Spectroscopic Analysis of Acylated and Deacylated Myelin Proteolipid Protein†

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ABSTRACT: The effect of covalently bound fatty acid on the conformation of the myelin proteolipid protein has been studied by ultraviolet and intrinsic fluorescence spectroscopy. With dimethyl sulfoxide used as a perturbant, the exposure of Trp and Tyr residues in various mixtures of chloroform–methanol was evaluated by difference spectroscopy of the proteolipid protein (APL) and its chemically deacylated form (d-APL). The fraction of chromophoric groups exposed increased with the proportion of chloroform with 25% of the groups exposed in 1:2 chloroform–methanol and 98% in 3:1 chloroform–methanol. These conformational changes correlate well with changes in intrinsic viscosity. Values for the deacylated form were indistinguishable from those of the acylated protein, suggesting that fatty acids do not affect protein conformation in organic solvents. In water, UV difference spectroscopy indicated that the number of Tyr and Trp groups exposed in both APL and d-APL was relatively small and was independent of the molecular size of the perturbant. However, differences in the environment of the Trp groups in the two forms of the protein could be demonstrated by intrinsic fluorescence. When the protein was excited at 295 nm, the maximum emission wavelength for the acylated protein was 330 nm, whereas it was 335 nm for the deacylated form. Furthermore, the Trp groups in d-APL were more easily quenched by acrylamide than in APL, indicating that they were more exposed, or in a more hydrophilic environment, following deacylation. Protein aggregation appears to be independent of the presence of fatty acids, suggesting that the fluorescence differences between APL and d-APL are related to factors other than aggregation. The presence of fatty acid may alter the folding of the protein in water with an accompanying change in the exposure of Trp groups. Finally, incorporation of either APL or d-APL into lipid vesicles produced an increase in fluorescence intensity with no change in emission maximum, indicating strong hydrophobic interactions between the protein and lipids.

Many hydrophobic membrane proteins have been shown to contain covalently bound long-chain fatty acids linked to specific amino acids (Magee & Schlesinger, 1982; Schmidt, 1983). Several hypotheses have been proposed for the function of the covalently bound fatty acid, including a role in intracellular transport of membrane proteins, membrane fusion, and stabilization of proteins in the membrane. In the vesicular stomatitis virus G glycoprotein, the human transferrin receptor, and the human histocompatibility antigen, the fatty acid is bound in a domain of the protein that interacts strongly with the lipid bilayer. In the gastric mucus glycoprotein, a secreted acylated protein, the fatty acid has been shown to contribute to its high intrinsic viscosity values, and an abnormally acylated form of the protein has been identified in cystic fibrosis (Murty et al., 1984). Thus, the acyl group may be associated with factors that are of physiological significance.

The bovine white matter proteolipid apoprotein (APL) was the first protein in which the occurrence of covalently bound fatty acid was recognized (Braun and Radin, 1969; Gagnon et al., 1971; Stoffyn & Folch, 1971). This hydrophobic protein is the major protein component of central nervous system myelin. The apoprotein, free of non covalently bound lipids, is soluble in organic solvents but can be transferred to a water-soluble form with an accompanying conformational change. The conformational characteristics of APL in both organic and aqueous solvents have been explored by spectroscopic techniques, viscosimetry, and analytical centrifugation, and these approaches have provided information on the secondary and tertiary structure of the protein and some insights into the forces that stabilize the two conformations [for a review see Lees and Brostoff (1984)]. However, no attention has been given to the effect of the covalently bound lipid on the protein. The fatty acids may be responsible, in part, for the unique solubility characteristics of the protein and may contribute to the stabilization of the protein in a specific conformation.

We have recently developed a mild procedure for the deacylation of the proteolipid apoprotein in which the protein retains its solubility properties (Bizzozero et al., 1985a). The deacylated form (d-APL) is suitable for physicochemical characterization and for comparison with the native (acylated) form of the protein. In the present study UV difference spectroscopy and intrinsic fluorescence studies, accompanied by acrylamide quenching experiments, have been used to determine the contribution of covalently bound fatty acids to the conformation of APL in aqueous and organic solvent mixtures and in lipid vesicles.

Materials and Methods

Materials. *N*-Acetyl ethyl esters of tyrosine (Ac-Tyr-OEt), tryptophan (Ac-Trp-OEt), and phenylalanine (Ac-Phe-OEt) and poly(ethylene glycol) (Carbowax 300) were from Sigma Chemical Co., St. Louis, MO. Acrylamide (electrophoresis grade, Bio-Rad Laboratories) was recrystallized from chloroform before use. All other solvents and reagents were spectroscopic quality of the best commercially available grade.

