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SOLUTION BEHAVIOR, CIRCULAR DICHROISM AND 220 MHz PMR STUDIES OF THE BOVINE MYELIN BASIC PROTEIN

LEONARD F. LIEBES*, ROBERT ZAND** and WILLIAM D. PHILLIPS

Biophysics Research Division and Department of Biological Chemistry, University of Michigan, Ann Arbor, Mich. 48105 (U.S.A.) and Central Research Dept., E. I. DuPont deNemours and Co., Wilmington, Del. 19889 (U.S.A.)

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SUMMARY

Bovine myelin basic protein has been investigated with regard to its solution behavior, circular dichroism and 220 MHz PMR spectral properties.

At pH 4.8 $\gamma/2 = 0.1$ acetate buffer, light scattering yielded a M_r of 17 700 and a virial coefficient of $1.0 \cdot 10^{-4}$ mol·ml/g^2. Above pH 7.0 the protein was found to aggregate to higher mol. wt species.

Sedimentation experiments at pH 4.8 yielded $s^\circ_{20,w}$ of 1.27 S at $\gamma/2 = 0.1$ and 1.46 S at $\gamma/2 = 0.35$. The diffusion coefficient determined from ultracentrifugal experiments was $7.25 \cdot 10^{-7}$ cm^2/s at $\gamma/2 = 0.1$ and 0.35. The value of f/f_0 from diffusion at pH 4.8 and $\gamma/2 = 0.35$ was 1.64, corresponding to an axial ratio of 11 to 1. The radius of gyration was calculated as 4.28 nm and the root mean square end to end distance was 10.5 nm. At pH 9.0, $\gamma/2 = 0.1$, $s^\circ_{20,w}$ was 1.71 S and $D^\circ_{20,w}$ was estimated at $7.4 \cdot 10^{-7}$ cm^2/s. The behavior at pH 9.0 reverted to the behavior at pH 4.8 when the pH was readjusted. The $E^{1\%}_{1cm} = 5.64$ at 276.4 nm and 225 at 196 nm.

Titration of the protein with trifluoroethanol elicited three distinct regions of conformational stability having increasing helical content as the mol fraction of trifluoroethanol increased.

The results of the present study have permitted some comparison of analogous properties and conformational behavior with the basic membrane protein cytochrome *c*.

INTRODUCTION

The myelin basic protein, also known as the A_1 protein or the encephalitogenic protein, is one of the few neural membrane derived proteins that has been purified [1–3] and sequenced [4–6]. Although extensively studied with regard to its antigenic behavior [7, 8] a comprehensive study of the biophysical chemistry of this protein has not been reported. Those few physical parameters that have been reported from different laboratories have often disagreed [9–11]. Aside from some early ORD measurements no studies of the effect of pH, ionic strength, and solvent on the secondary

* Current Address: Michigan Cancer Foundation ,110 East Warren Detroit, Michigan.
** Correspondence should be addressed to this author.

and tertiary structure of the protein have been reported. In order to study the conformation and perturbation of conformation of the bovine myelin basic protein, CD and 220 MHz PMR spectroscopy have been used as complementary methods to investigate this protein's conformational behavior. All of the constituent amino acid proton resonances were assigned by the methods of McDonald and Phillips [13]. Such a complete assignment of protein proton resonances is still quite rare and in this study provided a facile means of determining which residues were affected by a given change of condition. In addition, the PMR spectra provided an independent means of verifying our analyses of the CD results. Titration of the protein with trifluoroethanol showed three distinct regions of conformational stability rather than a continuous linear change to a structure of increasing α-helicity.

Light scattering and ultracentrifugal studies at pH 4.8 provided data that allowed for the calculation of mol. wt (a test of the correctness of certain parameters that were determined), a second virial coefficient, B; the sedimentation constant, $s^{\circ}_{20,w}$; diffusion constant, $D^{\circ}_{20,w}$; radius of gyration, R_g; and root mean square end to end distance, $<L^2>$. At pH values above seven, protein aggregation precluded a similar determination of these parameters. This investigation of the solution behavior and conformation of bovine myelin basic protein in conjunction with data from dye binding studies [27] has permitted a resolution of some of the conflicting data in the literature and has enabled some comparison of behavior with another membrane derived basic protein, cytochrome *c*.

MATERIALS AND METHODS

Preparation and Purification of the Myelin Basic Protein. The preparation of the crude protein extract from bovine central nervous system was carried out using the procedure of Eylar [2]. Purification of the protein was a modified combination of the methods of Eylar [2] and Nakao [1]. The first step in the purification incorporated the elution of the protein with a 0–0.6 M linear NaCl gradient in pH 4.6, 0.05 M acetate buffer from a 2.5 × 90 cm CM-52 column (Whatman microgranular). Additional purification was achieved by passage of the protein through a Sephadex G-50 (2.5 × 90 cm), Sephadex G-75 (2.5 × 90 cm) and BioGel P-10 (2.5 × 90 cm) series of columns eluted by 0.2 M acetic acid. The eluates were recycled on this column arrangement until pure by the criterion of disc gel electrophoresis.

