Intrinsic Fluorescence of a Hydrophobic Myelin Protein and Some Complexes with Phospholipids†

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ABSTRACT: The fluorescence characteristics of lipophilin, a proteolipid apoprotein from human myelin, were determined in aqueous and lipid environments. In all cases the tryptophan residues were located in buried hydrophobic sites of uniform, but limited, accessibility to the permeant quenching agent acrylamide; only in the helicogenic solvent 2-chloroethanol were the protein fluorophores exposed to the medium. Quantum yields were dependent on the state of aggregation of the protein in aqueous solution and increased considerably on treatment with lysolecithin micelles, or when the protein was combined with phosphatidylcholine by codialysis from 2-chloroethanol into water. Fluorescence titrations indicated that lipophilin bound to lysolecithin with an association constant greater than 10^6 L/mol. Radiationless singlet excitation energy transfer from tyrosine to tryptophan residues was found to decrease markedly when the protein was combined with lipids. When the protein was introduced into dimyristoylphosphatidylcholine vesicles, the tryptophan fluorescence did not detect any solid–liquid phase change. These results were consistent with strong hydrophobic interactions between lipophilin and phospholipids, which lead to conformational adjustments in the protein, and to the establishment of an immobilized layer of boundary lipid in bilayer systems.

Although extrinsic fluorescent probes have been used widely to investigate the architecture of natural and model membranes (Badley, 1976), intrinsic protein fluorescence has been applied comparatively rarely to the study of protein–lipid systems. The natural fluorescence of proteins arises from tryptophan and tyrosine residues, which thus act as "built-in" nonperturbing probes of their immediate environment, reporting in particular on polarity and accessibility to solvent and solutes. Some recent papers have indeed demonstrated that intrinsic fluorescence offers a sensitive technique for observing interactions between lipids and a wide range of proteins, including intrinsic membrane proteins (Dufourq et al., 1975; Gennis et al., 1976), apolipoproteins (Verdery and Nichols, 1974; Gwynne et al., 1975), and physiologically active peptides (Schneider and Edelhoch, 1972; Epand et al., 1977; Dufourq and Faucon, 1977).

The subject of the present investigation is the hydrophobic membrane protein lipophilin, isolated from the proteolipid of human myelin (Gagnon et al., 1971). When recombined with model phospholipid vesicles, this protein appears to be deeply embedded in the bilayer, with considerable effects on lipid organization (Papahadjopoulos et al., 1975; Boggs et al., 1976, 1977). Lipophilin has also been rendered water soluble by three dialysis procedures, yielding products differing in aggregation state and secondary structure; as described in the preceding paper (Cockle et al., 1978), it has a pronounced tendency to adopt conformations of high helical content, especially in the presence of phospholipids, when the proportion of α helix may be as high as 75%. To gain further information on the internal structure of the protein, we have now examined the intrinsic fluorescence of lipophilin alone and combined with lysolecithin micelles and phosphatidylcholine vesicles, with particular regard to variations in quantum yield, energy transfer, and susceptibility to fluorescence quenching. While characteristic differences exist between the water-soluble forms of the protein, the fluorescence of lipophilin is shown to be very sensitive to the binding of phospholipids. The induced changes are consistent with extensive hydrophobic interaction between the components, and permit protein–lipid stoichiometries to be estimated. Fluorescence spectra of an analogous bovine myelin proteolipid apoprotein, as well as of whole myelin itself, have been briefly described by other authors (Feinstein and Felsenfeld, 1975a,b; Crang et al., 1974).

Materials and Methods

Lipophilin and Lipids. Lipophilin was purified from normal human myelin by the procedure of Gagnon et al. (1971). The protein content of the lyophilized product was verified by amino acid analysis. The three water-soluble forms, denoted A, B, and C, were prepared as previously described (Moscarello et al., 1973; Cockle et al., 1978).

Lysolecithin was purchased from Sigma Chemical Co.; its purity was assessed as 98% by phosphate analysis (Bartlett, 1959), assuming a mean molecular weight of 510. Interaction with lipophilin was studied by mixing aqueous solutions of the components in suitable proportions. Lipophilin was combined with phosphatidylcholine (egg yolk lecithin; Serdary Research Laboratory, London, Ontario) by dialysis of a mixture containing protein and lipid in the ratio 3:1 by weight from 90% v/v 2-chloroethanol–water into 10 mM NaCl–1 mM EDTA at pH 7.4 and 4 °C (Cockle et al., 1978). A similar procedure was used to introduce the protein into dimyristoylphosphatidylcholine (Sigma) vesicles at 37 °C, starting with a protein/lipid ratio of 1:2 by weight.

Other Materials. 2-Chloroethanol (BDH Chemicals, Toronto) was purified by vacuum distillation over Tris[1] and Girards Reagent T (4 g of each per L) and used while fresh; the

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[1] Abbreviations used are: Tris, tris(hydroxymethyl)aminomethane; UV, ultraviolet; PC, phosphatidylcholine; CD, circular dichroism; DMPC, dimyristoylphosphatidylcholine; DPPC, dipalmitoylphosphatidylcholine.

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TABLE I: Absorption Properties of Lipophilin and Model Compounds.