Apoprotein Preparation. Bovine white matter was extracted with 19 volumes of chloroform–methanol (2:1 v/v) and the extract washed three times, as described by Folch and Lees (1951). The washed extract was concentrated under vacuum,

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acidified with 0.01 N HCl, and passed through a Sephadex LH-60 column (Pharmacia Fine Chemicals) (2 × 90 cm) equilibrated and eluted with chloroform–methanol–acetic acid (2:1:0.03 v/v/v). The protein, free of adventitious lipids (<0.02% w/w, lipid phosphorus) appears in the void volume. By gas chromatography, it was shown that APL contains 2.2% (w/w) of the following covalently bound fatty acids: palmitic (55%), oleic (26%), and stearic (19%) (Bizzozero et al., 1985a). The apoprotein was deacylated by hydroxylaminolysis in an alkaline organic medium as described by Bizzozero et al. (1985a). The deacylated apoprotein (d-APL), free of covalently bound fatty acids (<0.2% w/w), appears in the void volume. Total recovery was greater than 70%. Electrophoretic analysis on SDS gels showed that the deacylated, as well as the acylated, protein consists of a major band (PLP, 80%), a minor band (DM-20, 15%), and small amounts (5%) of lower and higher molecular weight bands (Bizzozero et al., 1985b). The proteins in the various bands are chemically and immunologically related. (Nicot et al., 1973; Agrawal & Hartman, 1980.)

To prepare samples for analysis, APL or d-APL was dissolved in chloroform–methanol–acetic acid (2:1:0.03 v/v/v), concentrated under vacuum, and precipitated by the addition of 5 volumes of cold acetone. After centrifugation at 4000*g* for 20 min, the pellet was dried under nitrogen and dissolved in 2-chloroethanol (2-CE) or in chloroform–methanol mixtures. To prepare the water-soluble form, the apoproteins were dissolved in 2-CE and dialyzed against distilled water for several days. Liposomes were formed with egg yolk L-α-phosphatidylcholine and cholesterol (4:1 w/w). Solutions of lipids and proteins in 2-CE were mixed (10:1 by weight) and dialyzed against 10 mM NaCl and 1 mM EDTA at pH 7.4 and at 4 °C (Cockle et al., 1978). The final protein concentration was 0.06 mg/mL. High-speed centrifugation of the liposomes indicated that >94% of the protein had been incorporated.

Ultraviolet Absorption. Spectra were recorded on a Cary 14 spectrophotometer at 25 °C, using 1-cm path length cells. Protein samples were centrifuged at 20000*g* for 20 min, and a correction for residual light-scattering was applied by using a linear plot of log (optical density) vs. log (wavelength). Protein concentration was determined by the procedure of Lowry et al. (1951), as modified for proteolipid by Lees and Paxman (1972), using bovine serum albumin as standard.

Difference Spectroscopy. Difference spectral measurements were carried out with a Cary Model 14 recording spectrophotometer equipped with a scale multiplier which permits expansion of the optical density scale by a factor of 10. Pairs of matched tandem double cells (0.4375 cm light path, Spectrocell, Inc.) were employed to subtract directly the solvent contribution to the difference spectrum. All measurements were performed at 25 °C, and the difference spectra were recorded between 250 and 320 nm by using a spectral bandwidth of 0.5 nm and a scanning speed of 0.5 nm/s. Measurements were made at least in triplicate and at three different optical densities. Preparation of solutions and addition of perturbants were carried out as described by Herskovitz and Laskowski (1962). The concentrations of the model compounds were determined spectrophotometrically by using the following molar extinction coefficients: 195 for Ac-Phe-OEt at 258.5 nm, 1340 for Ac-Tyr-OEt at 274.5 nm, and 5550 for Ac-Trp-OEt at 282 nm (Wetlaufer, 1962). Tyrosine and tryptophan exposure was estimated as described by Herskovitz (1967). Briefly, the solvent perturbation difference spectra of proteins were resolved into their constituent tyrosyl and tryptophanyl components by using appropriate tyrosyl and tryptophanyl model compound data and

$$\Delta E_{291-293}(\text{protein}) = a(\Delta E_{291-293}(\text{Trp})) + b(\Delta E_{291-293}(\text{Tyr}))$$

$$\Delta E_{286-288}(\text{protein}) = a(\Delta E_{286-288}(\text{Trp})) + b(\Delta E_{286-288}(\text{Tyr}))$$

where the coefficients a and b represent the apparent number of exposed tryptophanyl and tyrosyl residues in the protein and ΔE values refer to the molar absorptivity differences of the protein and the tryptophan and tyrosine model compounds at the 291–293- and the 286–288-nm difference spectral maxima. Values for APL or d-APL were calculated by using a molecular weight of 30000, and the fraction of Tyr or Trp exposed was calculated on the basis of 15 Tyr and 4 Trp groups per molecule of APL (Lees et al., 1983).