Routine disc gel electrophoresis of the column eluates utilized the method of Riesfield [14]. The method of Wray and Stubblefield [15] served as the final criterion of purity of the protein preparation.

Dry weight determinations were by the method of Hunter [16]. Light scattering measurements were made with a SOFICA light scattering instrument model 42000(701) equipped with a Heath Co. digital multimeter model IM-102. Light scattering cells (20 × 63 mm) were made to specification by Precision Cell Co. Calibration of the instrument was done with Ludox solutions in 0.05 M NaCl according to the method of Goring [17] and Kratohvil [18]. A further check of the calibration was made using monomeric bovine serum albumin purified by the procedure described by Janatova [19].

Sedimentation velocity and diffusion analyses were done using a Spinco Model E analytical ultracentrifuge equipped with the electronic speed control. The sedimentation runs utilized a synthetic boundary forming cell, a rotor speed of 60 000

rev./min and the schlieren optical system set at a 70° angle. Diffusion experiments employed a double rectangle, synthetic boundary forming cell designed according to the specifications of Dr J. L. Oncley and fabricated by Beckman Instruments, Palo Alto California. Use of this cell eliminated the need to employ corrections [11] for the asymmetry of the gradient curve which would result when a sector shaped cell is used. A rotor speed of 6000 rev./min was used.

Circular dichroism measurements were obtained with a JASCO-ORD-CD-5 instrument modified to the Sproul SS-20 configuration.

Absorption spectra and colorimetric assays were done with a Beckman ACTA III Spectrophotometer.

PMR spectra were obtained on a Varian Associates 220 MHz high resolution spectrometer. The polarizing field to 52 000 G was furnished by a superconducting solenoid. The temperature in the sample zone was maintained at $\pm$ 1 °C of the desired temperature by a stream of nitrogen. A Varian Associates computer of average transients (Model C 1024) was used when necessary to enhance the signal to noise ratio.

RESULTS

A protein that is to be used for physical biochemistry studies should be of the highest purity attainable. In our hands the usual methods for preparing central nervous system myelin basic protein required additional treatment to obtain a satisfactory degree of purification. Homogeneity of the final produce as ascertained by disc gel electrophoresis is shown in Fig. 1. The series of gels on the left side were prepared and destained by the procedure of Wray and Stubblefield [15] which provides a 100-fold increase in sensitivity of detection over a regular destaining procedure employing 7% acetic acid used for the gels on the right. By the former procedure several bands were visualized that migrated above and below the major basic protein band. Washing these same gels with 7% acetic acid resulted in the disappearance of the stain from contaminating bands leaving only the major band of the basic protein. The Wray and Stubblefield destaining procedure is capable of detecting less than 0.05 μg of protein and since we could detect these minor bands at a load of 4.4 μg of basic protein, we estimate that the amount of contaminating protein is of the order of 1% or less of the total purified protein.

The absorption maxima of the protein were observed (pH = 6.8) at 276.4 nm, $E^{1\%}_{1cm} = 5.64$ and 196 nm, $E^{1\%}_{1cm} = 225$. The $E^{1\%}_{1cm}$ at 276.4 is close to the value previously reported [10].

Light scattering studies at pH 4.8, $\gamma/2 = 0.1$ acetate buffer (Fig. 2) yielded a mol. wt of 17 700 and a virial coefficient, B, of $1.01 \cdot 10^{-4}$ mol·ml/g^2. The differential refractive increment for the basic protein in this buffer at 25 °C at a wavelength of 546 nm was determined to be 0.1876. The mol. wt obtained from light scattering differs by less than 3% from the mol. wt of 18 395 calculated from the amino acid composition of this protein [5]. This small deviation is within the experimental error inherent in the method (5%) and the excellent agreement between the experimental and calculated mol. wt provides independent verification of the homogeneity of the protein. Above pH 7.0 ($\gamma/2 = 0.35$) the base level of the 90° scattering exhibited by this protein is dramatically elevated (Fig. 3). A similar increase in turbidity as a function of pH has been reported for lysozyme [20]. However, only 1/68th of the amount of the latter