Material	λ_{max} (nm)	Extinction coefficients[a] $E(\lambda_{max})$	$E(280)$	$E(289)$	$E(295)$
Aqueous solutions					
Lipophilin	278	16.5 ± 0.3	16.4 ± 0.3		5.1 ± 0.2
Lipophilin–lysolecithin[b]	278	17.6 ± 0.3	17.5 ± 0.3		5.4 ± 0.2
2-Chloroethanol solutions					
Lipophilin	278	17.1 ± 0.3	16.9 ± 0.3	10.7 ± 0.2	4.3 ± 0.2
N-Acetyl-L-tyrosinamide	277	1645 ± 5	1530 ± 5	525 ± 5	40
N-Acetyl-L-tryptophanamide	281	6070 ± 15	6035 ± 10	5095 ± 10	2355 ± 5
L-Phenylalanine			3	3	2
L-Cystine			108	57	36

[a] $E_{1\text{-cm}}^{1\%}$ for lipophilin, and molar extinctions ϵ for the model compounds; mean values with their standard errors. [b] Measurements were made on several samples (water-soluble forms A,B, and C) in the presence of excess lysolecithin (lipid/protein ratio at least 5:1 by weight).

UV absorbance at 280 nm was <0.005. Acrylamide (electrophoresis grade, Eastman Kodak Co.) was recrystallized twice from chloroform and had a molar extinction coefficient of 0.231 at 295 nm. L-Tyrosine and L-tryptophan (Sigma) and *N*-acetyl-L-tyrosinamide and *N*-acetyl-L-tryptophanamide (Cyclo Chemical Corp.) were desiccated before use.

Amino Acid Analyses. Lipophilin (0.5 mg) was hydrolyzed in 1 mL of 6 N HCl under vacuum in a sealed tube at 110 °C for 24 or 48 h. Hydrolysates were evaporated to dryness and processed on a Technicon automatic amino acid analyzer, or assayed for tyrosine alone by the fluorimetric procedure of Udenfriend and Cooper (1952). Hydrolysis of the protein in 3 N mercaptoethanesulfonic acid (Penke et al., 1974) was found to be unsatisfactory for protecting tryptophan. Two colorimetric methods were adopted for in situ determination of tryptophan in lipophilin (Spies and Chambers, 1949, procedure N; Fischl, 1960); free tryptophan was the standard in each case.

Ultraviolet Absorption. Spectra were recorded on a Cary 118 spectrophotometer thermostated at 25 °C, using 1-cm path length cells. Protein samples were first centrifuged at 26 000*g* for 30 min to sediment any particulate matter, and a correction for residual light-scattering was applied by means of a linear plot of log(optical density) vs. log(wavelength) constructed from data in the range 340–400 nm.

Fluorescence. Measurements were made at 25 °C on a Perkin-Elmer MPF-44 fluorimeter, using 4-nm bandwidths. The instrument was routinely operated in the ratio mode, although excitation spectra were recorded in the corrected mode. Protein concentrations were in the range 25–55 μg/mL as determined from the absorbance at 280 nm; optical densities were then below 0.1 at the exciting wavelength (280 or 295 nm). To allow for attenuation of the incident light intensity by self-absorption of the sample, corresponding fluorescence and absorption measurements were made on a series of tryptophan solutions in the concentration range 10–120 μM, which established the operational relationship

$$\log K = 0.547A \quad (1)$$

between the inner filter correction factor K and the absorbance A at the wavelength of excitation; 0.547 cm should correspond closely to the incident light path (nominally one-half the width of a standard 1 cm cuvette). Integrated fluorescence intensities I were measured with a planimeter and expressed as relative quantum yields ϕ' with respect to a suitable standard (either free tryptophan in water at pH 7.0, or *N*-acetyltryptophanamide in 2-chloroethanol). The spectrum of each protein or protein–lipid system was generally determined at least three times with two or more preparations, and the results were averaged.

TABLE II: Tyrosine and Tryptophan Determinations on Lipophilin.[a]

Method	Tyrosine (μmol/g)	Tryptophan (μmol/g)	Tyr/Trp ratio
Amino acid analysis	478 ± 8		
Tyrosine fluorogen[b]	497 ± 11		3.1 ± 0.1
Tryptophan chromogen[c]		158 ± 2	
Tryptophan chromogen[d]		159 ± 2	
Ultraviolet absorption	459 ± 27	160 ± 9	2.9 ± 0.2

[a] Mean values with their standard errors. [b] Udenfriend and Cooper (1952). [c] Spies and Chambers (1949). [d] Fischl (1960).

Results and Discussion

Ultraviolet Absorption of Lipophilin. The aromatic absorption spectra of the three water-soluble forms of the protein were virtually indistinguishable, with a band maximum at 278 nm. Intensities were slightly enhanced on interaction with lysolecithin micelles, while the fine structure due to phenylalanine and tryptophan residues was perceptibly sharper when the protein was dissolved in 2-chloroethanol. Extinction coefficients, determined on the basis of protein concentrations obtained by amino acid analysis, are summarized in Table I.

Tyrosine and Tryptophan Content. Interpretation of the intrinsic fluorescence properties of a protein requires knowledge of the relative, or preferably the absolute, numbers of tyrosine and tryptophan residues. Lipophilin was assayed separately for these amino acids by several methods as outlined above, yielding the results in Table II. The estimated tyrosine/tryptophan ratio (3.1 ± 0.1) is somewhat lower than the value 3.7 ± 0.5 calculated from the published amino acid composition (Gagnon et al., 1971).