Intrinsic Fluorescence Measurements. Fluorescence measurements were carried out at 20 °C in an Aminco-Bowman spectrophotofluorimeter using a 4-mm bandwidth. The instrument was routinely operated in the ratio mode, and emission spectra were recorded between 450 and 250 nm. Typical protein concentrations ranged between 0.05 and 0.11 mg/mL. Corrections for inner filter effects were carried out as described by Cockle et al. (1978). Integrated fluorescence was calculated by measuring the area under the curve by weighing, and the values were expressed as relative quantum yields (Φ) with respect to the standard Ac-Trp-OEt. Spectra were recorded at least three times at two different protein concentrations. Trp fluorescence was determined by excitation of the protein at 295 mm. In some cases, tyrosine emission was also calculated by subtracting the emission spectrum at 295 nm from that at 280 nm, after appropriate normalization relative to the values of the two spectra at 360 nm, assuming that the spectral distribution of Trp fluorescence is independent of the excitation wavelength. Fluorescence quenching experiments were carried out by addition of small aliquots of an 8 M acrylamide solution to the sample.

Viscosity. Viscosity measurements were carried out in a calibrated kinematic viscosimeter, Canon-Fenske type (25 mL total volume). Constant temperature was obtained with a HETO thermostat (Denmark), maintained at 25.0 ± 0.1 °C. Solvent and protein solutions were filtered through a sintered glass filter prior to each experiment. Each flow time value represents the average of five measurements. Maximal deviation was ±0.5 s. Intrinsic viscosity was determined by extrapolation to zero protein concentration from the plots of reduced viscosities vs. protein concentration.

Results

Ultraviolet Absorption Spectra of APL and d-APL. The UV absorption spectrum of d-APL in chloroform–methanol (2:1 v/v) was identical with that of APL in the same solvent mixture (Figure 1). The shoulder at 290 nm is due to the Trp residues whereas the maximum at 279 nm and the shoulder at 283 nm correspond to the contribution of Tyr in an apolar environment. The minor peaks at 253, 260, 265, and 269 nm are visible only in organic solvents and represent the vibronic absorption band of Phe. The $E^{1\%}$ at 279 nm was 16.2 ± 0.3, which agrees with that calculated from the molar absorptivities of the model compound and the aromatic amino acid content (20 Phe, 15 Tyr, and 4 Trp) derived from the primary structure (Lees et al., 1983). For both APL and d-APL the $E^{1\%}$ at 279 nm was the same in 2-CE, water, or other mixtures of chloroform–methanol as in 2:1 chloroform–methanol. The absorption spectra in organic solvents were close to those of the model compound, indicating that the

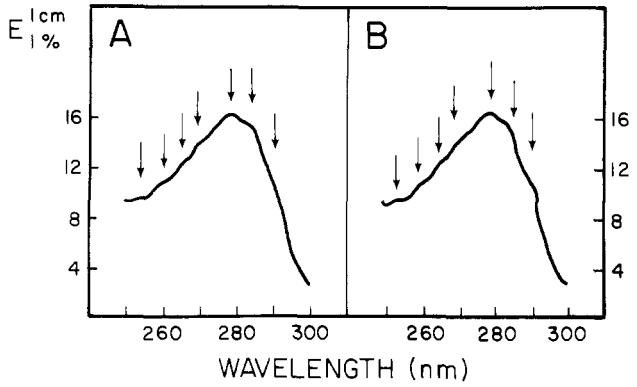

FIGURE 1: UV absorption spectra of APL (A) and d-APL (B) in chloroform–methanol (2:1 v/v). Arrows indicate absorption peaks contributed by aromatic amino acids, as discussed in the text.

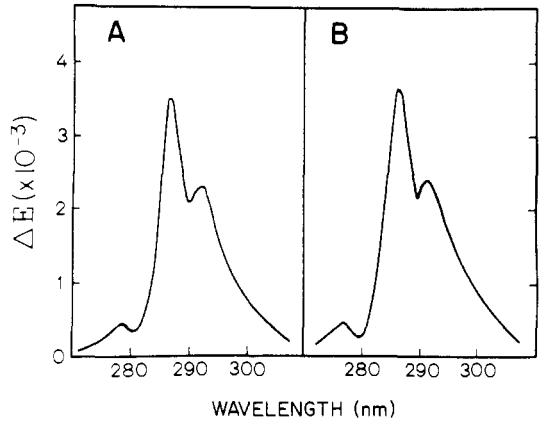

FIGURE 2: Dimethyl sulfoxide (20%) perturbation difference spectra of APL (A) and d-APL (B) in chloroform–methanol (2:1 v/v).

chromophoric groups were probably located at the protein surface in contact with the solvent. However, in water, spectra of APL and d-APL were red shifted with respect to the model compound, suggesting that in an aqueous medium the chromophoric residues were buried in internal hydrophobic regions of the protein.

