Biophysical Chemistry 19 (1984) 345–353
Elsevier

BPC 00855

INTRINSIC FLUORESCENCE OF A NON-MYELIN APOPROTEOLIPID AND EVIDENCE FOR THE EXISTENCE OF CONFORMATIONAL FLEXIBILITY

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Received 19th August 1983
Revised manuscript received 10th January 1984
Accepted 24th January 1984

Key words: Proteolipid; Fluorescence; Conformational flexibility

An extremely hydrophobic protein ($M_r = 16000$), which in its native form is only soluble in organic solvents and which differs from the myelin proteolipid ($M_r = 24000$), was purified to homogeneity. Intrinsic fluorescence studies on this apoproteolipid have revealed a large conformational flexibility. In the water-soluble form, the emitting residues appear to be buried in a hydrophobic core, while in organic solvents they are exposed to the external medium. Structural changes depending on the organic solvent are also observed. The emission characteristics of reconstituted proteoliposomes may be due to the formation of a membrane-linked complex between several proteolipid monomers.

1. Introduction

Proteolipids [1] are a family of intrinsic membrane proteins characterized by their solubility in organic solvents and their insolubility in water in their native forms. They occur as membrane components in animals, plants and microorganisms [2]. Their high hydrophobicity suggests that they may play a structural role within the membrane lipid bilayer and that their interactions with other membrane components may be important for membrane cohesion. Proteolipids may also be involved in many membrane-related functions, since some of them are associated with the energy-transducing H^+-ATPase complex as well as with active ion transport in mitochondrial, plasmic or sarcoplasmic membranes [3].

Most studies on proteolipids have so far been devoted to the white matter apoproteolipid or lipophilin ($M_r = 24000$), one of the two major proteins of myelin [4]. Its primary structure is now almost completely established [5]; many of its physicochemical properties have been elucidated, but very few studies have been devoted to its function in myelin.

For a better understanding of the structure and the biological function of proteolipids, we have undertaken a general study of myelin and non-myelin proteolipids and succeeded in showing that, after purification, some of them display single-channel phenomena [6], confirming their involvement in ion transport.

Another way to examine the role of proteolipids in membrane structure and functions is to study their conformational flexibility and aggregation properties. Such experiments are feasible, since the myelin apoproteolipid has been transformed into a water-soluble form [4], and preliminary studies have shown that apoproteolipids are soluble in several organic solvents with different polarities. A conformational study should clarify the contribution of the proteolipids to membrane lipid bilayer

fluidity and thus provide a model for examining intramolecular membrane interactions.

We therefore performed a detailed fluorescence study on a bovine brain non-myelin apoproteolipid of low molecular weight (16000). The emission characteristics due to tyrosine and tryptophan residues were determined in different solvents: methanol, butanol, 2-chloroethanol and water to compare the conformation of this proteolipid in organic and in aqueous media. The apoproteolipid was also incorporated into dimyristoylphosphatidylcholine (DMPC) vesicles to study its conformation in a lipid bilayer environment. This investigation on reconstituted proteoliposomes was particularly interesting, since lipid-protein or protein-protein interactions may play an important role in the possible biological function of proteolipids as transmembrane movement modulators.

2. Materials and methods

2.1. Proteolipid purification

Proteolipids were isolated from whole bovine brain homogenate by chloroform/methanol (2 : 1, v/v) extraction and fractionated by chromatography on methylated Sephadex into peaks I–IV as described previously [6]. Peak III (the proteolipid fraction ranging from M_r 16000 to 14000 was further purified by ion-exchange chromatography on a CM-Trisacryl column using the standard conditions described in ref. 6. The apoproteolipid of $M_r = 16000$ was eluted with 0.01 N ammonium acetate in chloroform/methanol/H_2O (4 : 4 : 1, v/v/v) and its homogeneity was checked by electrophoresis using the acrylamide system described by Laemmli [7] (fig. 1). Protein content was evaluated according to the method of Lowry et al. [8] as modified by Lees and Paxman [9]; bovine serum albumin was used as standard. Amino acid analysis was performed according to the standard procedure with a Durrum D 500 amino-acid analyser after hydrolysis of the sample (24 h and 72 h at 110°C in 6 N HCl); tryptophan was determined after treatment with methanesulfonic acid and cysteine after oxidation with performic acid.