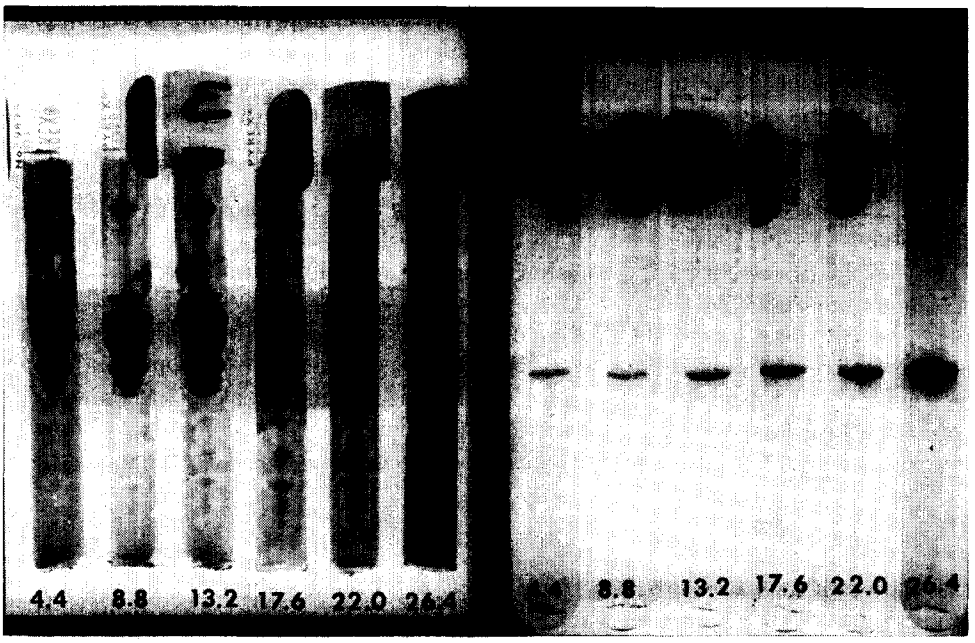

Fig. 1. Disc gel patterns of purified bovine myelin basic protein. 7% gels with urea/acetic acid at pH 2.5. The amount of protein applied (μg) is indicated beneath each gel. The gels were initially stained with amido black and destained according to Wray and Stubblefield [15] using a 1 M H_2SO_4 solution containing 3 M urea. These gels are shown in A. These gels are subsequently destained in successive washes of 7% acetic acid yielding gel patterns as shown in B. Minor components amounting to less than 1% that are visualized by the Wray and Stubblefield procedure are not evident after the standard 7% acetic acid destaining.

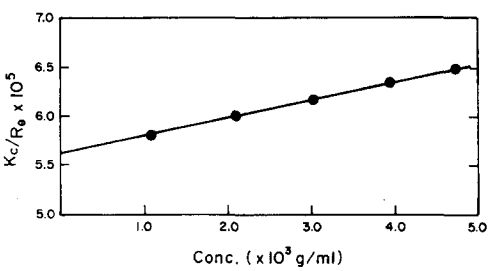

Fig. 2. Light scattering results from solutions of bovine myelin basic protein in acetate buffer pH 4.8, $\gamma/2 = 0.1$. The 90° scattering was obtained using 546 nm light and a temperature of 25 °C. The plot yields an intercept of $5.46 \cdot 10^{-5}$, slope of $1.01 \cdot 10^{-4}$ mol·ml/g^2 and a mol. wt of 17 700.

was required to obtain a comparable increase in turbidity. This increase in turbidity as a function of pH is attributed to aggregation of the protein and a concommitant reduction in solubility. This effect is completely reversible if the pH is lowered.

Sedimentation and Diffusion Behavior. The sedimentation coefficients corrected to 20 °C in water for the myelin basic protein in acetate buffer, pH 4.8, over a concentration range of 0.3–1.15% protein were fitted by a least squares line for each ionic strength. At $\gamma/2 = 0.1$, the intercept $s^\circ_{20,w}$ was equal to 1.27 S and k as defined by the

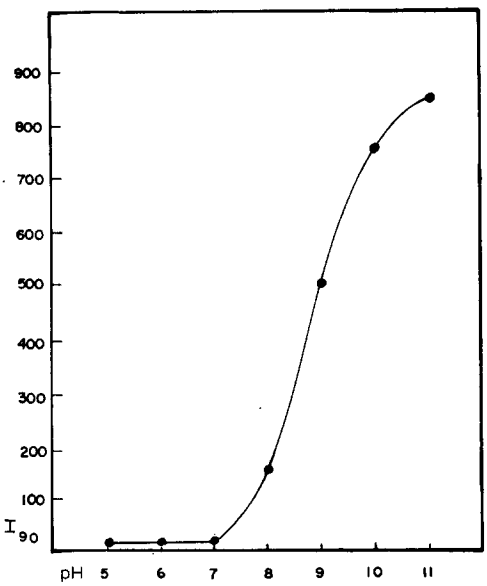

Fig. 3. A plot of the 90° scattering from a 0.072% solution of basic protein in 0.35 M KCl as a function of pH.

equation $s_{20,w} = s^\circ_{20,w}(1-kc)$ was determined to be 0.35 dl/g. At $\gamma/2 = 0.35$, $s^\circ_{20,w} = 1.46$ S and $k = 0.19$ dl/g. An analysis of the shape of the schlieren curves obtained at $\gamma/2 = 0.1$ and $\gamma/2 = 0.35$ gave a good fit between the calculated gaussian curves and the experimental curves. The value of $\bar{v}$ used in calculating $s^\circ_{20,w}$ was obtained from the amino acid sequence of the protein [5] according to the procedure of Cohn and Edsall [21]. The value obtained by this procedure is 0.720. The diffusion coefficients determined from ultracentrifugal experiments in the above buffer for protein concentrations of 0.3–1.25% yielded upon extrapolation a $D^\circ_{20,w}$ value of $7.25 \cdot 10^{-7}$ cm²/s for both 0.1 and 0.35 $\gamma/2$ buffer solutions.

