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Conformation Studies by Circular Dichroism and Fluorescence Spectroscopy of Myelin P2 Protein and Two of its Peptides

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Abstract: The conformation studies of myelin P2 protein and two of its major peptides were carried out using circular dichroism and fluorescence spectroscopy in water and in lipid environments. Significant conformational changes occur when the protein or peptides were bound to gangliosides. Similar effects were also found in trifluoroethanol solutions. The conformational features of P2 protein and its major peptides were discussed in relation to the environmental changes and the disease-inducing effects.
Key words: cicular dichroism, conformation, fluorescence, P2 protein, peptides.

P2 protein is a basic protein of low molecular weight (~15 kDa) localized in the peripheral nervous system myelin (Kitamura *et al.*, 1980; Ishaque *et al.*, 1982; Suzuki *et al.*, 1982). Interest in this protein has been stimulated by its function as an antigen in the induction of experimental allergic neuritis (EAN) (Brostoff *et al.*, 1977; Curtis *et al.*, 1979; Kadlubowski and Hughes, 1979; Hoffman *et al.*, 1980; Kadlubowski *et al.*, 1980; Rostami *et al.*, 1984). EAN is an experimental autoimmune demyelinating disease produced in test animals by the injection of the antigen with complete Freund's adjuvant (CFA), and is considered to be an appropriate model for a human demyelinating disease, the Guillain-Barre syndrome (Waksman and Adams, 1955).

The disease-inducing activity of P2 protein alone is much milder than that of whole myelin (Brostoff *et al.*, 1977; Curtis *et al.*, 1979; Kadlubowski and Hughes, 1979; Hoffman *et al.*, 1980; Kadlubowski *et al.*, 1980; Rostami *et al.*, 1984). Lipids such as phosphatidylserine (Ishaque *et al.*, 1981; Rostami *et al.*, 1982; Shin *et al.*, 1989), gangliosides (Nagai *et al.*, 1978) and galactocerebroside (Hughes and Powell, 1983) have been found to enhance the antigenic activity of the protein to a degree comparable with that of whole myelin. Myelin is known to be the strongest antigen for induction of EAN. It has been reported that CN1 peptide, comprising residues 21-113 of the complete 131 residue sequence of the P2 protein, when complexed with phosphatidylserine, also shows increased antigenic activity (Eylar *et al.*, 1980). Observations such as those have led to the speculation that a specific conformation, of importance for autoimmune disease induction, is produced when the protein interacts with lipids.

Consequently comparative conformation studies of the protein and two of its peptides, CN1 and BNPS1 (residues 9-97), bound to gangliosides, were carried out by circular dichroism (CD) and fluorescence spectroscopy. Gangliosides were chosen for this study as they are intrinsic constituents of myelin (Fong *et al.*, 1976) and give a visually transparent preparation, free of scattering artifacts. Comparative conformation studies were also carried out in trifluoroethanol (TFE), which is thought to approximate the microenvironment at the lipid membrane (Urry *et al.*, 1971).

Materials and Methods

Preparation of protein and peptides

P2 protein was prepared from bovine intradural spinal roots by the method of James and Moore (1980). Cleavage of P2 at the methionine residues with cyanogen bromide in 70% formic acid yields three peptides, designated CN1 (residues 21-113), CN2 (residues 114-131) and CN3 (residues 1-20), and the three peptides resulting were separated on G-50 and G-25 Sephadex columns as described by Weise *et al.* (Weise *et al.*, 1980). BNPS-skatole was used to cleave P2 protein at the COOH-tryptophanyl bond (Burnett and Eylar, 1971). After the reaction with thioglycollic acid, distilled water was added and the reaction mixture centrifuged at 20,000 rpm and 4℃ for 30 min. The lyophilized

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supernatant was dissolved in 0.2 M acetic acid and two peptides were isolated on a G-50 column. The peptides were labelled BNPS1 (residues 9-97), and BNPS2 (residues 98-131) in order of elution. Gangliosides (type II) purified from bovine brain were from Sigma (St. Louis, USA).

Circular dichroism measurement

Circular dichroism spectroscopy was performed as described previously (Shin and McFarlane, 1987). The mean residue ellipticities, in degree·cm^2 per dmol, were calculated from the relationship $[\Theta]=\theta_{obs}(MRW)/10lc$, where l is pathlength in cm, c is concentration in g/ml and qobs is the observed ellipticity. The mean residue weight (MRW) was calculated from the known primary sequence of the protein or peptide. No correction was made since the solutions are devoid of scattering.