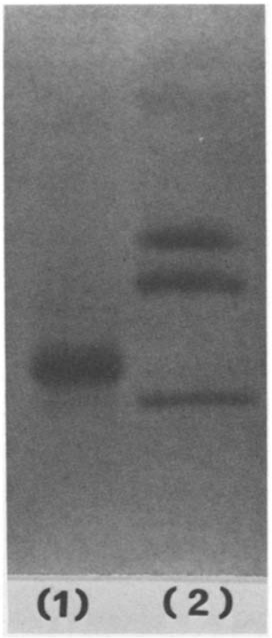

Fig. 1. SDS-polyacrylamide gel electrophoresis. Samples were resolved by discontinuous SDS-polyacrylamide gel as described by Laemmli [7]. The stacking gel was 6% in acrylamide and the separating gel was made up of a linear 10–20% concentration gradient of acrylamide. (1) The non-myelin apoproteolipid; (2) peak II α (M_r 20000), peak II β (M_r 24000) [6] and cytochrome *c* (M_r 12000) as standards.

2.2. Solutions

Reference: *N*-Acetyltyrosinamide and *N*-acetyltryptophanamide (Sigma) were dissolved in redistilled water or in the various organic solvents (spectroscopic grade, Merck).

Proteolipid solutions: After precipitation in diethyl ether and centrifugation, the proteolipid was dissolved in a small volume (10 μl) of formic acid and then diluted with the appropriate solvent. The water-soluble form was obtained by changing the solvent from chloroform/methanol (2 : 1, v/v) to water in a stream of nitrogen as described by Folch-Pi and Sakura [10]. Fluorescence measurements were made on protein solutions at a concentration of approx. 50 μg/ml.

of apoproteolipid and dimyristoylphosphatidylcholine (Sigma) in 2-chloroethanol or methanol was dialysed for 12 h against a buffer containing 10 mM NaCl and 1 mM EDTA at pH 7.4. Dialysis was carried out at 37°C, i.e., above the lipid phase transition temperature.

2.3. Methods

Absorption spectra were recorded with a Cary 219 spectrophotometer. They were corrected for light scattering by means of the formula $\log A_s = A \log(\lambda_{nm}) + B$, where A_s is the absorbance simulated by scattering light and A and B coefficients determined in the spectral region where protein does not absorb. Fluorescence spectra were obtained with an absolute spectrofluorimeter (FICA 55) and quantum yields (ϕ) were determined as previously described [12] taking free tyrosine and tryptophan as references [13]. Quantum yields were corrected by taking into account, on the one hand, the different indices of refraction, n, by using the ratio $\phi_1/n_1^2 = \phi_2/n_2^2$ and, on the other, the screening effect of the scattered light [14].

Fluorescence decay times (τ) were measured with the single photoelectron technique [15]. Excitation was performed at 295 ± 5 nm and fluorescence was observed at 342 ± 5 nm. The fluorescence decay profiles were analyzed statistically using the residual distribution form [16,17].

The degree of polarization (p) was obtained with an SLM 8000 spectrofluoropolarimeter. Solutions were excited at 295 nm and emission was observed at 342 nm. Low turbidity of the sample (less than 0.1 A units at 280 nm) precludes any correction for light scattering [18]. For polarization measurement, the cuvette temperature was adjusted to 18°C, the temperature within the cell being measured with a thermocouple to an accuracy of ±0.5°C.

3. Results

3.1. Tyrosine and tryptophan content

Tyrosine and tryptophan content was determined from the ultraviolet spectrum as described by Edelhoch [19]. It was evaluated from the proteolipid spectrum in 2-chloroethanol where the emitting residues were shown to be exposed to solvent (see section 4), using *N*-acetyltyrosinamide and *N*-acetyltryptophanamide as reference models to mimic the tyrosine and tryptophan residues. From the extinction coefficients (ϵ) of the protein at two wavelengths (280 and 289 nm) and of the model compounds in the same solvent [20], the concentration of the aromatic residue (C) in the protein was calculated from the equations:

$$E_p(280) = \epsilon_{Tyr}(280)C_{Tyr} + \epsilon_{Trp}(280)C_{Trp}$$

$$E_p(289) = \epsilon_{Tyr}(289)C_{Tyr} + \epsilon_{Trp}(289)C_{Trp}$$

where E_p represents the protein absorbance at 1 mg/ml concentration.