Since the values of $s^\circ_{20,w}$ differed at the two ionic strengths and it appeared that this difference was attributable to a primary charge effect, further calculations requiring s and D parameters used the values obtained from solutions at 0.35 $\gamma/2$. The mol. wt calculated from the expression $M_r = RTs/D(1-\bar{v}\varrho)$ was 18 200, in excellent, although perhaps fortuitous, agreement with the value of 18 395 obtained from the amino acid sequence.

The frictional ratio f/f_0 reflects the distortion of the protein shape from that of a perfect sphere. The value of f/f_0 was obtained from the relation [22]:

$$f/f_0 = D^\circ_{max}/D^\circ$$

The f/f_0 obtained for the basic protein in acetate buffer at pH 4.8 and $\gamma/2 = 0.35$ was 1.64. Assuming the shape of the protein to be that of an ellipsoid of revolution, the f/f_0 value obtained corresponds to an axial ratio (a/b) of 11 to 1 [21]. This value is in agreement with axial ratios determined from viscosity data by Eylar and Thompson [10] and Chao and Einstein [9].

The radius of gyration, R_g, and the root mean square end to end distance can be calculated from the relations: $R_g = kT/(6\pi\eta\S D^\circ$ and $R_g^2 = L^2/6$ (random coil). Employing these relations yields a value of 4.28 nm for the radius of gyration and 10.5 nm for the root mean square end to end distance.

Sedimentation velocity and diffusion experiments with the basic protein in carbonate buffer pH 9.0, $\gamma/2 = 0.1$ were complicated by protein association at this pH. This association was not characterized further other than to note that the sedimentation velocity behavior at pH 9.0 reverted to that observed at pH 4.8 when pH 9.0 solutions of protein were adjusted back to pH 4.8.

Circular Dichroism Studies. Solutions of basic protein at pH 4.8, 9.0 and 11.0 exhibited circular dichroic spectra characteristic of random coil polypeptide molecules (Fig. 4). Solutions of basic protein in varying concentration of trifluoroethanol exhibited CD spectra indicative of conformational changes with increasing trifluoro-

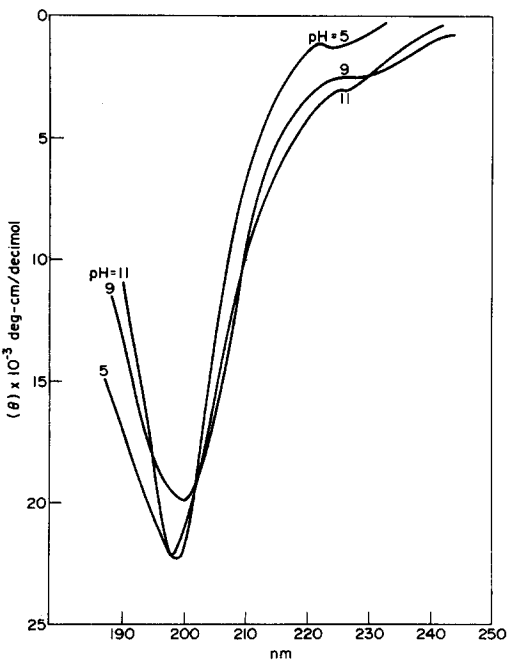

Fig. 4. CD spectra of basic protein solutions as a function of pH. Ionic strength is 0.1 for all buffers. Buffers were; acetate, pH 4.8 and carbonate, pH 9 and 11.

ethanol concentration (Fig. 5). From this family of curves, a plot of θ_{222} versus mol fraction of trifluoroethanol revealed a series of discrete conformational transitions rather than a simple linear change in conformation as a function of trifluoroethanol concentration (Fig. 6).

220 MHz PMR. spectra obtained from 10% protein solutions in ^2H_2O, pD_c = 4.8 exhibited sharp, well-defined resonance lines typical of a random coil protein (Fig. 7). These spectra were invariant over the temperature range of 4–67 °C. Further verification of the random conformation of the basic protein was provided by the

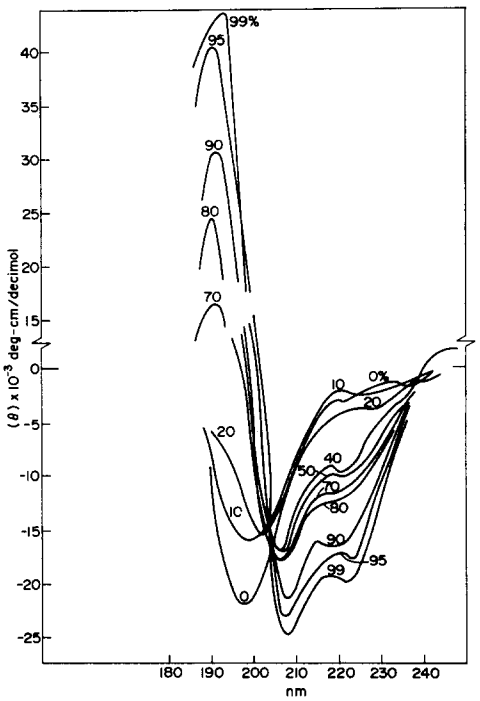

Fig. 5. CD spectra of basic protein as a function of trifluoroethanol concentration. The fraction of helical content was determined using the relationship proposed by Greenfield and Fasman [28].