The secondary structural components were analysed by the CONTIN procedure developed by Provencher and Glockner (1981). The analyses were of 51-point averaged data in the range 240~190 nm and the double precision procedure was used with weighting against data from below 205 nm.

Absorption and fluorescence measurements

Absorption was measured with a Philips PU8800 UV/Visible spectrophotometer. Fluorescence was measured at 20℃ with a SLM 8000 spectrofluorometer in the ratio mode by photo counting.

Fluorescence quenching

Fluorescence quenching measurements were carried out at 20℃ by adding aliquots of concentrated solution of acrylamide (3M) to the solutions of P2 protein. The excitation wavelength was 295 nm and the emission intensity was measured at 335 nm. The data were analyzed according to the Stern-Volmer equation (Lakowicz, 1983).

$$F_0/F=(1+K_{SV}\ [\Theta])e^{V[Q]} \quad (1)$$

where F_0 and F are the fluorescence intensities in the absence and presence of quencher, respectively. K_{SV} is the quenching constant and is denoted by $k_q\tau_o$ where k_q is the rate constant for quenching and to is the fluorescence lifetime of the fluorophore in the absence of quencher.

Results

Far UV-CD

The far-UV CD spectra of P2 protein, CN1 and BNPS1 peptides in water and in the presence of gangliosides (2 mg gangliosides per ml of water) are shown

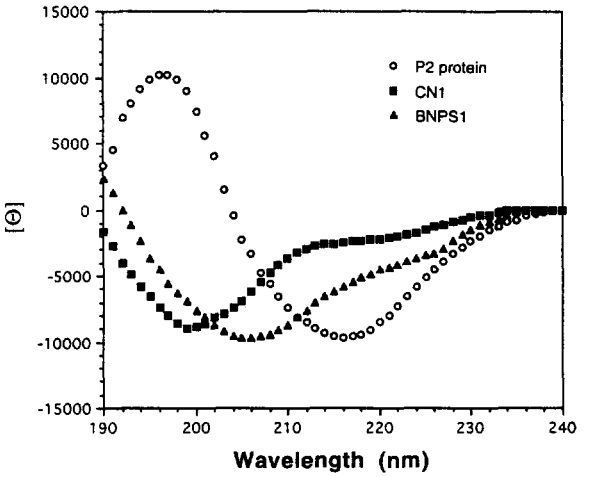

Fig. 1. Far-UV CD spectra of P2 protein, CN1 and BNPS1 peptides in water. All solutions were at pH 7.0. Protein concentrations were 0.2~0.3 mg/ml. Cell pathlength=0.1 cm, band width=1.0 nm, time constant=16 sec.

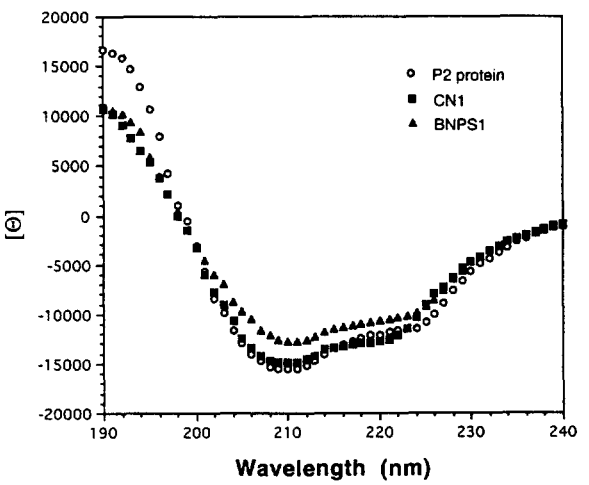

Fig. 2. Far-UV CD spectra of P2 protein, CN1 and BNPS1peptides bound to gangliosides. In all cases, lipid/protein or lipid/peptide ratio is 5/1 (w/w). All solutions were at pH 7.0.

in Figs. 1 and 2, respectively. The CD spectrum of P2 protein in water displays a typical circular dichroic pattern for a compact globular protein with high percentage of β-structure. CN1 peptide in water, on the other hand, with a strong negative peak centered at 200 nm and a shoulder in the 220 nm region, indicates the presence of a large amount of random structure. The small shoulder in the 220 nm region shows that there are still some residual secondary structures in CN1 peptide. BNPS1 peptide, comprising residues 9-97, displays a negative peak at 205 nm and a broad shoulder in the 220 nm region, indicating that it contains more α-helical structure than CN1 peptide (see Table 1). When P2 protein or the peptides bind with gangliosides, significant conformational changes were