Perturbation Studies in Organic Solvents. The degree of exposure of the chromophoric groups was studied by the perturbation technique of UV difference spectroscopy (Herskovitz & Laskowski, 1962). Polyethylene glycol, ethylene glycol, dioxane, glycerol, and Me_2SO (20% v/v) were tested as perturbants in organic solvents. Only Me_2SO gave well-resolved spectra, reproducible ΔE values, and relatively large red spectral shifts. The UV difference spectra produced by the addition of 20% Me_2SO to a chloroform–methanol (2:1 v/v) solution of the protein showed well-resolved maxima at 287 and 293 nm, which were the same for APL and d-APL (Figure 2). In both forms of the protein, 62% of the Tyr (9.2 residues) and 73% of the Trp (2.9 residues) were exposed to the solvent. The addition of SDS (1% w/v) or acetic acid (1% v/v) to the chloroform–methanol (2:1 v/v) led to exposure of the remaining chromophoric groups in both APL and d-APL, and they reverted to their original conformation after removal of the additives by chromatography on Sephadex LH-60. Exposed aromatic groups increased with increasing chloroform concentration from 25% of the Trp (1.0 residue) and 23% of the Tyr (3.4 residues) in chloroform–methanol (1:2 v/v) to complete (98%) exposure of both Trp and Tyr in chloroform–methanol (3:1 v/v) (Figure 3). In the helix-promoting solvent 2-CE, the aromatic groups were largely exposed. However, neither the Tyr nor Trp groups were fully exposed, suggesting residual tertiary structure. The presence of well-resolved tyrosyl and tryptophanyl peaks, the absence of spectral distortions, and the close fit of the protein and the model data in all solvents studied suggest that the effect of Me_2SO on the protein structure per se was minimal. The $\Delta E/E$ values were linear with Me_2SO concentration up to 30% (v/v), indicating that the number of groups exposed is independent of Me_2SO concentration (data not shown). Furthermore, the intrinsic viscosity of both forms was the same and did not change upon addition of Me_2SO.

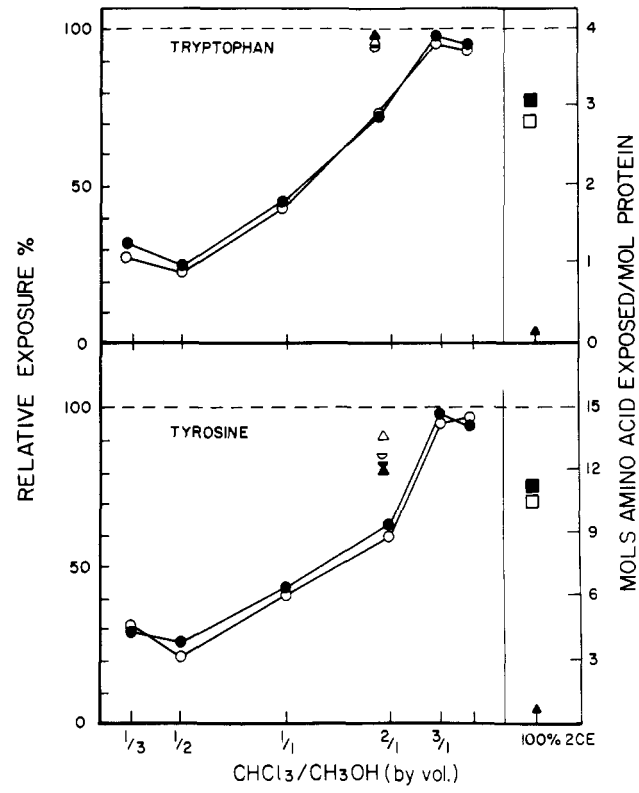

FIGURE 3: Fraction of Trp and Tyr groups exposed in APL and d-APL in organic solvents by UV difference spectroscopy. APL, d-APL, or model compounds were dissolved in different chloroform-methanol mixtures or in 2-CE and the ΔE values produced by the addition of 20% Me_2SO determined as described under Methods and used to calculate the relative exposure. Protein concentration ranged between 1 and 2 mg/mL. Closed symbols, APL; open symbols, d-APL; (●, ○), chloroform–methanol mixtures; (▲, △) chloroform–methanol (2:1 v/v) + 1% SDS; (▼, ▽) chloroform–methanol (2:1) + 1% acetic acid; (■, □) 2-chloroethanol.

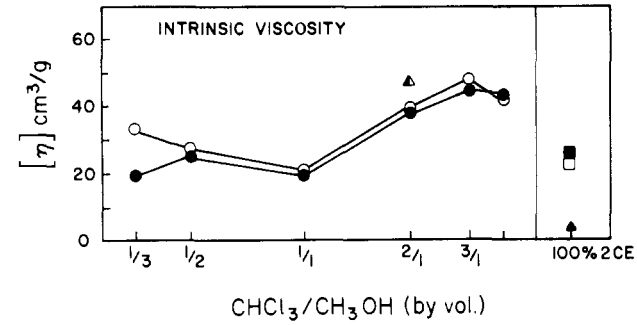

FIGURE 4: Intrinsic viscosity of APL in organic solvents in the presence (open symbols) or absence (closed symbols) of 20% Me_2SO: (●, ○) chloroform–methanol mixtures; (▲, △) chloroform-methanol (2:1 v/v) + 1% SDS; (■, □) 2-chloroethanol.