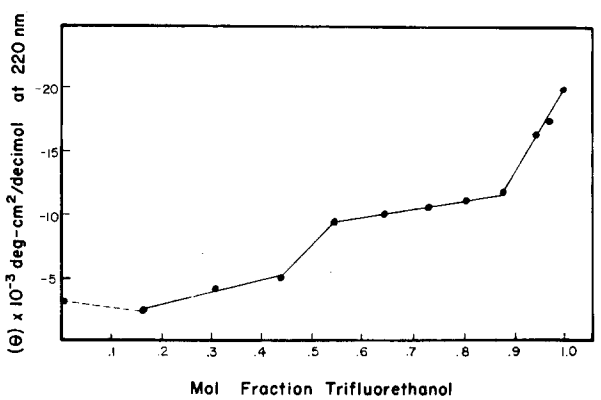

Fig. 6. Plot of θ_{222} as a function of the mol fraction of trifluoroethanol.

degree to which computed spectra agreed with experimental data. Figs 8 and 9 show a comparison of the high and low field regions of calculated and observed basic protein spectra at pH 7.0. The calculated spectra were obtained via the procedure of McDonald and Phillips [13], using the amino acid composition reported by Eylar et al. [5]. The region from 800 to 1000 Hz did not lend itself to assignment of proton resonances since this region contains the envelope of C - α proton and H_2O spinning side band resonances which cannot be evaluated in any predictable manner.

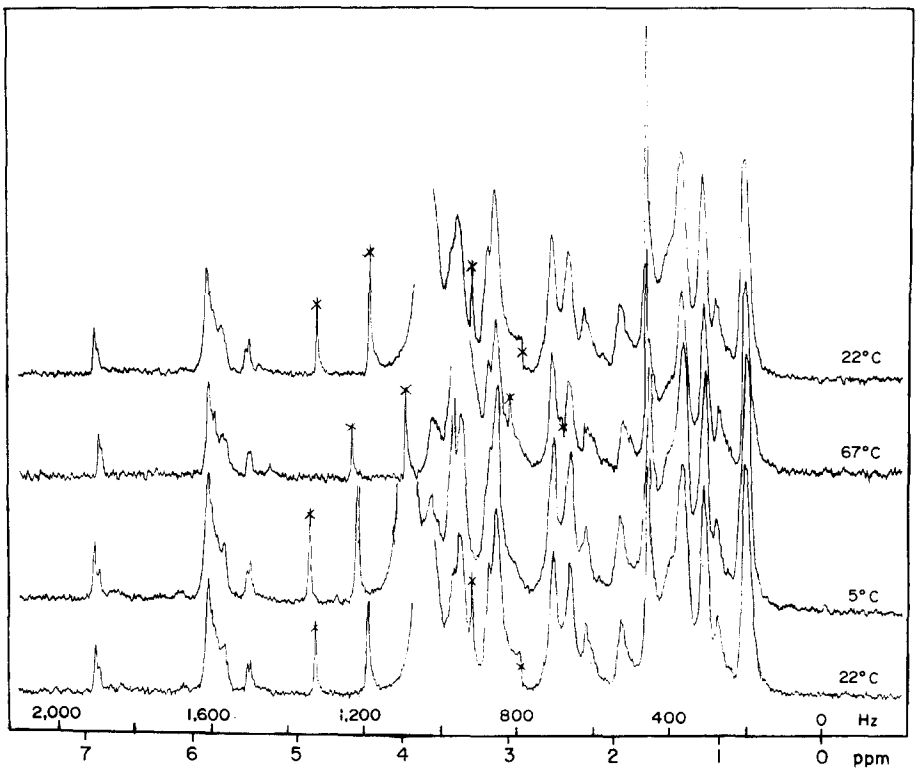

Fig. 7. 220 MHz PMR spectra of a 10% basic protein solution at a pD_c = 5.0 as a function of temperature. X'd peaks denote the spinning side bands of the water proton resonance.

The effect of pH on solutions of basic protein in ^2H_2O and DMSO-d_6 are shown in Figs 10 and 11. In Fig. 10, resonance shifts attributable to the loss of protons from the histidine residues are readily discernible at 1900→1700 Hz (his imidazole C_2H), 1570→1530 Hz (his imidazole C_4H) and 700→650 Hz (his-CH_2). Some broadening of the aliphatic proton resonance lines (400–200 Hz) is apparent as the pD is raised to 9.2. Similar changes in the histidine and aliphatic protons are seen in DMSO-d_6, at pD_c 10.9, and a more striking change in the aromatic proton resonance envelope is observed in the low field region of the spectrum (Fig. 11). This shift can be attributed to the shielding and subsequent upfield shift of the tyrosyl proton resonances.

PMR spectra in varying concentrations of trifluoroethanol are shown in Fig. 12. Increasing trifluoroethanol concentration results in a broadening of the protein resonance peaks to the extent that in 99% trifluoroethanol, all resonances have merged except for those attributable to the valine, leucine and isoleucine resonance envelope at 200 Hz. The amide nitrogen proton resonance at 1800 Hz also increases with increasing trifluoroethanol concentration. The broadening of most of the resonances and the growth of the amide nitrogen proton resonances is explicable in terms of the increase in helicity of the molecule in this solvent.