Results from this procedure showed that the tyrosine concentration was about twice that of tryptophan. Since the protein extinction coefficient at 280 nm was approx. 26 000 M^{-1} cm^{-1}, the protein must contain five tyrosine and three tryp-

Table 1

Amino acid composition of apoproteolipid

Amino acid	Residues/ 100 residues	mol/mol proteolipid [a]
Asp	4.63	7
Thr	8.60	13
Ser	5.96	9
Glu	3.97	6
Pro	3.97	6
Gly	7.94	12
Ala	13.24	20
Val	7.28	11
Ile	4.63	7
Leu	11.26	17
Tyr	3.31	5
Phe	6.62	10
His	2.65	4
Lys	4.63	7
Arg	2.65	4
Trp	1.98	3
Cys	3.97	6
Met	2.65	4
Total hydrophilic [b]	34.41	
Total hydrophobic [c]	65.53	

[a] Based on M_r 16000.
[b] Sum of Arg, Asp, Glu, Lys, Pro, Ser, Thr.
[c] Sum of Ala, Cys, Gly, His, Ile, Leu, Met, Phe, Trp, Tyr, Val.

tophan residues. This spectroscopic determination agreed well with values obtained for amino acid analysis (table 1).

3.2. Spectral characteristics

Besides water, the organic solvents methanol, butanol and 2-chloroethanol were also used, since they solubilize proteolipids and are transparent in the far-ultraviolet region. Chloroform could not be used because it induces significant quenching of the tryptophan and tyrosine fluorescence emission.

In all these media (water and organic solvents), the absorption spectra (fig. 2) were very similar, with a maximum at 282 nm and a marked shoulder at 292 nm, the position of which is characteristic of tryptophan in a non-polar environment [15]. For the proteolipid dissolved in organic solvents, the spectra were close to those observed for the model *N*-acetyltryptophanamide in alcohols, which indicated that the tryptophan residues were probably located at the protein surface in contact with the surrounding solvent molecules. In contrast, in the water-soluble form of the proteolipid, the absorption spectrum was red shifted with respect to the model in water, suggesting that the residues were buried in internal hydrophobic regions of the proteolipid.

Fluorescence spectra were obtained after excitation at 280 nm where both tyrosine and tryptophan residues absorb and at 295 nm where tryptophan is selectively excited. Emission maxima