Circular dichroism studies have demonstrated that lipophilin exists as extended helical chains in 2-chloroethanol (Cockle et al., 1978). Such a structure should ensure that the aromatic chromophores are largely exposed to solvent, experiencing a uniform environment similar to that of free amino acids. It should therefore be possible to determine the tyrosine–tryptophan composition directly from the UV spectrum in this medium, using suitable monomers for reference (cf. Edelhoch, 1967). *N*-Acetyltyrosinamide and *N*-acetyltryptophanamide were chosen to represent the tyrosine and tryptophan residues, which account for virtually all the absorption above 270 nm.

TABLE III: Fractional UV Absorbance of Aromatic Residues in Lipophilin.[a]

Residue	$f(280)$	$f(295)$
Tyrosine	0.43	0.05
Tryptophan	0.56	0.94
Others	0.01	0.01

[a] Results refer to lipophilin dissolved in 2-chloroethanol and are calculated from the equation $f_i(\lambda) = [n_i\epsilon_i(\lambda)]/[\Sigma_i n_i\epsilon_i(\lambda)]$, where i = tyrosine, tryptophan, phenylalanine, cystine. The relative numbers of residues n_i are respectively 12:4:20:3, and the molar extinction coefficients ϵ_i are taken from Table I.

Extinction coefficients were determined at 280 and 289 nm, which are convenient wavelengths for the protein and the tryptophan model, and are reported in Table I. The amino acid contents c (mol/g) were calculated from the simultaneous equations

$$E_p(\lambda) = \epsilon_{Tyr}(\lambda)c_{Tyr} + \epsilon_{Trp}(\lambda)c_{Trp} \quad (2)$$

at 280 and 289 nm, where E_p is the protein absorbance at 1 mg/mL concentration, and the ϵ's are molar extinctions.

The results from this procedure agreed well with those derived otherwise (Table II), and we conclude that the tyrosine/tryptophan ratio in lipophilin should be taken as 3.0. Allowance for the slight absorption due to phenylalanine and cystine residues (cf. Table I) had a negligible effect on this value. A model protein spectrum constructed from the appropriate mixture of monomers was almost coincident with the authentic spectrum above 265 nm (not shown). A total of 12 tyrosines and 4 tryptophans per protein subunit would correspond to a molecular weight around 25 000, which is close to estimates of 24 000 and 28 000 obtained by equilibrium centrifugation (Moscarello et al., 1973).

Fluorescence of Lipophilin in 2-Chloroethanol. The emission spectrum of lipophilin in 2-chloroethanol excited at 280 nm contains prominent features from both tyrosine and tryptophan residues and compares well with a composite spectrum obtained from a mixture of tyrosine and tryptophan monomers in the ratio 3:1 found in lipophilin (not shown). Since the protein fluorescence produced by excitation at 295 nm is due almost entirely to tryptophan, subtraction of this spectrum from the 280 nm spectrum after appropriate normalization at 380–400 nm, where only tryptophan fluoresces, yields the profile of the tryosine emission. It must be assumed that the spectral distribution of tryptophan fluorescence is independent of excitation wavelength, but this is assured if the population of chromophores is homogeneous. With the aid of the fractional absorbances in Table III, individual quantum yields for the tyrosine and tryptophan residues may then be calculated, for example

$$\phi'_{Tyr}(280) = \frac{I(280) - I(295)}{f_{Tyr}(280)A(280)} \Big/ \frac{I_{Std}(280)}{A_{Std}(280)} \quad (3)$$

where the fluorescence intensities I are normalized. Numerical data on the protein and the model compounds *N*-acetyltyrosinamide and *N*-acetyltryptophanamide are compared in Table IV.

Both absorption and fluorescence measurements on lipophilin in 2-chloroethanol appear consistent with a protein conformation in which the side chains are accessible to the medium, as anticipated for an extended helix with little tertiary folding (Cockle et al., 1978). Nevertheless, some additional information is available regarding the surroundings of the aromatic residues. Despite the similarity in intensity and bandwidth of the tryptophan fluorescence to that of *N*-acetyltryptophanamide, the blue shift of 4 nm in λ_{max} shows that the residues are not fully exposed. The direction of the shift suggests a local decrease in polarity (Teale, 1960), which might be due to the presence of apolar residues at neighboring sites in the amino acid sequence. The lack of dependence of tryptophan quantum yield on excitation wavelength indicates that resonance energy transfer between tyrosine and tryptophan is effectively absent; individual pairs of residues are probably separated by at least six other amino acids (see Edelhoch et al., 1968). Tyrosine fluorescence from proteins is generally characterized by small quantum yields (Longworth, 1971); for lipophilin in 2-chloroethanol, the yield is 86% that of *N*-acetyltyrosinamide, emphasizing the high degree of exposure to the solvent.

Fluorescence of Water-Soluble Lipophilin. For each of the three aqueous forms A, B, and C of lipophilin, the fluorescence is dominated by tryptophan, as generally occurs with undenatured proteins (Longworth, 1971). With 280-nm excitation, band maxima occur at 328–329 nm but form B is distinguished from A and C by a markedly higher intensity. On excitation at 295 nm, the emission bands are slightly narrower and shifted about 3 nm to the red (Table V). Such changes are consistent with a weak tyrosine contribution to the 280-nm spectrum, but could in principle arise from non-uniform absorption and fluorescence properties among the total population of tryptophan residues. Such heterogeneity has been demonstrated for a few proteins (e.g., papain; Weinryb and Steiner, 1970), but fluorescence quenching studies described later exclude this possibility in lipophilin. Moreover, pairs of emission spectra normalize satisfactorily between 380 and 400 nm, giving difference spectra representative of tyrosine fluorescence, as illustrated for lipophilin form A in Figure 1A. Relative quantum yields for the tyrosine and tryptophan residues (Table V) were estimated on the basis of the previously determined fractional absorbances for the protein dissolved in 2-chloroethanol.

