contains a heat-labile, 80 000-dalton calmodulin-binding protein ($CaM\text{-}BP_{80}$) which inhibits the calmodulin-dependent activities of cyclic 3′,5′-nucleotide phosphodiesterase, adenylate cyclase, and Ca^{2+}-ATPase in vitro. $CaM\text{-}BP_{80}$ is composed of two polypeptides (60 000 and 18 500 daltons) present in a 1:1 ratio. An antibody directed against $CaM\text{-}BP_{80}$ was raised in rabbits, and a radioimmunoassay was developed, having a sensitivity of 60 fmol of $CaM\text{-}BP_{80}$. Using the radioimmunoassay, we determined the levels of $CaM\text{-}BP_{80}$ in various bovine tissues. The protein was found primarily in the brain, present in particularly high levels in the neostriatum. These results, together with immunohistochemical localization of $CaM\text{-}BP_{80}$ at the postsynaptic densities and the microtubules of postsynaptic dendrites [Wood, J. G., Wallace, R., Whitaker, J., & Cheung, W. Y. (1980) *J. Cell Biol. 84*, 66–76], suggest that the protein may have a role in the cerebrum at the site of neurotransmitter action and at the level of microtubular function.

Adenosine 3′,5′-monophosphate (cAMP)[1] is a versatile regulator, modulating many cellular functions and processes. Regulation of the cellular level of the nucleotide involves two complex enzyme systems, the synthetic enzyme adenylate cyclase and the degradative enzyme phosphodiesterase. In mammalian brain, both enzymes require Ca^{2+} for maximal activity. Activation by Ca^{2+} is mediated through calmodulin, a Ca^{2+}-dependent modulator protein ubiquitous in eucaryotes (Cheung et al., 1975b; Waisman et al., 1975).

Regulation of Ca^{2+}-activatable adenylate cyclase (Brostrom et al., 1975, 1976, 1977; Cheung et al., 1975a, 1978; Lynch et al., 1977), phosphodiesterase (Cheung et al., 1975a, 1978; Kakiuchi & Yamazaki, 1970; Kakiuchi et al., 1975; Wolff & Brostrom, 1974; Brostrom & Wolff, 1974, 1976; Teo & Wang, 1973; Wang et al., 1975), and Ca^{2+}-Mg^{2+}-ATPase (Gopinath & Vincenzi, 1977; Jarrett & Penniston, 1977, 1978; Hanahan et al., 1978; Lynch & Cheung, 1979) by calmodulin has been extensively studied. In the presence of Ca^{2+}, calmodulin undergoes a change toward a more helical conformation, which is the active species, and interacts with an apoenzyme to form the active holoenzyme (Liu & Cheung, 1976; Klee, 1977; Dedman et al., 1977). Calmodulin also regulates the activities of skeletal muscle phosphorylase kinase (Cohen et al., 1978), myosin light chain kinase (Waisman et al., 1978; Dabrowska & Hartshorne, 1978; Dabrowska et al., 1978; Sherry et al., 1978; Yagi et al., 1978), NAD kinase (Anderson & Cormier, 1978; D. Epel, R. W. Wallace, and W. Y. Cheung, unpublished experiments), phospholipase A_2 (Wong & Cheung, 1979), Ca^{2+} transport in erythrocytes (Hinds et al., 1978; Larsen & Vincenzi, 1979) and sarcoplasmic reticulum (Katz & Remtulla, 1978), phosphorylation of membranes (Schulman & Greengard, 1978a,b), and the disassembly of microtubules (Marcum et al., 1978). Thus, calmodulin appears to be a major receptor or mediator of Ca^{2+}, regulating many cellular functions [for reviews, see Wolff & Brostrom (1979), Cheung (1980), Wang & Waisman (1980), and Klee et al. (1980)].

In bovine brain, there are several proteins that bind calmodulin in a Ca^{2+}-dependent manner; the biological activities of these proteins have yet to be identified (Cheung et al., 1978; Wang & Desai, 1976, 1977; Klee & Krinks, 1978; Wallace et al., 1978a, 1979; LaPorte & Storm, 1978; Sharma et al., 1978, 1979). One of these proteins is heat labile and has a molecular weight of 80 000 ($CaM\text{-}BP_{80}$) (Cheung et al., 1978; Klee & Krinks, 1978; Wallace et al., 1978a, 1979; Wang & Desai, 1976). $CaM\text{-}BP_{80}$ contains two subunits, 60 000 and 18 500 daltons, in equivalent molar ratios (Wallace et al., 1979). However, Sharma et al. (1979) reported the molar ratio of the large and small subunits to be 1:2. The protein

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‡ Recipient of U.S. Public Health Service Research Fellowship AM 05689.

[1] Abbreviations used: cAMP, adenosine 3′,5′-monophosphate; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N*′-tetraacetic acid; NaCl–P_i, phosphate-buffered saline [0.15 M NaCl and 20 mM sodium phosphate (pH 7.2)]; $CaM\text{-}BP_{80}$, heat-labile calmodulin-binding protein.

0006-2960/80/0419-1831$01.00/0