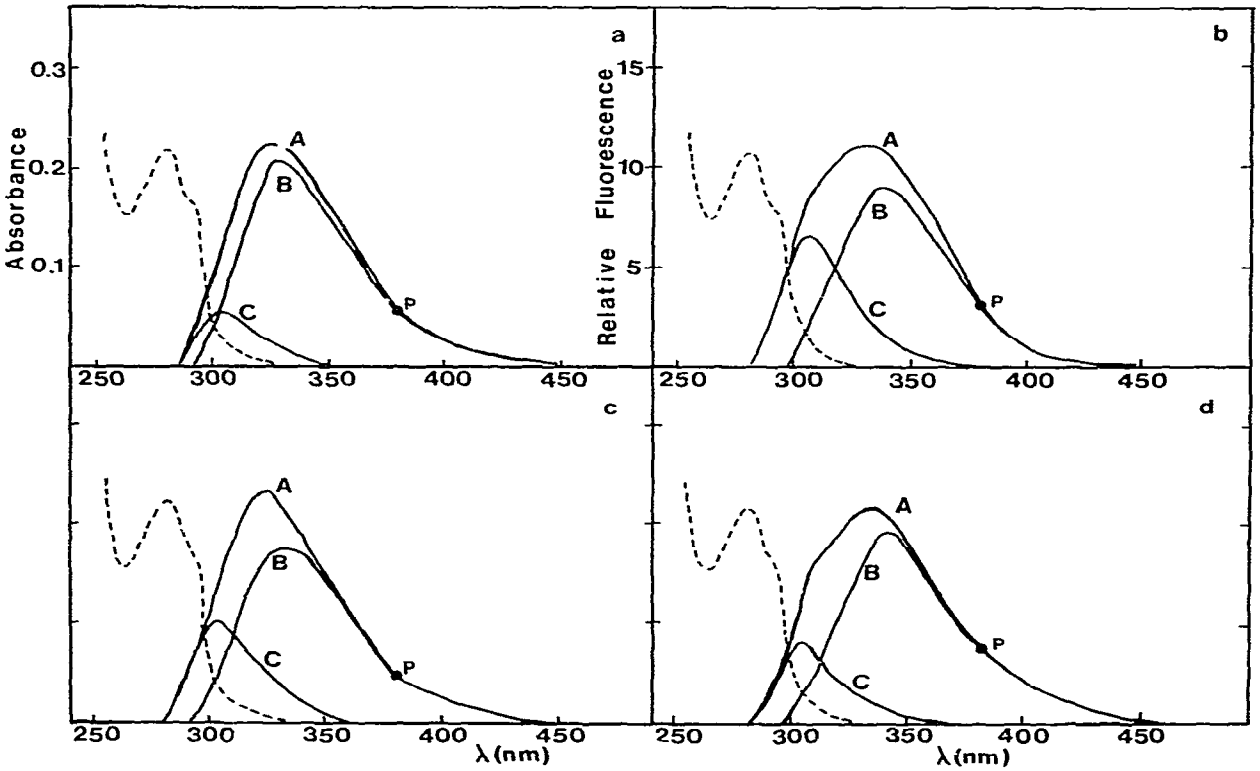

Fig. 2. Absorption and emission spectra of the apoproteolipid in various solvents. Absorption (------) and fluorescence spectra (———) for excitation at 280 nm (curve A) and 295 nm (curve B) and difference spectrum (curve C) between curve A and B normalized at 380 nm (point P) for the apoproteolipid in water (a), methanol (b), butanol (c) and 2-chloroethanol (d).

Table 2

Fluorescence characteristics of the proteolipid apoprotein in the various solvents and in the lipid bilayer

	λ_{exc} (nm)	λ_{max} (nm)	$\Delta\lambda$ (nm)	ϕ_p	$\phi_{Trp}(280)$	$\phi_{Tyr}(280)$	$\langle\tau\rangle$ (ns)	ω
Water	280	324	55	0.05	0.075	0.01		
	295	325	53	0.05			2.5	0.71
Methanol	280	335	69	0.09	0.12	0.04		
	295	338	54	0.11			3.0	0.35
Butanol	280	326	62	0.10	0.11	0.08		
	295	330	55	0.10			3.6	0.61
2-Chloro-	280	334	73	0.13	0.15	0.10		
ethanol	295	338	57	0.15			2.8	0.10
Liposomes	280	330		0.02				
	295	333					3.0	0.90

(λ_{max}) and half-maximum spectral width ($\Delta\lambda$) (table 2) in all cases revealed a contribution of the tyrosine residues to total fluorescence, tyrosine emission being lower in water. For tryptophan fluorescence, except in water, emission maxima were close to those of reference compounds in the corresponding solvents (table 3) which indicated that these residues were largely exposed to the external medium. The small blue shift ($\simeq 5$ nm) as compared with the models could be explained, as proposed by Moscarello and co-workers [20] for lipophilin, by the presence of apolar residues near the tryptophan in the amino acid sequence, which increased the hydrophobicity of the tryptophan residue microenvironment. In water, the tryptophan spectral parameters ($\lambda_{max} = 325$ nm, $\Delta\lambda = 53$ nm) were typical of residues in a hydrophobic environment and therefore buried inside the protein, which was confirmed by the fact that an efficient fluorescence quencher such as acrylamide had a very small effect on apoproteolipid emission. Moreover, it is important to note that the fluorescence spectrum of the proteolipid in water was not affected by adding 6 M guanidinium chloride or by high temperatures (80°C).