Although the position of the emission maximum is not always a reliable measure of "exposure" of tryptophan residues (Kronman and Holmes, 1971), the fluorescence of water-sol-

TABLE IV: Fluorescence Characteristics of Lipophilin and Model Compounds in 2-Chloroethanol.

Material	λ_{max}[a] (nm)	$\Delta\lambda$[a] (nm)	$\phi'(280)$[b]	$\phi'(295)$[b]
N-Acetyl-L-tyrosinamide	302	30	0.71	
N-Acetyl-L-tryptophanamide	343	57	1.00	1.00
Lipophilin {Tyr component	301	31	0.61	
Lipophilin {Trp component	339	56	0.96	0.94

[a] The reproducibility of band maxima (λ_{max}) and width at half-height ($\Delta\lambda$) was ±1 nm. [b] Quantum yields (ϕ') are expressed relative to *N*-acetyltryptophanamide.

TABLE V: Fluorescence Characteristics of Lipophilin and Lipophilin–Phospholipid Complexes in Water.

Protein system[a]	Excitation at 280 nm[b] λ_{max} (nm)	$\Delta\lambda$ (nm)	ϕ'_{Tyr}	ϕ'_{Trp}	Excitation at 295 nm[b] λ_{max} (nm)	$\Delta\lambda$ (nm)	ϕ'_{Trp}	e[c]
Aqueous solutions								
Form A	329	59	0.11	0.44	331	56	0.34	0.35
Form B	329	60	0.13	0.63	333	56	0.48	0.40
Form C	328	59	0.10	0.41	331	57	0.33	0.30
Lysolecithin complexes								
Form A	325	64	0.33	0.70	332	56	0.61	0.20
Form B	327	63	0.27	0.73	333	58	0.63	0.20
Form C	325	65	0.41	0.82	332	57	0.71	0.20
PC vesicles	328	59	0.15	0.50	330	56	0.44	0.20

[a] The medium was distilled water in all cases, except for the PC vesicles, which were suspended in 10 mM NaCl. Sample compositions are referred to in the text. [b] The reproducibility of band maxima (λ_{max}) and width at half-height ($\Delta\lambda$) was ±1 nm. Quantum yields (ϕ') are expressed relative to tryptophan in water at pH 7.0; average deviations from the mean were within ±6%. [c] Efficiency of Tyr → Trp singlet resonance energy transfer. Average deviations from the mean were around ±10%, but the overall uncertainty is probably no better than ±20%.

uble lipophilin appears characteristic of tryptophan in a buried, hydrophobic environment (Teale, 1960; Burstein et al., 1973). This would be anticipated in the folded protein if, as suggested above, tryptophan is located amongst apolar residues in the primary sequence. The quantum yield of buried tryptophans is controlled mainly by static quenching processes which depend on the proximity and orientation of such perturbing groups as disulfide, sulfhydryl, and amine (Cowgill, 1968b). The variations in yield among the three aqueous forms of lipophilin may therefore reflect not only the known conformational differences, but also a greater structural rigidity in the interior of the highly aggregated form B relative to the trimeric forms A and C (see Cockle et al., 1978). In contrast, buried tyrosine residues are usually thought to be nonfluorescent, largely owing to hydrogen bonding to peptide and other polar groups (Cowgill, 1968a). However, in a truly hydrophobic environment, fluorescence enhancement would be anticipated, and such a situation has recently been described for the single tyrosine of calf thymus histone H1 (Giancotti et al., 1977). While the tyrosine yield in lipophilin is not insignificant (about 10% of that of free tyrosine at pH 7.0), it is therefore difficult to draw any firm conclusion regarding the disposition of these residues.

The dependence of tryptophan quantum yield on excitation wavelength provides evidence that resonance energy transfer from tyrosine can occur in the folded protein, contrary to the situation in 2-chloroethanol solution. The overall efficiency of energy transfer may be estimated from the expression

$$f_{Trp}(280)[\phi'_{Trp}(280) - \phi'_{Trp}(295)] = ef_{Tyr}(280)\phi'_{Trp}(295) \quad (4)$$

where $\phi'_{Trp}(295)$ is regarded as the unsensitized, wavelength-independent quantum yield of tryptophan, and e represents the fraction of tyrosine excitation energy transferred to tryptophan (cf. Eisinger, 1969). It is unlikely that transfer takes place uniformly between the considerable numbers of tyrosine and tryptophan residues present, though the small variations in e among the forms A, B, and C of lipophilin may reflect the differences in three-dimensional folding previously demonstrated by circular dichroism (Cockle et al., 1978).

