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The Folded Conformation of the Encephalitogenic Protein of the Human Brain†

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ABSTRACT: The encephalitogenic protein from human myelin was shown to have a nonrandom structure in aqueous solution on the basis of its intrinsic viscosity and from the observed change in the far-ultraviolet circular dichroism between a solution of the protein in 6 M guanidine hydrochloride and in the absence of denaturing agents. This nonrandom structure was shown to be stable to large variations of pH on the basis of circular dichroism and viscosity, suggesting that this protein has a specific folded structure. A folded structure is also expected on the basis of a β-bend analysis. Viscosity, sedimentation velocity, low-angle X-ray scattering, and electron microscopy results are all consistent with a prolate ellipsoid model for the shape of this protein with approximate dimensions of 15 × 150 Å.

There are a small number of major protein components of the myelin membrane. One of these proteins, the encephalitogenic protein, accounts for approximately 30% of the protein content of myelin. An injection of as little as 1 μg of this protein in complete Freund's adjuvant into guinea pigs will induce experimental autoimmune encephalomyelitis, a demyelinating disease (Oshiro and Eylar, 1970). The encephalitogenic protein is thus of interest because of its potent biological activity and because of its importance as a major constituent maintaining the structure of the myelin membrane.

Until now it has been assumed by most workers that the encephalitogenic protein is largely devoid of an ordered structure (Eylar and Thompson, 1969; Choa and Einstein, 1970). This conclusion was based mainly on the high intrinsic viscosity of the protein and on its rotatory properties. This protein was found to be an excellent substrate for a neural acid proteinase (Einstein *et al.*, 1972). A theory of myelin breakdown was advanced (Einstein *et al.*, 1970, 1972) in which this protein in a highly open structure was readily hydrolyzed by neural acid proteinase.

The intrinsic viscosity and optical rotatory data (Eylar and Thompson, 1969; Choa and Einstein, 1970) are also consistent with a highly ordered protein having a specific tertiary structure which is asymmetrical and relatively devoid of α helical or β structures. It is the purpose of the present study to distinguish between these two alternatives, *i.e.*, between a largely random, highly solvated structure and an ordered, compact and asymmetric structure.

Methods

Isolation of the Acid-Soluble Encephalitogen. Myelin was prepared from normal human white matter and the acid-soluble encephalitogen was isolated (Lowden *et al.*, 1966). The protein was further purified by chromatography on a Celex-P column (Eylar *et al.*, 1969).

Concentration and Purity of Protein Solutions. Concentrations of protein solutions were determined by amino acid analysis with a Beckman 120C analyzer using an internal standard of norleucine.

Viscosity Measurements. Viscosities were measured with Cannon-Ubbelohde semimicro viscometers having flow times of 70 and 250 sec with solvent at 25.000 ± 0.005°. Protein solutions of concentrations of 0.2–1% were used.

Circular Dichroism Measurements. These measurements were performed with a Cary, Model 61, spectropolarimeter calibrated according to the values given by Cassim and Yang (1969). The temperature of the sample was controlled by means of a thermostatable cell holder attached to a circulating constant-temperature bath. A mean residue weight of 108.5 was calculated from the amino acid composition (Eylar, 1970; Carnegie, 1971).

Ultracentrifugal Studies. These studies were performed at 20° with a Spinco Model E ultracentrifuge using sapphire windows. A double-sector, capillary-type, synthetic boundary cell was used for the sedimentation velocity runs with the schlieren optical system and a speed of 60,000 rpm.

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TABLE 1: Intrinsic Viscosity and Sedimentation Constant.

Solvent	Intrinsic Viscosity (ml/g)	$s^0_{20,w}$ (S)
0.2 M acetic acid + 0.1 M NaCl	11.1 ± 0.3	1.60
0.01 M Tris-HCl buffer (pH 8.0) + 0.1 M NaCl	11.2 ± 0.3	
0.01 M Tris-HCl buffer (pH 8.0) + 6 M guanidine hydrochloride	23.2 ± 0.7	

Small-Angle X-Ray Scattering. The small-angle X-ray scattering measurements were performed with a Kratky camera. The incident irradiation was monochromatized by a Ni filter in connection with a proportional counter and a pulse-height analyzer. The scattered intensity of the solutions and the solvent was measured under identical conditions in a thin glass capillary; the excess scattering was obtained by subtraction.