In contrast to the absence of effects of Me_2SO, intrinsic viscosity increases as a function of the proportion of chloroform in the solvent mixture (Figure 4), and correlates well with the increase in the number of exposed groups (cf. Figures 3 and 4). At the concentration of protein used for the determination

Table I: Intrinsic Fluorescence Characteristics of APL and d-APL[a]

	N-Ac-Trp-OEt		APL			d-APL		
solvent	λ_{max} (nm)	$\Delta\lambda$ (nm)	λ_{max} (nm)	$\Delta\lambda$ (nm)	Φ(Trp(295))	λ_{max} (nm)	$\Delta\lambda$ (nm)	Φ(Trp(295))
2-CE	343	59	339	62	0.94	338	62	0.96
water	350	59	330	57	0.33	335	59	0.27
vesicles			330	57	0.42	329	56	0.43

[a] Values represent the mean of three to four experiments. The reproducibility of λ_{max} (at 295 nm) and the width of the half-height ($\Delta\lambda$) was ±1 nm. The tryptophan quantum yields (Φ) were expressed relative to Ac-Trp-OEt in the same solvent, and average deviations of the mean were within ±3%.

of intrinsic viscosity (3–7 mg/mL), no polyelectrolyte effects were observed. Thus, the changes in intrinsic viscosity with the chloroform:methanol ratio represent true protein conformational changes. Similarly, addition of SDS also increases the intrinsic viscosity. As expected from the helix-promoting properties of the organic solvent mixtures, high [η] values were obtained, suggesting that a large proportion of the molecule is in helical conformation.

Perturbation Studies in Water. In water, the number of Tyr and Trp groups exposed in both APL and d-APL was relatively small (40% and 30%, respectively) and was independent of the size of the perturbant, between 4.0 and 9.2 Å (data not shown). The low exposure is compatible with a model in which the aromatic groups are buried in the hydrophobic internal folds of the protein far from the hydrophilic surface. As for the protein in chloroform–methanol, the inaccessibility of the groups to the perturbant does not appear to be due to a rigid structure formed by disulfide bridges, since addition of SDS (1% v/w) led to the exposure of all of the Tyr and Trp groups. Again, the well-resolved spectral maxima, the absence of distortions, and the constant number of chromophores exposed at different perturbant concentrations indicate that the perturbants do not affect the protein conformation.

Viscosity measurements could not be performed in aqueous solvents since the proteins precipitated irreversibly at the concentration required for accurate measurements (3-7 mg/mL).

Intrinsic Fluorescence Studies in 2-CE. Because of the quenching properties of chloroform, intrinsic fluorescence studies in organic media were carried out only in 2-CE. The emission spectra of APL and d-APL in this solvent are indistinguishable, and upon excitation at 280 nm, prominent features of both Tyr and Trp residues appear (Figure 5). The efficiency of energy transfer from Tyr to Trp is decreased, and consequently the Tyr emission is clearly visible. Furthermore, the high quantum yield obtained and the similarity in λ_{max} emission of the Trp component with respect to the Ac-Trp-OEt (Table I) support the conclusion (see below) that the side chains are accessible to the medium.

Intrinsic Fluorescence Studies in Water. The fluorescence spectra of the water-soluble forms of APL and d-APL were characteristic of Trp residues in a hydrophobic environment since there is a large blue shift in the λ_{max} and a significant decrease in the Trp quantum yield with respect to the free Ac-Trp-OEt (Table I). In contrast to the observations in organic solvents, the Tyr quantum yields in water were small and much lower than that of the free Ac-Tyr-OEt (data not shown). Despite the presence of 15 Tyr compared with only 4 Trp residues per 30000 dalton monomer, the intrinsic fluorescence of the protein in water was predominately due to Trp. On excitation at 280 nm, a shoulder for the Tyr emission at 305–310 nm was not observed, whereas the Trp quantum yield at 280 nm was higher than at 295 nm, suggesting a high energy transfer from Tyr to Trp. After deacylation, the emission maximum was shifted 5 nm to longer

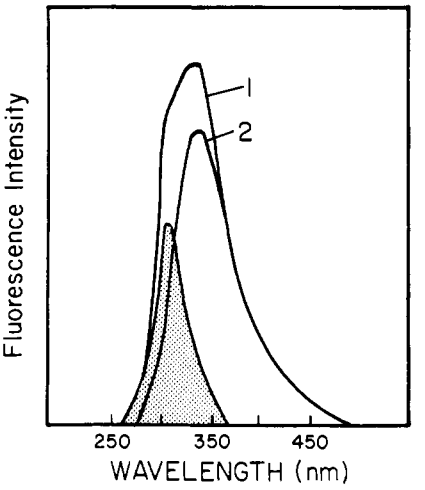

FIGURE 5: Emission spectra of APL (50 μg/mL) in 2-chloroethanol, normalized as described under Methods, produced by excitation at 280 nm (1) and 295 nm (2). The difference in emission spectra 1 and 2 is the shaded area.

wavelengths and there was a small decrease in the quantum yield, suggesting small conformation differences between APL and d-APL (Table I).