Interaction with Lysolecithin. Addition of lysolecithin to aqueous lipophilin gave rise to enhanced fluorescence intensity, saturable at small lipid/protein ratios. Analogous effects of lysolecithin on the CD spectra of lipophilin were reported

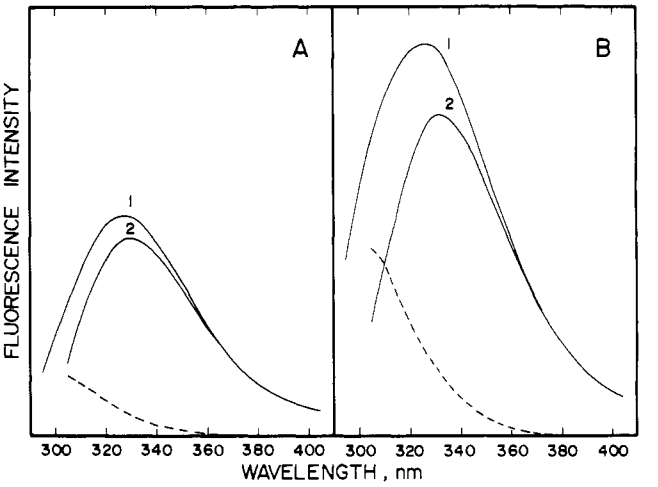

FIGURE 1: Normalized fluorescence spectra of lipophilin form A (35 μg/mL) alone (A), and in the presence of 170 μg/mL lysolecithin (B) at 25 °C; emission excited at 280 nm (1) and 295 nm (2), and their difference (- - -).

previously (Cockle et al., 1978). Titration curves were obtained by monitoring the tryptophan emission excited at 295 nm during the addition of small aliquots of a 2.5 mg/mL stock solution of lysolecithin and demonstrate appreciable differences between the three forms of the protein (Figure 2).

In aqueous solution, lysolecithin exists as micelles comprising 170–190 monomer units, with a critical micelle concentration below 120 μM, or 60 μg/mL (Lewis and Gottlieb, 1971). Schneider and Edelhoch (1972) have shown that, when lysolecithin is diluted to concentrations well below this value, some hours are required for dissociation into monomers. The protein–lipid interactions were therefore analyzed on the basis of a formal equilibrium between lipophilin monomers (molecular weight about 26 000) and intact micelles (particle weight about 92 000 for 180 molecules), described by the standard mass action law

$$\nu = nK[P]/(1 + K[P]) \quad (5)$$

Each micelle is assumed to bind up to n lipophilin monomers with a common intrinsic association constant K; [P] is the concentration of free protein in solution and ν is the average mole ratio of bound protein to lipid micelles. K and n are

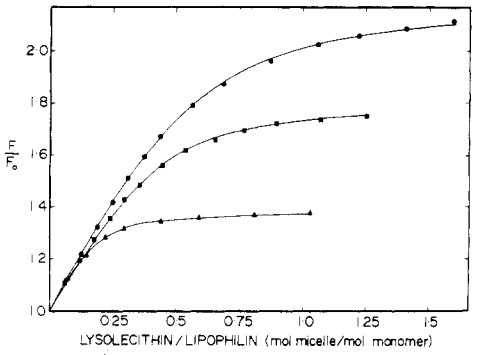

FIGURE 2: Fluorescence titrations of lipophilin (32–39 μg/mL) with lysolecithin at 25 °C. The points are experimental fluoresence intensities at 335 nm, with excitation at 295 nm: lipophilin forms A (■), B (▲), and C (●). The solid lines are theoretical curves generated from the values of K and n in Table VI.

conveniently determined from a Scatchard plot ($\nu/[P]$ vs. ν). To calculate the variables ν and [P], the amount of bound lipophilin was assumed to be directly proportional to the increase in fluorescence intensity F. The limiting intensity corresponding to complete binding of protein was obtained by plotting $1/F$ vs. $1/[\text{lipid}]$ (cf. Weber and Young, 1964).

This treatment appears to account satisfactorily for the observed behavior, yielding the binding parameters listed in Table VI; theoretical titration curves based on these values agree closely with the experimental data (Figure 2). Although the mean association constants are essentially the same for all three water-soluble forms of the protein, forms C and A evidently bind about three times as much lysolecithin per mole as form B. This result correlates with the known variations in particle size of the free protein. If some allowance is made for uncertainties in the mean aggregation number of lysolecithin before and after complexation, it is quite possible that forms C and A combine as the trimeric unit that exists in free solution. In contrast, the polymeric form B undoubtedly undergoes appreciable dissociation, the six monomers absorbed per micelle perhaps corresponding to one-third of an aggregate with molecular weight around 500 000. This interpretation is consistent with our previous analysis of the conformational changes that take place when lipophilin interacts with lysolecithin; the major effect with forms A and C is an elongation of helical segments with little increase in the total amount of helix, whereas the opposite is the case for form B (Cockle et al., 1978).

Fluorescence of Lysolecithin Complexes. Emission spectra of the three complexes with lysolecithin were studied in more detail using samples containing excess lipid (protein 30–50 μg/mL, lysolecithin 100–200 μg/mL). The data are collected in Table V, and normalized spectra obtained from lipophilin form A are illustrated in Figure 1B. Since band maxima and widths with 295-nm excitation remain unaffected on interaction of protein and lipid, no polarity change in the environment of the tryptophan residues is detected. However, the marked increases in quantum yield imply greater shielding from the polarizable groups that are normally responsible for tryptophan quenching. This would be expected if the complexes are stabilized primarily by hydrophobic interactions between the micelle interior and the many apolar amino acid side chains, rather than simply by charge neutralization.

The blue shift in the emission excited at 280 nm in relation to free aqueous lipophilin is due to a larger tyrosine contribution (compare Figures 1A and 1B). Since the λ_{max} of tyrosine is not environmentally sensitive, it is difficult to assess the

TABLE VI: Binding Parameters of Lipophilin–Lysolecithin Complexes.