Data for the proteolipid-DMPC recombinants are included in table 2. The fluorescence emission spectrum ($\lambda_{exc} = 280$ nm) had its maximum at 330 nm, which is commonly observed for proteins incorporated in liposomes [21]. Concerning the fluorescence of the tryptophan residues (observed

Table 3

Fluorescence characteristics of model compounds in various solvents

Solvent	Residue	λ_{max} (nm)	$\Delta\lambda$ (nm)	Quantum yield (ϕ)	Lifetime (τ) (ns)
Water	Nac Tyr am	303	30	0.065	1.6
	Nac Trp am	352	62	0.14	3
Methanol	Nac Tyr am	301	30	0.17	3.2
	Nac Trp am	342	59	0.16	2.9
Butanol	Nac Tyr am	301	30	0.19	3.8
	Nac Trp am	336	55	0.25	3.6
2-Chloroethanol	Nac Tyr am	301	30	0.09	2.7
	Nac Trp am	343	57	0.13	2.7

after excitation at 295 nm), the emission spectrum ($\lambda_{max} = 333$ nm) was characteristic of residues located in a low-polarity environment, which is probably due to the proximity of aliphatic hydrocarbon chains.

3.3. Fluorescence quantum yields and decay times

In each solvent, the emission quantum yield was determined for excitation at 280 nm (total quantum yield ϕ_p^{280}) and at 295 nm where only tryptophan emits (ϕ_p^{295}). In comparison with model systems (table 3), tryptophan quantum yields of the proteolipid (table 2) were rather low, which showed that efficient quenching processes were occurring. The quantum yield in water ($\phi = 0.05$) was compared to the reference yield of *N*-acetyltryptophanamide in dioxane ($\phi = 0.30$) [15], which represented the tryptophan residues in a buried environment. A higher tryptophan yield was observed when proteolipid was dissolved in 2-chloroethanol ($\phi_p^{295} = 0.15$).

Further interpretation of the fluorescence properties requires evaluation of the quantum yields of the tyrosine and tryptophan components. Since protein emission upon excitation at 295 nm was entirely due to tryptophan, the tyrosine emission profile was obtained by subtracting the emission spectra excited at 280 and 295 nm, respectively, and normalized at 380 nm where only tryptophan fluoresces (curves A and B in fig. 2). Fractional yields are given by

$$\phi_p^{280}(\mathrm{Trp}) = \phi_p^{280}\frac{S_B}{S_A}$$

$$\phi_p^{280}(\mathrm{Tyr}) = \phi_p^{280}\frac{S_C}{S_A}$$

where S denotes the area under the curve indicated by the subscript.

Assuming that the proteolipid contained three tryptophan and five tyrosine, it was necessary to take into account fractional absorption (f) of both kinds of residue at 280 and 295 nm. The yields, ϕ_{Tyr} and ϕ_{Trp}, were deduced from the following equations:

$$\phi_{Tyr} = \frac{\phi_p^{280}(\mathrm{Tyr})}{f_{Tyr}^{280}} \text{ and } \phi_{Trp} = \frac{\phi_p^{280}(\mathrm{Trp})}{f_{Trp}^{280}}$$

$$f_{Tyr} = \frac{n'\epsilon'}{n'\epsilon' + n\epsilon} \text{ and } f_{Trp} = 1 - f_{Tyr}$$

when ϵ' and ϵ are the molar extinction coefficients of *N*-acetyltyrosinamide and *N*-acetyltryptophanamide, respectively, in the considered solvent, and n the number of residues, the prime referring to tyrosine. We found, for example, $f_{Tyr} = 0.30$ and $f_{Trp} = 0.70$ for the proteolipid in 2-chloroethanol.

In table 2, we have summarized total quantum yields and the respective contributions of the tyrosine and tryptophan residues. Quantum yields for the tyrosine emission from proteolipid were lower than those of *N*-acetyltyrosinamide (table 3) or of similar magnitude in the case of proteolipid in 2-chloroethanol. In the water-soluble form, the tyrosine contribution was low but not negligible as is often the case for proteins containing these two emitting residues. On the other hand, tryptophan quantum yields depended on excitation wavelength, which provided evidence of resonance energy transfer between tyrosine and tryptophan.

No such spectral analysis was possible for the proteolipid incorporated in phospholipid bilayers due to considerable scattered light in these samples. However, the global quantum yield was very low ($\phi = 0.02$) which indicated that the vesicular insertion of the proteolipid was accompanied by strong quenching of the emitting residues.