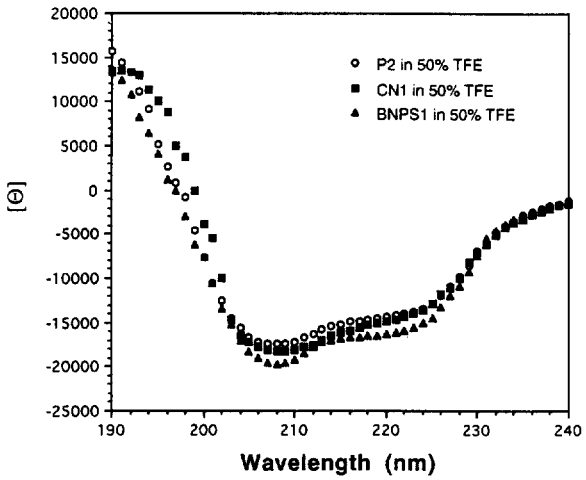

Fig. 3. Influence of TFE on the conformation of P2 protein, CN1 and BNPS1 peptides. The CD spectra were measured in 50% TFE solutions.

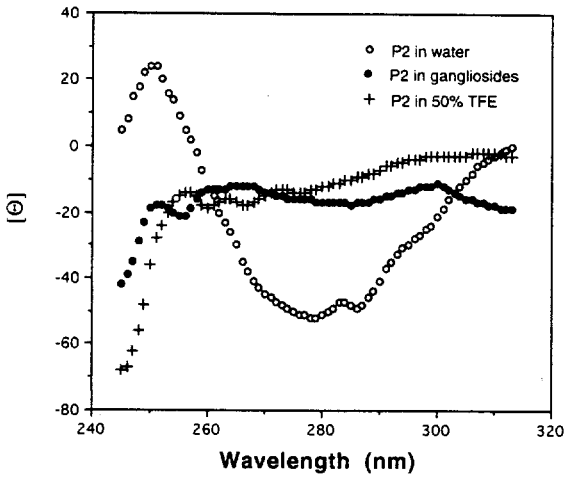

Fig. 4. Near-UV CD spectra of P2 protein in water (○), P2 bound to gangliosides (●) and P2 in 50% trifluoroethanol (+).

Table 1. The relative amount of each type of secondary structure of P2 protein, CN1 and BNPS1 peptides in the presence of gangliosides and in various concentrations of trifluoroethanol (TFE) solutions

Sample	%α-helix	%β-structure	%Remainder
P2 in water	20	55	25
P2+gangliosides	41	29	30
P2 in 10% TFE	24	48	29
30%	39	27	34
50%	49	22	29
70%	55	8	37
90%	83	0	17
CN1 in water	8	45	47
CN1+gangliosides	41	14	46
CN1 in 10% TFE	17	38	45
30%	34	32	33
50%	54	28	18
70%	61	16	23
90%	69	4	27
BNPS1 in water	22	40	39
BNPS1+gangliosides	37	16	46
BNPS1 in 10% TFE	25	41	34
30%	47	11	42
50%	52	6	42
70%	66	14	20
90%	81	0	19

The analyses were of 51-point averaged data in the range of 240 nm~190 nm and Provencher's CONTIN procedure was applied.

observed. The spectra of these complexes, with troughs at 209 nm and 222 nm, suggest the presence of a moderate amount of α-helix (Fig. 2 and Table 1). In all cases, the relative amount of α-helix was 39± 2%. However regarding β-structure and the remainder (β-turn and random), differences were found between the protein and the peptides. When the structures were titrated with trifluoroethanol (TFE), which is an effective structure-supporting solvent (Urry *et al.*, 1971), a substantial increase in the fraction of *a*-helix was observed (Fig. 3). As the concentration of TFE increases, the *a*-helicity also increases (Table 1).

Near-UV CD

The near-UV CD spectra often reveal differences in tertiary structure and in particular the microenvironments of aromatic residues. The near-UV CD spectra of P2 protein in three different environments are shown in Fig. 4. The spectrum of P2 protein in water is similar to that previously reported (Weise and Brostoff, 1982) with two minima at 282 nm and 288 nm and a maximum at around 250 nm. The positive ellipticity in the 250 nm region can be reasonably assigned to the disulphide group (Sears and Beychock, 1973), whereas the broad negative ellipticity in the 260~310 nm region is due to the contributions from phenylalanine, tyrosine and tryptophan. On binding the protein to gangliosides, a negative peak appears at 250 nm and the intensities of the two minima at 281 nm and 288 nm decrease considerably. Similar changes were found in 50% trifluoroethanol solution.