Lipophilin	K (μM^{-1})	n
Form A	4.5	2.3
Form B	4.1	5.7
Form C	4.1	1.8

causes of this enhancement, although one likely possibility would be the transfer of some of the tyrosine residues to a more shielded, apolar environment, as for tryptophan. Part of the enhancement can certainly be attributed to a decrease in resonance energy transfer to tryptophan. The efficiency of transfer is determined by the distance between the fluorophores and also by the relative orientation of their transition dipoles (Steinberg, 1971), both of which might be influenced by the conformational changes we have previously described (Cockle et al., 1978). Thus, we suggested that certain irregularities in the CD spectra of lipophilin–lysolecithin mixtures might be due to Cotton effects of the aromatic chromophores themselves, implying that the mutual orientation of these residues might be influenced by the phospholipid.

Fluorescence of Lipophilin in Phosphatidylcholine Vesicles. Lipophilin and egg yolk phosphatidylcholine were combined in the same proportions, 3:1 by weight, as used previously to obtain optically clear samples for circular dichroism (Cockle et al., 1978). The data included in Table V demonstrate that the overall response of the fluorophores appears intermediate between that of aqueous lipophilin in form C, similarly prepared by dialysis from 2-chloroethanol, and the complex of form C with lysolecithin. The tryptophan fluorescence reports a typical apolar environment, with unchanged λ_{max} and a 30% higher yield than found in form C, while the extent of tyrosine–tryptophan energy transfer is reduced relative to the free protein. These results are consistent with a hydrophobic interaction between lipid and protein as discussed in the preceding paragraphs.

To investigate whether the protein fluorescence was sensitive to phase changes in the lipid bilayer, lipophilin was introduced into dimyristoylphosphatidylcholine vesicles, for which the gel–liquid transition conveniently occurs at 24 °C; egg yolk PC exists in a fluid state above −10 °C (Ladbrooke and Chapman, 1969). The protein/lipid ratio was 1:2 by weight for this experiment. When incorporated into dipalmitoylphosphatidylcholine vesicles, lipophilin immobilizes about half its own weight fraction of lipid (Papahadjopoulos et al., 1975), so by analogy, most of the DMPC molecules in the present case should be available to undergo the phase transition characteristic of the free phospholipid. Samples of this composition were appreciably turbid, though less so than a suspension of the lipid alone prepared in the same manner, and were therefore examined in a 4-mm path length cell to reduce light scattering losses. The tryptophan fluorescence intensity was similar to that observed in the PC vesicles of higher protein content, but the monotonic variation with temperature in the interval 5–45 °C was indistinguishable from that of lipophilin form C alone. Accordingly, any conformational perturbation of the protein induced by lipid phase change had no significant effect on the tryptophan quantum yield.

Excitation Spectra of Lipophilin. The dependence of tryptophan quantum yield on excitation wavelength has been discussed implicitly in terms of resonance energy transfer from tyrosine residues. A simple method of confirming that sensitization is in fact due to tyrosine is to measure the fluorescence

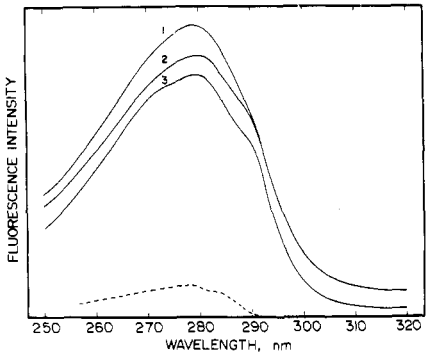

FIGURE 3: Normalized corrected excitation spectra at 25 °C of lipophilin form C (50 μg/mL) alone (1), and in the presence of 330 μg/mL lysolecithin (2), and their differences (- - -); excitation spectrum of lipophilin in 2-chloroethanol (3), displaced with respect to the vertical axis. Emission was measured at 380 nm.

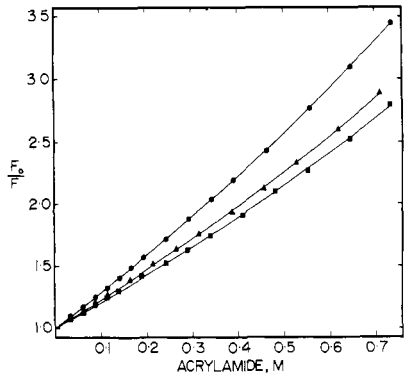

FIGURE 4: Stern-Volmer plots for acrylamide quenching of tryptophan fluorescence in lipophilin at 25 °C. The points are experimental intensities at 335 nm, with excitation at 295 nm: lipophilin forms B (●) and C (■), and lipophilin in PC vesicles (▲). The solid lines are best-fit curves according to eq 7.

excitation spectrum of the protein at a wavelength where only tryptophan emits, and establish a contribution from tyrosine. Figure 3 shows corrected excitation spectra for three lipophilin systems, recorded with emission wavelength 380 nm and a wide emission slit width (10 nm) to improve sensitivity. The spectrum of lipophilin in 2-chloroethanol, for which no energy transfer was previously detected, closely resembles an undistorted tryptophan absorbance. The spectra of water-soluble form C in the absence and presence of lysolecithin have been normalized at 295–310 nm, where tyrosine has almost no absorption. An additional component is detected in the spectrum of the protein alone, which largely disappears on interaction with lysolecithin; the difference spectrum has the features of a tyrosine absorption with band maximum at 278 nm. Thus the tryptophan fluorescence in the free aqueous protein is indeed sensitized by tyrosine. The same conclusion was reached on examination of fluorescence polarization spectra (Weber, 1960).