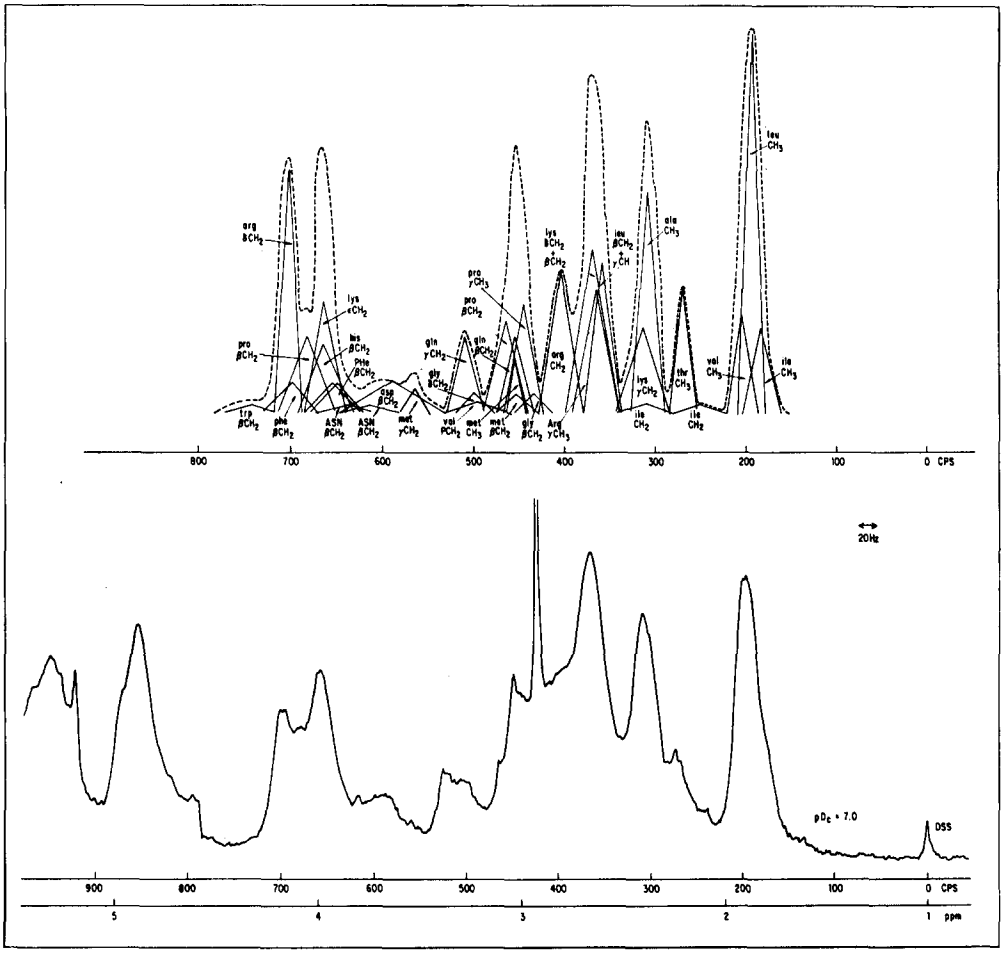

Fig. 8. Experimental and computed 220 MHz PMR spectra of basic protein in the high field region, pD_c = 7.0. The dashed lines correspond to the summation of specific amino acid proton resonances arrived at by means of a DuPont 310 curve resolver tuned for Lorenztian curves.

DISCUSSION

The results of this investigation have demonstrated that the correct $s^{\circ}_{20,w}$ value for bovine myelin basic protein in the pH region of 4.8 at $\gamma/2 = 0.35$, is 1.47 S. The previously reported values of 1.33 S at pH 4.6 $\gamma/2 = 0.15$, [9] 1.27 S pH 2.6, $\gamma/2 = 0.25$, [10] and 1.34 S, pH 2.6, $\gamma/2 = 0.4$ [11] are not valid but reflect perturbations by the primary charge effect [23].

Charge effects and pH play a major role in the solution behavior, conformation and stability of this unique protein. Thus, at mildly acidic conditions (pH 4–5) where the molecule has a net charge of 30, it behaves in the expected way in solution. However, reduction of the net charge below 14 leads to a diminished inter- and intra-