Additional information on the quenching processes occurring in the protein was obtained by comparative analysis of protein quantum yield and decay times with those for the model system in the same solvent. Dynamic quenching due to a diffusion-controlled encounter between the excited fluorophore and some quenching groups of the protein was evidenced by lifetime values being lower for the residue than for the model (*N*-acetyltryptophanamide in the appropriate solvent). Static quenching, related to a permanent contact between the chromophore and the quenching group, can be deduced from the ϕ/τ ratios, since it induces a quantum yield decrease without modification of decay time values. The efficiency (ω) of this process can be estimated from ϕ/τ ratios of the protein and model system, since the fraction of fluorescent residues submitted to this process is

given by:

$$\omega = 1 - \frac{\phi/\tau_p}{\phi/\tau_m} \quad [15]$$

where p and m refer to proteolipid and models in the same solvent.

The values of ω (table 2) obtained for the proteolipid in water, methanol and butanol (ω = 0.71, 0.35, 0.61) indicated rather high probability of contact with a quenching group in these conformational states. Analysis of decay times, which fitted best with a double-exponential model, also showed that the three tryptophans were not equivalent. The result was noticeably different for the proteolipid in 2-chloroethanol, where ω was very low ($\omega = 0.10$). In that solvent, both tryptophan and tyrosine residues seemed to be in a special environment, partially sheltered from the quenching process, perhaps a helical region since it has been shown [22] that tryptophan and tyrosine emission yields are unusually high in helical parts of proteins. This is consistent with circular dichroism studies which demonstrate that 2-chloroethanol induces the formation of large helical segments in other proteolipids [23]. In contrast for the proteolipid incorporated in liposomes, the evaluation of ω showed that only 0.1 residue was involved in the fluorescence ($\omega = 0.9$). This quenching of tryptophan fluorescence might have resulted from conformational changes in the proteolipid incorporated in lipid vesicles.

3.4. Tyrosine → tryptophan energy transfer

The dependence of tryptophan quantum yield on excitation wavelength indicated that resonance energy transfer was occurring. The efficiency, η, of this process was deduced from

$$\phi_p^{280}(\mathrm{Trp}) = \phi_{\mathrm{Trp}}\left(f_{\mathrm{Trp}}^{280} + \eta f_{\mathrm{Tyr}}^{280}\right)$$

and the mean distance (R) between the two chromophores was evaluated using the relation

$$R = \left(\eta^{-1} - 1\right)^{1/6} R_0$$

where R_0 is the Förster critical distance for the tyrosine → tryptophan system. The average value of the orientational factor κ^2, i.e., $\kappa^2 = 2/3$, was adopted as is usual for a random distribution of the chromophores. This is appropriate in the case of the indole and phenol rings, since indole is characterized by two linear transition moments and phenol by a plane or partially plane one, which minimizes the cases where κ^2 is very small. Under these conditions, the distance R_0 between tyrosine and tryptophan has been calculated to be 12–15 Å depending on the donor fluorescence quantum yield [24]. The results reported in table 4 indicate that resonance energy transfer took place in all solvents but with varying efficiencies. The mean distance R between the different donors and acceptors was evaluated but it should be borne in mind that R represents only an average distance which, however, can give global information on the possible structure of the protein.

Similar values of η corresponding to an average distance of approx. 18–20 Å between the tyrosine and tryptophan rings were found for the proteolipid dissolved in organic solvents. If we assumed that the radius of a globular protein with a molecular weight of 16 000 was roughly 20 Å, our results implied a rather stretched conformation of the proteolipid in methanol, butanol and 2-chloro-

Table 4

Resonance energy transfer and polarization data in various solvents and in lipid bilayers

	Transfer efficiency (η)	Distance (R) (Å)	Polarization (p) (λ_{exc} = 295 nm)	Average lifetime ($\langle\tau\rangle$) (ns)	Relaxation time (ρ) (ns)
Water	0.90	9	0.21	2.5	9.5
Methanol	0.10	20	0.10	3.0	3.0
Butanol	0.20	18	0.15	3.6	6.7
2-Chloroethanol	0.10	20	0.14	2.8	4.7
Liposomes	–	–	0.18	3.0	7.8

ethanol. Energy transfer was much more efficient for the proteolipid dissolved in water where the mean distance between donors and acceptors was reduced to 9 Å, which was consistent with a globular conformation of the protein in the water-soluble form.