Electron Microscopy. Samples were prepared according to the technique of Brenner and Horne (1959) except that freshly prepared, saturated, aqueous uranyl formate was used as the negative stain. Both control and sample grids were pretreated with 0.01% ovalbumin so as to enable the dilute protein solutions to wet the grid. Samples were placed on carbon-covered, 400 mesh, copper grids and stained with uranyl formate and the excess was drawn off with filter paper. An internal marker of ferritin (0.125 mg/ml) was added to the protein solution. As a control, grids treated in an identical fashion but without the encephalitogenic protein were used. The specimens were examined in a Phillips EM-300 operated at 60, 80, or 100 kV.

Results

Intrinsic Viscosity. The intrinsic viscosity and sedimentation constant are shown in Table I.

Circular Dichroism. The circular dichroism (from 250 to 210 nm) of this protein showed a dependency on solvent composition (Figure 1) and on temperature (Figure 2). The spectra shown in Figure 1 were taken at 25° at a protein concentration of 0.03% in 0.2 M acetic acid, 0.01 M sodium hydroxide, and 6 M guanidine hydrochloride in 10^{-2} M Tris buffer (pH 8.0). The spectrum in acid was very similar to that in alkali, and different from that in the denaturing solvent.

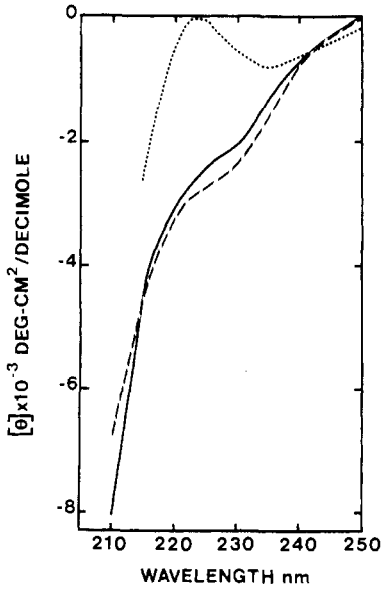

FIGURE 1: Circular dichroism of the human encephalitogenic protein of myelin at 25°, protein concentration 0.03%: (—) 0.2 M acetic acid, (— —) 0.1 M sodium hydroxide, and (----) 6 M guanidine hydrochloride–10^{-2} M Tris-HCl buffer (pH 8).

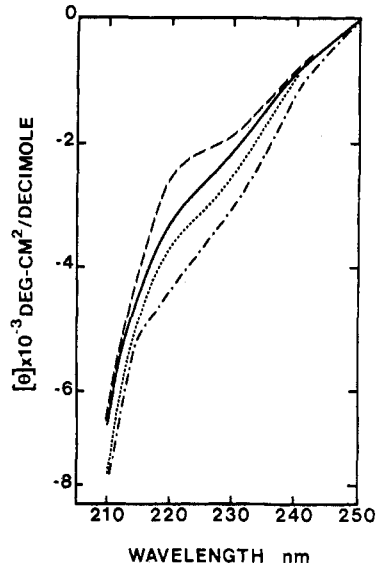

FIGURE 2: Temperature dependence of the circular dichroism of the human encephalitogenic protein of myelin in 0.2 M acetic acid–0.1 M sodium chloride, protein concentration 0.05%: (— —), 0°; (——), 25°; (-----), 45°; (— - — -), 80°.

The temperature dependence from 0 to 80° of the spectra is shown in Figure 2. As can be seen, the spectra were not drastically changed by a large change in temperature. The relatively small changes observed were completely reversible.

Multiple Sclerosis Myelin. Viscosity and circular dichroism measurements of the encephalitogenic protein obtained from the myelin of patients with multiple sclerosis demonstrated that the conformational property of this protein was the same as that from normal individuals.