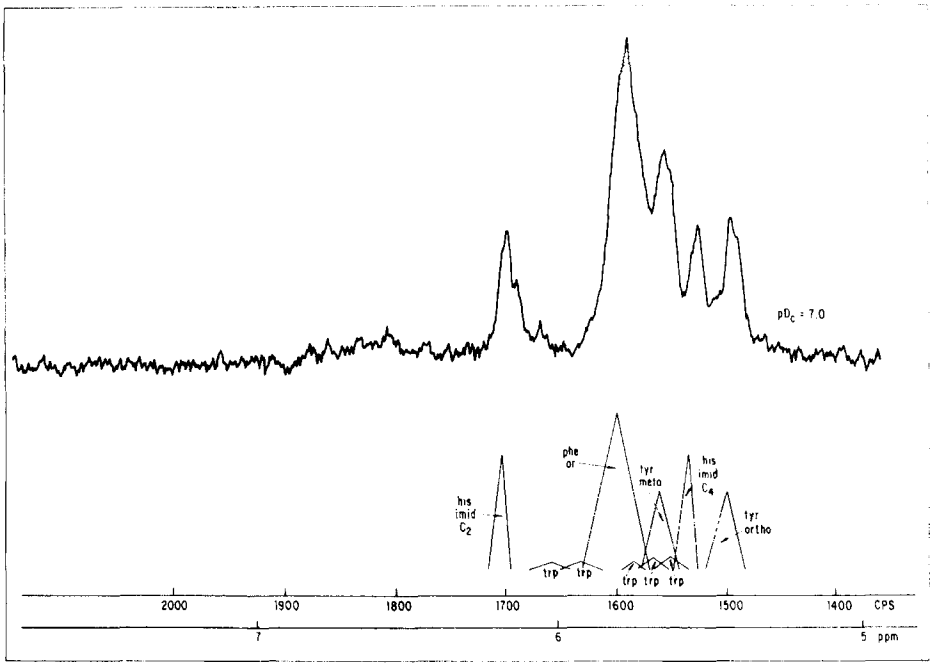

Fig. 9. Experimental and computed 220 MHz PMR spectra of basic protein in the low field region.

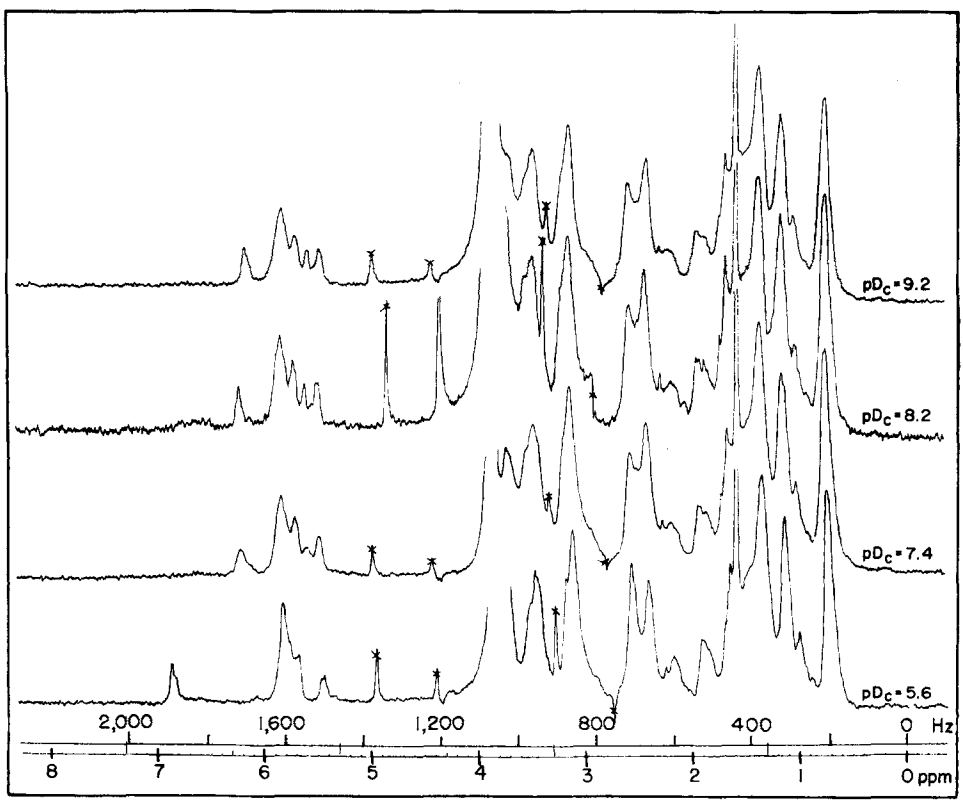

Fig. 10. 220 MHz PMR spectra as a function of pD_c. The concentration of basic protein was 10%