Fluoroscence spectroscopy

Emission spectra of P2 protein were measured at 20℃ under various solvent conditions (Fig. 5). 295 nm was selected for excitation wavelength in order to avoid the fluorescence of tyrosine. The emission spectrum in 50 mM phosphate buffer (pH 7.0) showed a maximum at 335 nm, in agreement with a previous

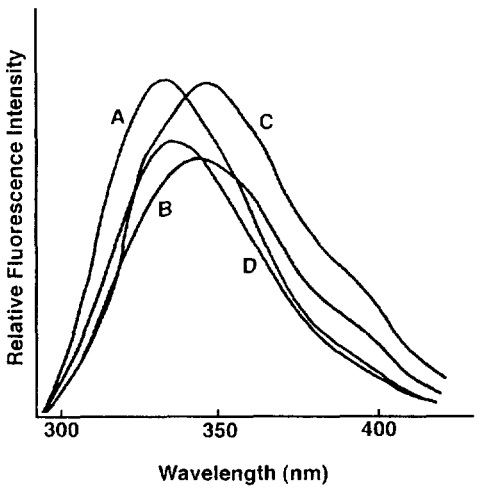

Fig. 5. Emission spectra of P2 protein. (A) P2 in water (pH 7.0); (B) P2 in water (pH 3.0); (C) P2 in 50% trifluoroethanol; (D) P2 bound to gangliosides. Excitation wavelength was 295 nm. In all cases, protein concentration was 0.15 mg/ml.

report (James and Moore, 1980). The addition of gangliosides up to a 7 : 1 weight ratio in 20 mM phosphate buffer (pH 7.0) did not change the emission spectrum. On the other hand, the maximum shifted to 345 nm in 50 mM glycine buffer (pH 3.0) and 350 nm in 50% trifluoroethanol solution, indicating that tryptophan is exposed to the solvent.

The protein contains two tryptophans at positions 7 and 96. Fluorescence quenching has been widely applied to obtain information concerning the exposure and microenvironment of tryptophanyl residue in proteins (Lakowicz, 1983; Efftink and Ghiron, 1981). Fluorescence quenching measurements were carried out at 20℃ using acrylamide as quencher. Fig. 6 shows the Stern-Volmer plot of data from quenching experiments with acrylamide, under the various solvent conditions described above. Identical volumes of the sample were placed in two fluorimetric cuvettes. A small volume of 3 M acrylamide solution was added to one cuvette and the same volume of water to the other, then the ratio of the fluorescence intensities was measured. The Stern-Volmer plot of data measured in 50 mM phosphate (pH 7.0) showed an upward curve, indicating that the solvent accessibilty of the two tryptophans is nearly identical. At pH 3.0, the solvent accessibilty of the two tryptophans increased considerably, indicating that P2 protein is partially unfolded. When analyzed according to equation 1, the data for P2 protein in water yield $V=0.5\ M^{-1}$ and $K_{sv}=3.6\ M^{-1}$ at pH 7.0 and $V=1.0\ M^{-1}$ and $K_{sv}=7.3\ M^{-1}$ at pH 3.0. However in the presence of gangliosides, the Stern-Volmer plot was linear, suggesting that the relative accessibility of the acrylamide quencher to the two tryptophans is slightly different. The quenching constant K_{sv}(eff)

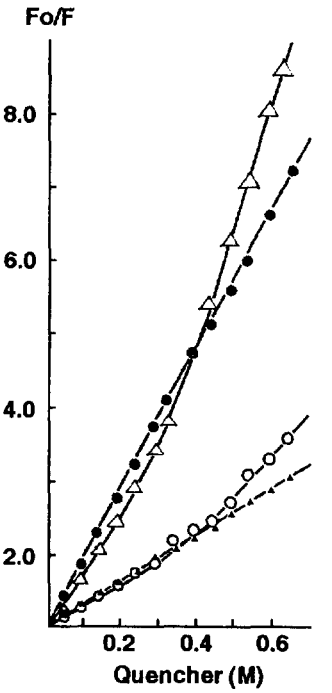

Fig. 6. Stern-Volmer plot of the data from quenching experiments on P2 protein at pH 7.0 (○), P2 at pH 3.0 (△), P2 in 50% trifluoroethanol (●), and P2 bound to gangliosides (▲) by acrylamide.

for the P2-ganglioside mixture was calculated to be 2.8 M^{-1}. The quenching constant K_{sv}(eff) of P2 protein in 50% TFE increased to 8.8 M^{-1}, indicating that the two tryptophans are more exposed in 50% TFE compared to the P2 protein in water at pH 7.0.