Low-Angle X-Ray Scattering. Low-angle X-ray scattering measurements were performed on a 1 and a 2% solution of the encephalitogenic protein in 0.2 M acetic acid–0.1 M NaCl. The scattering intensity is related to the radius of gyration, R, by the formula

$$I = I_0 e^{-1/3 R^2 S^2}$$

where I is the intensity of the scattered radiation, I_0 the intensity of the incident radiation, and $S = (4\pi/\lambda) \sin \theta/2$, λ

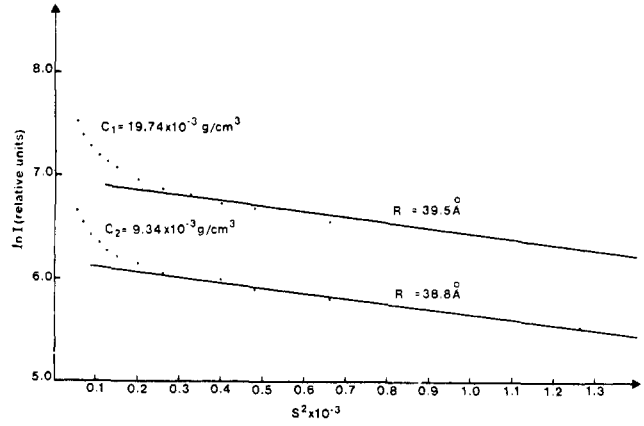

FIGURE 3: The X-ray scattering from solutions of the human encephalitogenic protein in 0.2 M acetic acid–0.1 M sodium chloride in the region of the Guinier approximation.

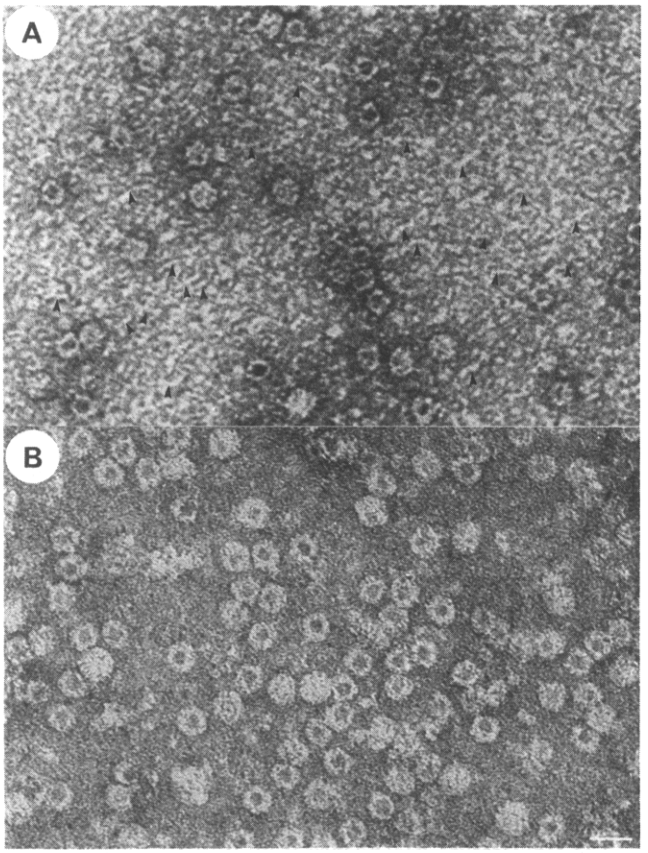

FIGURE 4: (A) Electron micrograph of a mixture of the encephalitogenic protein and ferritin negatively stained with uranyl formate. Total magnification 277,500 ×. Arrows show encephalitogenic protein in A. (B) Ferritin treated in an identical manner in the absence of the encephalitogenic protein. The bar in the lower right-hand corner of the picture measures 200 Å.

being the wavelength of radiation used (1.54 Å) and θ is the scattering angle. The radius of gyration can thus be obtained from the slope of a plot of ln I *vs.* S^2 (Figure 3). The deviations from a straight line at $S^2 < 0.2 \times 10^{-3}$ indicate the presence of a higher molecular weight component. This may be caused by the presence of some turbidity which was not removed because of the limited amount of sample available. From the linear portion of the plot a radius of gyration of 39 ± 2 Å was calculated.