Incorporation of APL into phosphatidylcholine–cholesterol vesicles resulted in an increase of the fluorescence intensity and a reduction in the Tyr to Trp energy transfer without a change in the λ_{max} emission (Table I). These results are consistent with strong hydrophobic interactions betweeen the lipid and protein. The fluorescence parameters for d-APL and APL incorporated in lipid vesicles were indistinguishable.

Acrylamide Quenching. The acrylamide quenching data were analyzed by the Stern–Volmer equation (Weller, 1961): $F_o/F = 1 + K_{sv}(Q)$, where F_o/F is the ratio of the fluorescence intensity in the absence and presence of a given concentration of quencher (Q), and K_{sv} is the quenching constant. The Stern–Volmer plots (F_o/F vs. (Q)) for APL and d-APL in different media are shown in Figure 6. In all cases, upward curves (positive deviations) were obtained. Assuming that all the Trp residues fluoresce, the results indicate that all the Trp groups are equally accessible (Eftink & Ghiron, 1976). The curves also suggest that both static and collisional (dynamic) processes contribute to the quenching. To allow for a static quenching component, the Stern–Volmer equation has been modified by Birks (1970):

$$F_o/F = (1 + K_q(\text{eff})(Q))\ \exp(V(Q))$$

where the parameter V expresses the probability of finding a quencher molecule in contact with a fluorophor at the instant of excitation. K_q(eff) represents a crude measure of the exposure of Trp and can be calculated from the slope of the curves at low acrylamide concentrations. However, the true measure of kinetic exposure requires knowledge of the rate constant of the collisional component, k_q(eff), and that can be calculated from $K_q(\text{eff}) = \tau k_q(\text{eff})$, where τ is the average lifetime of the excited state. In the case of multitryptophan

Table II: Acrylamide Quenching Parameters for APL and d-APL[a]

	N-Ac-Trp-OEt	APL			d-APL		
solvent	K_q(eff) (M^{-1})	K_q(eff) (M^{-1})	K_q(eff)/Φ(Trp) (M^{-1})	λ_{max}[b] (nm)	K_q(eff) (M^{-1})	K_q(eff)/Φ(Trp) (M^{-1})	λ_{max}[b] (nm)
2-CE	4.8	3.0	3.2	0	3.1	3.3	0
water	17.0	1.3	3.9	−1	2.2	8.1	−2
vesicles		0.5	1.1	0	0.5	1.2	0

[a] Values represent the mean of four experiments. Average deviations of the mean were within ±15%. [b] Shift in λ_{max} at 50% quenching.

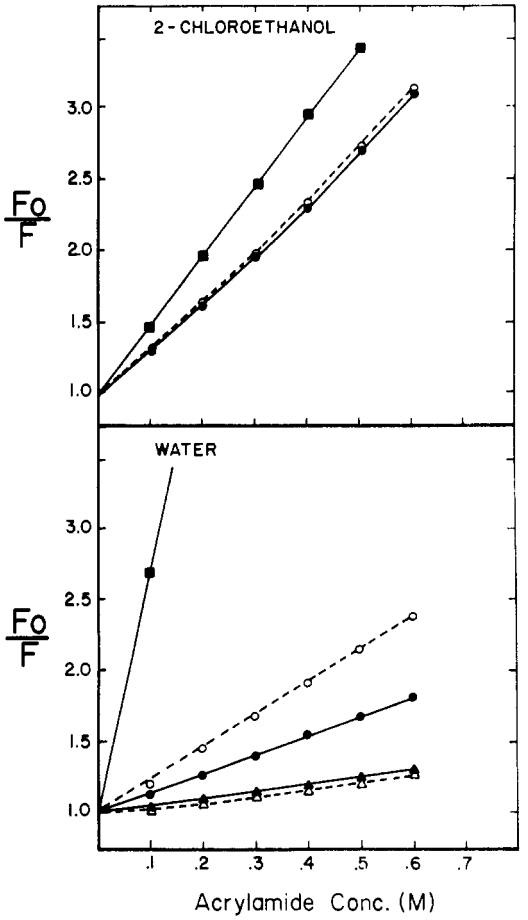

FIGURE 6: Stern–Volmer plots for acrylamide quenching of tryptophan fluorescence in APL and d-APL at 25 °C. The points were calculated from the experimental intensities at λ_{max} emission, with excitation at 295 nm. (■) Trp; (●) APL; (○) d-APL; (▲) APL–phosphatidylcholine vesicles; (△) d-APL–phosphatidylcholine vesicles.

proteins, k_q(eff) can be taken as an estimate of the average exposure of the fluorescing residues. In the absence of fluorescence decay measurements, we used the approach of Cockle et al. (1978), and calculated the ratio K_q(eff)/Φ(Tyr (295)) assuming that τ is proportional to the observed relative quantum yield. Therefore, this ratio will provide an approximate measure of the quenching rate constant k_q(eff), and will reflect solely changes in the rate of diffusion of the acrylamide through the protein or protein–lipid matrix. In 2-CE, as anticipated from the λ_{max} emission and Trp quantum yield values, the Trp fluorescence was easily quenched by acrylamide in both forms of the protein (Table II). In water, the ratio of K(eff)/Φ(Trp) was low with respect to the free Trp for both APL and d-APL, indicating that, on average, these groups are buried. However, a large difference was found between the values for APL and d-APL, indicating that d-APL has a matrix more permeable to acrylamide and that the groups are on average in a less polar environment than APL.