3.5. Polarization data

Steady-state polarization measurements were performed to obtain information on the motion of tryptophan residues. Relaxation times (ρ) were deduced from fluorescence polarization values using Perrin's relation:

$$\left(\frac{1}{p}-\frac{1}{3}\right)=\left(\frac{1}{p_0}-\frac{1}{3}\right)\left(1+\frac{3\tau}{\rho}\right)$$

where ρ is the relaxation time, τ the fluorescence lifetime, and p and p_0 the measured and the fundamental polarization degrees, respectively.

Considering that intrinsic chromophores are characterized by short lifetimes (3–4 ns), the protein relaxation times are presumably representative of the dynamics in the close environment of the tryptophan residues while only a very small fraction of these rotational amplitudes may result from overall motion of the macromolecule. This means that Perrin's equation can be used to deduce the relaxation times of aromatic fluorophores if we assume that they could be considered as small globular particles, which seems reasonable as a first approximation. Moreover, the values of ρ derived from the degree of polarization of the tryptophan fluorescence are significantly lower than those calculated for a globular protein of 20 Å radius ($\rho = 24$ ns) or for a long prolate ellipsoid ($\rho > 100$ ns in the orientation of the long axis), which indeed indicates that the fluorophores rotate independently of the protein matrix. The analysis of ρ, which in the presence of several fluorophores represents a weighted average over the various tryptophan environments, allowed us nevertheless to appreciate the variations of overall mobility of the emitting residues and to obtain consequently some information on the protein conformation.

The polarization data (see table 4) obtained for the water-soluble form enabled us to conclude that tryptophan residues were rigidly attached to the protein, as shown by the long relaxation time. The rotational mobility of the emitting residues increased in organic solvents which was in agreement with their high degree of exposure in these solvents. We also observed that the emissive tryptophan residues remained relatively mobile in the lipid environment.

4. Discussion

Our purified non-myelin apoproteolipid has a large conformational flexibility. Fluorescence studies show a major structural difference between its organic solvent- and water-soluble forms.

Fluorescence parameters show that in the water-soluble form, the emitting residues are buried in a hydrophobic core where they have little freedom of motion. This structure is firmly held by hydrophobic interactions, since no conformational change was detected upon the addition of 6 M guanidinium chloride, which is known to disrupt hydrogen bonds. Such a rigid conformation with interactions between large non-polar areas of the apoprotein [25] is to be expected for this extremely hydrophobic protein when transferred to aqueous solution.

When the proteolipid was dissolved in organic solvents, hydrophobic segments of the apoprotein were, as expected, exposed to the external medium, as shown by the spectral characteristics of the fluorophores. Furthermore, the structural changes observed with different organic solvents were noteworthy. More extensive helical arrangements were found in 2-chloroethanol than in butanol and methanol. On the other hand, polarization results and quenching data (ω) suggest particular folding of the apoprotein in methanol.

For the interactions of the purified proteolipid with DMPC in liposomes, spectroscopic changes (shift in emission peak, variations in quantum yield and decay times) are consistent with protein insertion in the phospholipid bilayer. This is corroborated by the fact that the lipid phase transition temperature of the acyl chain (24°C for DMPC) is strongly influenced by association with proteolipid, which is in line with the embedding of

the protein in the lipid bilayer. For the apoproteolipid incorporated in vesicles, we observed a marked decrease in quantum yield ($\phi = 0.02$) as compared with soluble protein. This result is surprising, since the formation of lipid complexes generally gives rise to enhanced fluorescence intensity related to an increase in the helical conformation which facilitates insertion into the hydrophobic membrane environment [21]. Here, in contrast, the low quantum yield denotes a very efficient static quenching, which could be due to the formation of a membrane-linked complex between several protein monomers. In such a structure, we imagine that the tryptophan residues would interact with surrounding quenching groups in the contact area between protein monomers. Such a situation, involving additional quenching upon protein aggregation, has already been described for plant virus proteins [26].

Finally, our results concerning the proteolipid incorporated in liposomes suggest a protein aggregation but further studies on the proteolipid-proteolipid interactions are necessary to determine the molecular composition of the membrane complexes.

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