Fluorescence Quenching by Acrylamide. Polarizable ionic quenchers such as I^- and Cs^+ have been used widely to study the degree of exposure and heterogeneity of protein fluorophores (e.g., Burstein et al., 1973) but offer no selectivity if all the tryptophan residues lie below the surface. To investigate possible heterogeneities within and variations between the internal tryptophans of the various lipophilin systems, we have instead employed acrylamide; this is an extremely efficient collisional quencher of indole derivatives, capable of permeating the protein matrix and reporting on the "depth of burial" of the tryptophan residues (Eftink and Ghiron, 1975, 1976).

Experiments were carried out by titrating samples of aqueous protein or protein-lipid complex with small aliquots of 8 M acrylamide, to a final acrylamide concentration of around 0.7 M. The sample was excited at 295 nm, in order to monitor only tryptophan emission and also to minimize the amount of light absorbed by the quencher (although an inner filter correction was applied as described in Materials and Methods). Data were analyzed in terms of the classical Stern-Volmer law for collisional quenching

$$F_0/F = 1 + K_q[Q] \quad (6)$$

where F_0 and F are the fluorescence intensities in the absence and presence of quencher at concentration [Q], and the quenching constant $K_q = k_q\tau_0$, the product of a rate constant and the unquenched excited state lifetime τ_0. This relation predicts a linear plot of F_0/F vs. [Q] if all the fluorophores are equally accessible, but in the general case a downward curvature is expected, since subpopulations of fluorophores will tend to be quenched in the order of decreasing K_q. To allow for a possible static quenching component, Birks (1970) has introduced the modified equation

$$F_0/F = (1 + K_q[Q])\exp(V[Q]) \quad (7)$$

where the parameter V expresses the probability of finding a quencher molecule in contact with a fluorophore at the instant of excitation. A full description of the quenching processes in multi-tryptophan proteins therefore becomes very complex; however, since the exponential factor predisposes toward an upward curvature in the Stern-Volmer plot, useful qualitative information is often forthcoming.

TABLE VII: Acrylamide Quenching Parameters for Lipophilin and Lipophilin-Phospholipid Complexes in Water.

Protein system	K_q(eff) (M^{-1})	V(eff) (M^{-1})	K_q(eff)/$\phi'_{Trp}(295)$
Aqueous solutions			
Form A	2.2 ± 0.1	0.0	6.3
Form B	2.5 ± 0.0	0.24 ± 0.01	5.1
Form C	1.7 ± 0.1	0.27 ± 0.03	5.0
Lysolecithin complexes			
Form A	2.3 ± 0.1	0.05 ± 0.02	3.8
Form B	2.2 ± 0.0	0.08 ± 0.01	3.5
Form C	2.7 ± 0.1	0.07 ± 0.03	3.8
PC vesicles	2.0 ± 0.1	0.24 ± 0.03	4.5

Representative Stern-Volmer plots for the various lipophilin systems are presented in Figure 4; these illustrate not only the narrow range of the response to acrylamide, but also the general tendency toward a positive deviation from linearity. Although it is not possible to differentiate a priori the opposing effects of static and selective collisional quenching on the final curvature, experience has shown that acrylamide is predominantly a collisional quencher (Eftink and Ghiron, 1976). Thus the curves imply that the tryptophan residues in each lipophilin system are quite uniformly accessible and so presumably occupy similar environments (discounting the chance that the fluorescence is always dominated by a small proportion of the tryptophans). On this basis, the experimental data were fitted to eq 7 using a nonlinear least-squares curve-fitting computer program, to provide some numerical comparison among the different states of the protein. The resulting "effective" parameters are listed in Table VII.

V(eff) serves as an index of curvature in the Stern–Volmer plot, but the physical significance of the variations observed is impossible to decide; the values themselves are comparable to those reported for single tryptophan proteins (Eftink and Ghiron, 1976). K_q(eff) can be regarded less ambiguously as a mean quenching constant, the results being appropriate for tryptophans buried in apolar sites (Eftink and Ghiron, 1976). However, a true measure of kinetic exposure requires knowledge of the average lifetime τ_0 of the excited state, which is unlikely to remain constant for all forms of the protein. In the absence of fluorescence decay measurements, we will assume that τ_0 is in fact proportional to the observed relative quantum yield $\phi'_{Trp}(295)$ (Table V). Accordingly the ratio $K_q(\text{eff})/\phi'_{Trp}(295)$ (Table VII) should provide an approximate measure of the quenching rate constant k_q. Since all encounters between acrylamide and an excited fluorophore result in quenching (Eftink and Ghiron, 1976), changes in k_q solely reflect changes in the rate of diffusion of the reagent through the protein or protein–lipid matrix. While the variations in conformation and aggregation state among the water-soluble forms of lipophilin exert some influence on the average exposure of fluorescing tryptophans, interaction between protein and lipid clearly results in considerable shielding of these fluorophores from acrylamide. This we infer is due to a close association of the lipid with the hydrophobic tryptophan residues, aided by the helical conformations adopted by the protein (Cockle et al., 1978). The screening effect appears more marked for lysolecithin micelles than for phosphatidylcholine vesicles.

For most of the lipophilin systems examined, the fluorescence quenching was accompanied by a small blue shift of 1–2 nm in λ_{max}. A similar effect has even been detected in certain single-tryptophan proteins (Eftink and Ghiron, 1976) and presumably indicates some slight environmental heterogeneity; "blue" tryptophans are likely to be more buried and therefore less easily quenched. The absence of a red shift on quenching provides strong evidence that acrylamide does not denature the protein since denaturation usually leads to a greater average exposure of tryptophan residues.