Incorporation of either APL or d-APL into lipid vesicles clearly results in an almost complete shielding of the quencher, presumably due to the close association between lipid and protein. The absence of spectral inflections and a large red shift on quenching provide strong evidence that acrylamide does not denature this protein.

Table III: Effect of Treatment with Hydroxylamine or Triethylamine on Fluorescence Characteristics of APL in Water[a]

treatment (h)	fatty acid content (% w/w)	λ_{max} (nm)	K_q(eff)/Φ(Trp-(295)) (M^{-1})
hydroxylamine			
0 (APL)	2.23	330	3.9
2 (d-APL)	0.18	335	8.1
4	0.20	336	7.4
16	0.24	337	7.8
0.01 M triethylamine (pH 8)			
16	1.79	329	4.3

[a] Values represent the mean of two to four experiments. Average deviations for the acrylamide quenching constants were within ±15%.

Effect of Different Hydroxylamine Treatments on the Fluorescence of APL in Water. To rule out that the differences in the fluorescence emission characteristics of the proteins were due to the nature of the chemical treatment (e.g. pH, temperature, ionic strength), APL was incubated with hydroxylamine for different periods of time and the fatty acid content and fluorescence characteristics of the resultant preparations were determined (Table III). Complete deacylation was obtained at the earliest time point tested (2 h). Treatment with hydroxylamine for longer periods did not result in any change in the fluorescence characteristics. Furthermore, incubation of APL with triethylamine for 16 h produced no change in either fatty acid content or fluorescence. These results indicate that the absence of the fatty acids rather than the conditions used for their removal is responsible for the observed conformational changes.

Discussion

Fatty acid acylation is the only known posttranslational modification of the myelin proteolipid protein, and since this protein can be obtained in large quantities, it provides a useful model to study the effect of covalently bound fatty acid on protein structure. The acylated form of the protein displays the unusual property of solubility in both organic and aqueous solvents, and its conformational characteristics have been studied extensively in both types of solvents. Intrinsic fluorescence (Cockle et al., 1978), extrinsic fluorescence (Feinstein & Felsenfeld, 1975), and CD spectroscopic studies (Sherman & Folch, 1970; Moscarello et al., 1973) have demonstrated conformational flexibility of the protein as a consequence of the transfer from one medium to another. Thus, while the protein is largely monomeric inorganic solvents with most of its segments in a helical configuration (Moscarello et al., 1973; Nguyen-Le et al., 1976), the water-soluble form occurs as a polydisperse solution (Gagnon et al., 1971; Nguyen-Le et al., 1976) in which most of the hydrophobic side-chain amino acids are buried in the internal folds of the protein.

In the present report we have studied the conformation of the acylated and the chemically deacylated apoprotein by spectroscopic techniques. In chloroform–methanol (2:1 v/v), the solvent most commonly used for the extraction of pro-

teolipids, Tyr and Trp groups were exposed to the medium to a major extent. Contrary to the general concept that only a single conformation of the protein exists in organic solvents, different degrees of chromophoric exposure and different values for intrinsic viscosity were found when the chloroform–methanol ratio was changed. Similarly, Lux et al. (1984) have reported different conformations for the non-myelin proteolipid in 2-CE, butanol, and methanol. As determined by intrinsic viscosity and ultraviolet and fluorescence spectroscopy, no differences were found between APL and d-APL in different organic solvents. In these solvents, the hydrophobic side chain amino acids are in contact with the solvent and little tertiary structure is present. Consequently, the fatty acids are probably exposed to the medium and exert no effect on the protein conformation. In water, as measured by UV difference spectroscopy, only a small number of chromophoric groups were exposed to the medium and no differences were found between the acylated and deacylated forms, even though perturbants of different sizes were used.

On the other hand, intrinsic fluorescence studies of the protein in water revealed differences in conformation between the two forms. Although neither the emission maximum nor the quantum yield can be a simple function of the degree of exposure of Trp residues (Weinryb & Steiner, 1970), the combination of the shift in the λ_{max} and a decrease in the Trp quantum yield argues that Trp residues buried in APL were exposed to a greater extent or are located in a more hydrophilic environment following deacylation. This was supported by the acrylamide quenching experiments, in which the K(eff) for d-APL is higher than for APL. Since acrylamide is able to penetrate the protein matrix and report on the depth of the fluorophor, the differences can be attributed to a more compact structure in the presence of the long-chain fatty acid. It was initially thought that the hydrophobic nature of this protein and its aggregation in water were due, in part, to the presence of the fatty acids. However, both the sedimentation analysis and gel filtration studies show that aggregation occurs independently of the presence of covalently bound lipid (data not shown). Similarly, the self-aggregation of the native murein lipoprotein was originally attributed to its bound fatty acids (Braun & Bosch, 1972), but a mutant precursor form lacking fatty acids (We et al., 1977) also aggregates. The conformation of the apoprotein is known to be affected by prior treatment. Three forms of the protein have been shown by intrinsic fluorescence measurements and acrylamide quenching, and the magnitude of the changes were similar to those in the present study (Cockle et al., 1978). However, it should be emphasized that in the present study we demonstrate that the changes are related to the absence of fatty acid per se and not to the procedure required for their removal.