Discussion

From inspection of the CD spectra of P2 protein it is clear that it adopts a conformation different to that in water when it is bound to gangliosides (Figs. 1 and 2). The major change is that about 20% of the β-structure undergoes a transition to α-helix on binding. Application of the Chou-Fasman secondary structure prediction method (Chou and Fasman, 1978) indicates that as much as 70% of the protein has a certain propensity to form α-helix (Martenson, 1983; Shin and McFarlane, 1987). Thus one is tempted to speculate that, although β-structure dominates in aqueous solution, those regions with helix-forming propensity tend to be stabilized in the a-helical conformation when the protein is bound to the gangliosides.

The protein contains 18 Lys and 7 Arg residues out of a total of 131, and consequently it is highly basic (pI=9.8). Therefore it is very likely that the positively charged side chains of basic amino acid residues in the protein bind to the negatively charged sialic acid residues of gangliosides by electrostatic interactions.

And this type of interaction probably caused transition from the β-structure to α-helix in the protein structure. But other types of interactions such as hydrogen bonding and hydrophobic interactions cannot be totally excluded.

CN1 and BNPS1 in aqueous solution show less structured features in their CD spectra compared to that of P2 protein, CN1 with a more denatured spectrum than BNPS1 (Fig. 1). About 20 residues (22%) of the BNPS1 sequence are calculated to be in α-helical segments, compared to 7 residues (8%) of CN1 (Table 1). BNPS1 contains 12 more residues in the more hydrophobic N-terminal part and 16 less residues in the more hydrophilic C-terminal part, compared to CN1. So the hydrophobic N-terminal part of the protein is considered to be important in maintaining the α-helical structure. It is interesting to see that these two peptides also show a gain of up to 41% α-helix when they are bound to gangliosides. CN1 and BNPS1 also contain a high percentage of basic amino acid residues, giving high isoelectric points, 10.9 and 10.5, respectively. So a similar explanation can be applied to these peptides when bound to gangliosides, as in the case of P2-ganglioside mixture.

All current CD analyses of protein conformation are empirical and there is no guarantee that the estimated conformation of an unknown protein will be always correct. At present the estimate of α-helix can be viewed with some confidence, but the estimates of the β-sheets and β-turns still seem to be uncertain. This is true when we look at the CD spectrum of CN1 in water. The peptide displays a random type circular dichroic pattern with a negative peak centered at 200 nm even though there is a small shoulder in the 220 nm region. But when analysed using Provencher's CONTIN program (Provencher and Glockner, 1981), the peptide turned out to have 45% β-structure. Therefore it is more rational to consider that the amount of β-structure is overestimated in the case of CN1 peptide.

The CD spectra in the near-ultraviolet region for the protein in three different environments showed that the microenvironments of the aromatic side-chains and disulphide bond change considerably when the protein is in 50% TFE or bound to gangliosides. The reduction of ellipticities in the range of 260~310 nm shows that the mobility of the aromatic side chains increases in the 50% TFE or in the ganglioside environment. Previously Weise and Brostoff (1982) assigned 283 and 289 nm peaks, which appear at 282 and 288 nm in our case, to be the two tyrosine residues in the two different environments. If this is correct, the two tyrosines lose their ellipticities in 50% TFE or in a ganglioside environment due to the increased mobility, that is, they are in a position highly exposed to the solvent. This means that the regions occupied by the two tyrosines are not involved in the binding regions with gangliosides.

Fluorescence quenching measurements showed that the two tryptophanyl residues of P2 protein adopt different environments when the protein is bound to gangliosides. One tryptophan displays similar accessibility to quencher but the other tryptophan shows less accessibility to quencher compared to those in aqueous solution. Two possible explanations arise; 1) the buried tryptophan is involved in the binding site of P2-ganglioside mixture. 2) the binding between P2 and gangliosides causes the conformational change of the protein so the tryptophan becomes more buried. At present it is not possible to assign the tryptophans due to the limited data available. Clearly, more data regarding the conformation of P2 protein are needed to fully understand the behavior of the two tryptophans in different environments.

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