Electron Microscopy. The protein may be visualized by electron microscopy. All electron micrographs of the encephalitogenic protein showed rod-shaped particles of approximately the same dimensions. Some of the rods appear slightly curved or twisted. These particles were absent in control grids not containing the encephalitogenic protein. The photograph shown in Figure 4 was taken by staining a grid in the presence (A) or absence (B) of 10^{-4} mg/ml of the encephalitogenic protein and containing 0.125 mg/ml of ferritin negatively stained with saturated uranyl formate. The dimensions of the protein were calculated to be 130 × 13 Å by comparison with the diameter of the ferritin molecule.

Discussion

Polypeptides and proteins with no disulfide bonds behave as random coils in 6 M guanidine hydrochloride. Under these conditions, the intrinsic viscosity of a wide variety of proteins can be described by the Mark–Houwink relationship $[\eta] = 0.716n^{0.66}$, where n is the number of monomer units per polypeptide chain (Tanford *et al.*, 1967). From this relationship

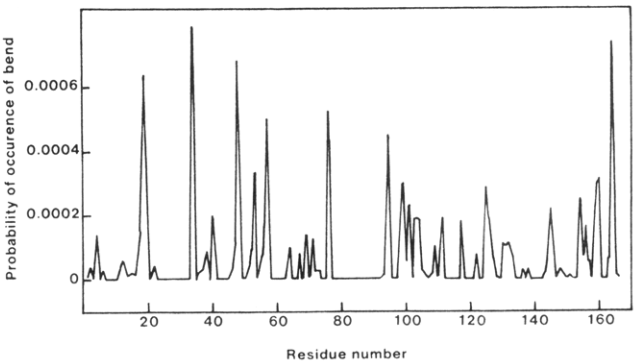

FIGURE 5: Folding probability of the encephalitogenic protein, calculated by the method of Lewis *et al.* (1971).

we can predict that a randomly coiled chain of 169 residues, the size of the encephalitogenic protein, would have an intrinsic viscosity of 21.2 ml/g in good agreement with our measured value of 23.2. The observed $[\eta]$ of this protein in the absence of denaturing agents, 11 ml/g, is significantly lower than the value for a random coil and indicates that the protein is somewhat folded in aqueous solution. This value lies between those reported by Eylar and Thompson (1969) and Choa and Einstein (1970), who reported values of 9.3 and 16.9 ml per g, respectively, for the intrinsic viscosity of the bovine encephalitogenic protein in aqueous solution. In addition, a difference between the randomly coiled conformation of the protein in 6 M guanidine hydrochloride and the conformation in the absence of denaturing agents is indicated by a change in the far-ultraviolet Cotton effects (Figure 1).

Recently Lewis *et al.* (1971) proposed a method for calculating the probability of occurrence of a bend in a protein based on the frequency that an amino acid residue in proteins of known crystal structure was found in a particular position of a tetrapeptide β bend. Applying this approximate analysis to the published amino acid sequence for the encephalitogenic protein (Carnegie, 1971) it is observed that several regions of the protein have high probabilities for having a bend (Figure 5). Taking an arbitrary cutoff point at 10^{-4} for the probability of a bend, Lewis *et al.* (1971) found 80% agreement with proteins of known structure. Analysis of the encephalitogenic protein indicates a large number of bends whose probability is greater than 10^{-4}. Thus, there is an *a priori* reason to expect this protein to have a highly ordered and folded conformation in aqueous solution.

Intrinsic viscosity is a sensitive indicator of molecular conformation. Our results demonstrate that the intrinsic viscosity of the protein does not change over a wide range of pH. The $[\eta]$ of a flexible molecule would be expected to depend on its state of ionization and solvation which would be pH dependent. The sedimentation coefficient of the bovine encephalitogenic protein was also found to be independent of pH (Eylar and Thompson, 1969). We find that the circular dichroism spectra of the human protein also indicate its conformational stability over a very wide range of pH (Figure 1). In addition, large temperature changes do not drastically affect the circular dichroism (Figure 2). The variation of the spectra with temperature is suggestive of an inverted transition with more unfolding occurring at lower temperatures. A similar effect of temperature of the circular dichroism of glucagon has also been observed over a wide range of temperatures (Epand, 1972).