Recently, it was reported that the native and the chemically deacylated G glycoproteins from vesicular stomatitis virus interact differently with membrane lipid after reconstitution into liposomes (Schlesinger & Magee, 1982). Furthermore, whereas water-soluble proteins are not usually incorporated into liposomes, complete incorporation of IgG (Huang et al., 1980) and α-bungarotoxin (Babbitt & Huang, 1985) has been demonstrated after chemical palmitoylation, indicating that the addition of fatty acids makes these proteins interact hydrophobically with lipid bilayers. Studies on the primary structure of the bovine myelin proteolipid protein have identified Thr-198 as an attachment site for fatty acid. In two proposed models for the orientation of the protein in myelin (Stoffel et al., 1983; Laursen et al., 1984), this residue is located within a hydrophilic segment exposed to an aqueous environment. It has been speculated that the fatty acid at that location may promote interaction of this domain with the opposite membrane (Laursen et al., 1984). This region may interact differently with lipids depending on the presence or absence of fatty acid; however, no such indication is given from the present fluorescence experiments on the intact protein. Both forms are easily incorporated into phosphatidylcholine–cholesterol vesicles and both exhibit strong hydrophobic interaction with lipids.

The most striking differences observed between APL and d-APL were in their spectroscopic properties in water. It can be inferred from these results that the fatty acid in the myelin proteolipid influences the protein folding in aqueous media. In view of the location of the fatty acid at the aqueous interface, this may have significant implications for the maintenance of the lamellar structure of the normal myelin sheath.

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N-Terminal Domain of the Bacteriophage λ Repressor: Investigation of Secondary Structure and Tyrosine Hydrogen Bonding in Wild-Type and Mutant Sequences by Raman Spectroscopy†

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ABSTRACT: Laser Raman spectroscopy has been employed to investigate structures of the λ repressor N-terminal fragment, which recognizes operator DNA. Examination of repressor fragments containing deuterated amide groups and specifically labeled deuteriotyrosines has enabled the assignment of many of the conformation-sensitive Raman bands. By use of Fourier deconvolution and signal averaging techniques, the spectra of both wild-type and mutant sequences have been obtained as a function of the total protein concentration in aqueous solution over the range 5–100 mg/mL. This analysis has permitted monitoring of the monomer–dimer association of the repressor fragment and determination of the effects of dimerization upon individual side-chain interactions and main-chain secondary structure. The spectra are interpreted to reveal the hydrogen-bonding environments of four tyrosines of the N-terminal fragment (Y22, Y60, Y85, and Y88). The fifth tyrosine (Y101) is known from NMR experiments to be exposed to solvent molecules. The results show that in the dimer Y22 and Y85 are each acceptors of a strong hydrogen bond from a positive donor group, while Y88 is the donor of a strong hydrogen bond to a negative acceptor and Y60, like Y101, is involved in both a donor role and an acceptor role. Y60, Y85, and Y88, which are all near the dimer interface, undergo a collective change in hydrogen-bonding environment with dissociation of the dimer. The net effect of this change is the conversion of one acceptor tyrosine, deduced to be Y88, to a combined donor and acceptor role. The Raman results also indicate a predominantly α-helical structure for the N-terminal fragment in aqueous solution, with 70 ± 4% of the residues incorporated into helical domains. The amount of α-helix determined from the Raman spectrum is consistent with X-ray and prediction results and is altered neither by the mutations C85 → Y85 and C88 → Y88 nor by dissociation of the dimer.

The bacteriophage λ repressor is a DNA-binding protein that contains 236 amino acids and comprises two structural domains. The N-terminal domain binds specifically to the λ operators, and the C-terminal domain contains dimer and higher order contacts (Pabo et al., 1979; Sauer et al., 1979). The crystal structure of an N-terminal fragment of λ repressor (residues 1–92) has been determined at 3.2-Å resolution (Pabo & Lewis, 1982). The secondary structure of this fragment consists of five α-helices, as shown in Figure 1, and the tertiary structure is compatible with binding to double-stranded B DNA. A detailed model of the complex of λ repressor and operator DNA of the B form has been proposed (Lewis et al, 1983). Major features of the model have been supported by studies on genetically altered repressors (Hecht et al., 1983; Nelson et al., 1983; Eliason et al., 1985; Nelson & Sauer, 1985).

The active species in operator binding is a dimer (Chadwick et al., 1970). Because dimerization and DNA binding are coupled equilibria, dimerization contributes to the apparent operator affinity of the repressor. Both C-terminal and N-

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