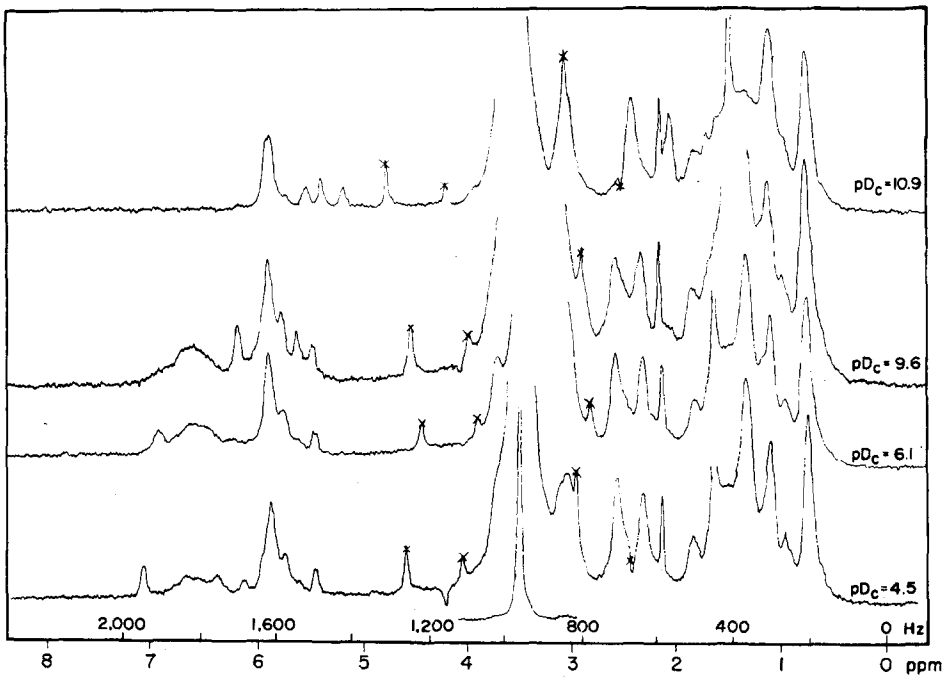

Fig. 11. 220 MHz PMR spectra of basic protein in DMSO-d_6 as a function of p*D*. The concentration of protein was 10% (w/v).

molecular repulsion and subsequent compaction of the structure accompanied by aggregation.

The aggregation of the protein at pH values greater than 7.0 is reflected in the somewhat broadened but still clearly resolved peaks in the PMR spectra. Proteins that contain any appreciable amounts of secondary or tertiary structure do not exhibit the kind of spectral resolution shown by the myelin basic protein even at very basic pH conditions [13]. The CD spectra at basic pH values show minimal variations in trough intensity and the wavelength of the minimum. These changes are within the experimental error of the measurement but may also contain a slight component of distortion arising from the aggregation.

Such charge effects are known to exert a major influence on the conformation of the basic protein, cytochrome *c* [24]. However, the myelin basic protein lacks the heme moiety, present in cytochrome *c*, so that the influence of pH and ionic strength on conformation is less readily discernible by spectroscopic techniques. Nevertheless, both proteins, because of their high isoelectric points exhibit many common solution behavior and conformational properties. Cytochrome *c*, upon binding cardiolipin, is known to undergo conformational changes [25]. Analogous conformational alterations have been observed when myelin basic protein binds lipids [26] or related amphipathic molecules [27]. We have also observed that heme is bound by the bovine myelin basic protein [27]. Both proteins are known to form aggregates at elevated pH. The role of such basic proteins in contributing to the structural integrity of the membranes from which they derive has not been ascertained. However, the current

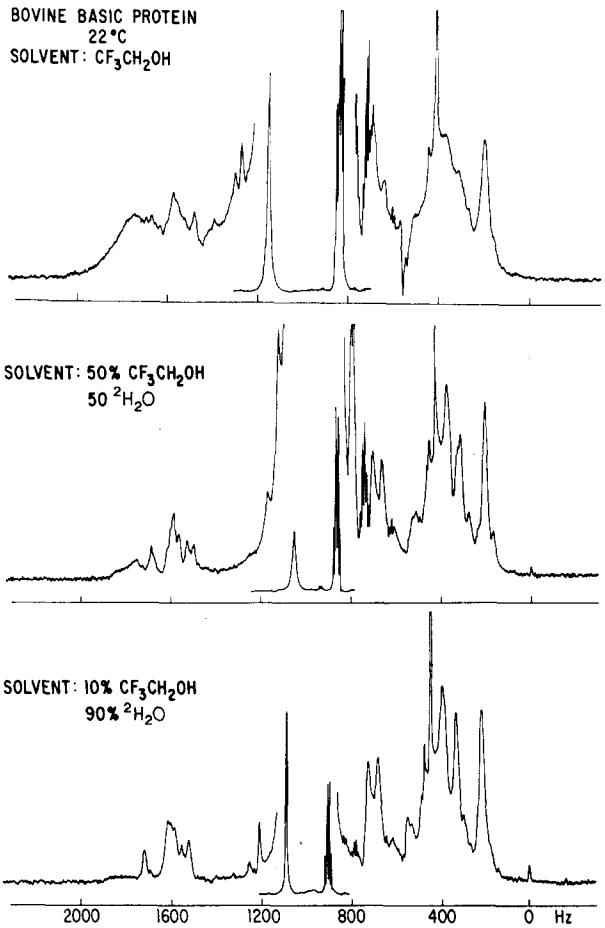

Fig. 12. 220 MHz PMR spectra of basic protein as a function of trifluoroethanol concentration. Note the growth of the N-H proton resonance at 1800 Hz and the broadening of the aliphatic proton resonances between 200 and 600 Hz with increasing concentrations of trifluoroethanol.

evidence does imply that when these proteins are in their membrane environments, their properties and conformations may be somewhat altered by the presence of and interactions with the surrounding lipids. The CD and PMR data are complementary and augmentative in clearly demonstrating changes in the myelin basic protein conformation under specified conditions and, in specific instances, elucidating those amino acid residues primarily involved in conformational alterations. These assignments are very useful in understanding why changes in pH, ionic strength and solvent composition give rise to altered behavioral and conformational states of the molecule.

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