The high intrinsic viscosity of the encephalitogenic protein compared to a value of approximately 3 ml/g for globular proteins is indicative of either an asymmetric structure or an

expanded, flexible chain. These two alternatives can be distinguished by comparing the radius of gyration calculated from hydrodynamic measurements with that obtained directly from low-angle X-ray scattering measurements.

The relationship between the intrinsic viscosity, $[\eta]$, and the radius of gyration, R, for a flexible chain is given by the equation $\eta = (10\pi N/3M)\xi^3 R^3$, where N is Avogadro's number, M is the molecular weight of the polymer, and ξ is a dimensionless quantity relating the radius of the equivalent hydrodynamic sphere to the radius of gyration. Using the experimental values of $[\eta]$ and R, we calculate $\xi = 0.815$. This is within the range of 0.775–0.86 found for other flexible polymers (Krigbaum and Carpenter, 1955) and demonstrates that these data are consistent with a flexible coil model.

For an unhydrated polymer $[\eta] = \nu\bar{v}_2$, where ν is Simha's factor (Simha, 1940) and $\bar{v}_2$ is the partial specific volume of the protein which we calculated to be 0.72 ml/g based on its amino acid composition by the method of Cohn and Edsall (1943). The value of Simha's constant corresponds to a prolate ellipsoid of axial ratio 10.7:1. An oblate ellipsoid model would lead to a somewhat larger axial ratio, but the calculated radius of gyration is lower than the experimentally determined value. The volume, v_h, of the protein molecule can be calculated from M and $\bar{v}_2$. From this the lengths of the major semiaxis, a, and the minor semiaxis, b, of the prolate ellipsoid model can be obtained from the relationship $v_h = (4/3)\pi ab^2$. For this model we find $a = 86$ Å and $b = 7.8$ Å. The radius of gyration of an ellipsoid of these dimensions is equal to $[(1/5)(a^2 + 2b^2)]^{1/2}$. The value calculated from this equation agrees exactly with the experimentally determined value of 39 Å. This analysis does not depend on assumptions concerning the extent of solvation of the polymer (Tanford, 1961).

A similar analysis can be made using the sedimentation velocity results combined with the X-ray scattering data. From the relationship $s^0 = M(1 - \bar{v}_2\rho_0)/6\pi\eta N\xi R$, where ρ_0 and η are the solvent density and viscosity, respectively, we obtain a value of $\xi = 0.72$. This is larger than the value of 0.665 predicted for a random chain by Kirkwood and Riseman (1948) but is within the range of values found experimentally (Mandelkern *et al.*, 1952).

For an asymmetric molecule we can calculate from the sedimentation coefficient the ratio of the observed frictional coefficient to the minimum possible frictional coefficient for an unhydrated sphere to be 1.62. This value corresponds to a prolate ellipsoid of axial ratio 12, in good agreement with the results from viscosity measurements. The combined results from hydrodynamic measurements and X-ray scattering measurements eliminate an oblate ellipsoid as a possible model for the shape of the encephalitogenic protein.

The viscosity and sedimentation velocity data may also be combined as shown by Scheraga and Mandelkern (1953) to give an equation in which the parameter β is indicative of molecular shape. From $[\eta]$ and $s^0_{20,w}$ determined in 0.2 M acetic acid–0.1 M sodium chloride, we calculate $\beta = 2.35 \times 10^6$. This corresponds to a prolate ellipsoid of axial ratio 8:1. The agreement of the axial ratio calculated by the Scheraga–Mandelkern equation and that calculated by the combination of hydrodynamic and X-ray scattering data give strong evidence that a prolate ellipsoid is a good model for the shape of the encephalitogenic protein.

Finally electron microscopy reveals a particle of dimensions and shape similar to that which we have calculated from various physical measurements. These results indicate that the molecule is folded in a specific compact and asymmetrical conformation. This finding was predicted on the basis of the β-bend analysis of Lewis *et al.* (1971) and explains the invariance of the intrinsic viscosity and circular dichroism with pH.

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