Conclusion

This investigation demonstrates that intrinsic fluorescence provides a useful complement to circular dichroism in structural studies on lipophilin, particularly with regard to conformational changes induced by interaction with phospholipids. The technique also enjoys the important advantage that particulate and aggregated samples do not normally present problems of spectrum interpretation. The fluorescence behavior of lipophilin shows that the tryptophan residues are probably located among apolar amino acids in the primary sequence and are thus buried in internal hydrophobic regions when the protein is solubilized in water. The resulting tryptophan sites appear to be remarkably uniform in view of the response to the permeant quenching agent acrylamide. Folding of the protein results in appreciable tyrosine–tryptophan energy transfer not observed when the protein is dissolved in 2-chloroethanol, when the conformation is entirely helical with little or no tertiary structure. In comparison with these results, Feinstein and Felsenfeld (1975b) have reported that the tryptophans in bovine myelin proteolipid apoprotein are also buried in hydrophobic sites virtually inaccessible to the surface quencher iodide.

Treatment of aqueous lipophilin with lysolecithin, or combination of the protein with phosphatidylcholine, gives rise to large increases in fluorescence intensity without change in λ_{max} when the relative tryptophan and tyrosine contributions are taken into account. In this respect, the behavior of lipophilin resembles that of cytochrome b_5 (Dufourq et al., 1975) and apoprotein A-I of serum high density lipoprotein (Verdery and Nichols, 1974). We conclude that hydrophobic interaction between protein and lipid serves to diminish the exposure of tryptophan fluorophores both to intrinsic quenching groups and to acrylamide, while increases in the average helical segment length (Cockle et al., 1978) reduce the extent of energy transfer between tyrosine and tryptophan residues. The fluorescence enhancement provides a convenient means of evaluating the stoichiometry of lipophilin–lysolecithin binding, which appears to be dependent on the initial aggregation state of the protein. The estimated binding constants (approximately 4 μM^{-1}) are about one order of magnitude smaller than for apolipoprotein A-I, a molecule of similar size, but not self-associated under the experimental conditions (Gwynne et al., 1975).

From the variation of the fluorescence intensity of the lipophilin–DMPC complex with temperature, it is evident that the protein fluorophores do not detect any melting phenomenon in the bulk lipid. This is consistent with the presence of a layer of immobilized boundary lipid around the protein which is no longer able to participate in solid–liquid phase changes. Such an immobilized component has been demonstrated by calorimetric (Papahadjopoulos et al., 1975) and spin label (Boggs et al., 1976) studies on lipophilin–PC and lipophilin–DPPC systems. Moreover, recent calorimetric work on the interaction of bovine myelin protelipid apoprotein with DMPC (Curatolo et al., 1977) has indicated that the ordering effect of this protein is so great as to embrace all the lipid at protein concentrations greater than 20 wt %. In a number of other cases, the tryptophans of lipid-associated proteins and peptides have been shown to be markedly sensitive to lipid phase changes (cytochrome b_5, Dufourq et al., 1975; melittin, Dufourq and Faucon, 1977; glucagon, Epand et al., 1977). Both situations are of course compatible with the intimate association of the membrane components, the differences presumably relating to the presence or absence of substantial boundary lipid.

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Mechanism of Ethanol-Induced Changes in Lipid Composition of *Escherichia coli:* Inhibition of Saturated Fatty Acid Synthesis in Vivo†

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ABSTRACT: The in vivo effects of ethanol on lipid synthesis in *Escherichia coli* have been examined. Under conditions which uncoupled fatty acid synthesis from phospholipid synthesis, ethanol decreased the amount of saturated fatty acids synthesized but had little effect on the selectivity of their incorporation into phospholipids. In the absence of fatty acid degradation and unsaturated fatty acid synthesis, *E. coli* was still able to adapt its membrane lipids to ethanol, while the inhibition of total fatty acid synthesis eliminated this response. During growth in the presence of ethanol, strain K1060 (an unsaturated fatty acid auxotroph) incorporated an increased amount of exogenous heptadecanoic acid (17:0) to compensate for the reduction in palmitic acid (16:0) available from biosynthesis. Thus, our results indicate that the reduced levels of saturated fatty acids observed in the phospholipids of *E. coli* following growth in the presence of ethanol result primarily from a decrease in the amounts of saturated fatty acids available for phospholipid synthesis.

In a previous report from our laboratory it was shown that *Escherichia coli* will alter its membrane fatty acid composition when grown in the presence of straight chain alcohols (Ingram, 1976). These alcohol-induced fatty acid changes are similar to those induced by changes in growth temperature (Marr & Ingraham, 1962). Short chain alcohols such as ethanol cause a decrease in the amounts of saturated fatty acids, similar to a shift down in growth temperature. Long chain alcohols such as hexanol induce changes analogous to a shift up in temperature, an increase in the proportion of saturated fatty acids (Ingram, 1976). Like temperature adaptation, these changes in fatty acid composition are independent of major changes in phospholipid composition (Ingram, 1977a). These results with alcohols have been extended to show that a wide variety of other lipophilic agents (organic solvents and food preservatives) also induce fatty acid changes in *E. coli* (Ingram, 1976). The diverse structures of these compounds as well as their lipophilic nature support the theory that these changes in fatty acid composition result from a general interaction with a hydrophobic site, rather than a result of specific catabolic processes (Ingram, 1977b). The intercalation of these agents into the membrane would be expected to alter membrane fluidity. The

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