Fluorescence Studies on a Membrane-Embedded Peptide from the Carboxy Terminus of Lipophilin†

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ABSTRACT: Fluorescence of an intramembranous polypeptide (T-3) derived from the carboxy-terminal sequence of lipophilin was studied in aqueous solution, detergent micelles, and lipid vesicles. In all cases, the fluorescence of the only Trp (211) was indicative of a hydrophobic, buried residue. Addition of lysophosphatidylcholine (LPC) or phosphatidylcholine (PC) gave Trp-211 a more hydrophobic, less quenching environment as compared to that in aqueous solution. Energy transfer between Trp and Tyr observed in aqueous solution was decreased by the addition of lipid or detergent. There was limited quenching by acrylamide both in the aqueous and in the lipid or detergent environments. However, PC or LPC further decreased this quenching. Cs^+ and I^- were even less accessible than acrylamide to Trp, further proving that the Trp was located inside the lipid bilayer. The quenching indicated that I^- binds to positive charges of the protein located on the surface of the membrane. This, combined with knowledge of the sequence of lipophilin, suggested that Trp-211 was located within the membrane but was close to amino acid residues that are external to the bilayer.

Lipophilin is an intrinsic protein of the myelin membrane of the human central nervous system. In model membrane studies in which lipophilin was incorporated into phosphatidylcholine vesicles, it was shown to span the bilayer by both X-ray diffraction (Brady et al., 1979) and labeling with a membrane-impermeant reagent (Wood et al., 1980). These studies demonstrated clearly that segments of the protein were exposed to the hydrophilic environment, although most of it was embedded in the hydrophobic region of the bilayer. From its sequence, this proteolipid was predicted to have four large domains embedded in the bilayer with segments exposed outside the membrane (Stoffel et al., 1983; Larsen et al., 1983).

The organization of lipophilin in the membrane is an important question since this protein is a major component of myelin and presumably plays an important role in myelin function. In a recent study, using a membrane-permeant reagent, 3-(trifluoromethyl)-3-(*m*-[^{125}I]iodophenyl)diazirine (^{125}I-TID), that labeled the intramembranous portions of lipophilin, we were able to isolate and identify by sequence studies four labeled peptides (Kahan & Moscarello, 1985). One of these peptides represented the carboxy-terminal sequence (residues 205–268) of the protein.

When this peptide (previously referred to as T-3) was incorporated into vesicles of phosphatidylcholine, it was found to be highly α-helical (unpublished results). In order to extend our studies of the intramembranous organization of this carboxy-terminal fragment, we took advantage of the fact that it contained a single Trp residue at position 211 of the peptide chain. In this way we could follow the fluorescence changes that occurred at this site when T-3 interacted with lipids.

In an earlier study, the fluorescence characteristics of human lipophilin in both aqueous and lipid environments were studied (Cockle et al., 1978). The Trp residues were located in hydrophobic sites, in agreement with findings on the bovine myelin proteolipid apoprotein (Feinstein & Felsenfeld, 1975a,b; de Foresta et al., 1979). Since lipophilin has four Trp residues in its sequence, individual Trp residues could not be studied. Peptide T-3 thus provided us with the opportunity to study the specific site (position 211) near the carboxy terminus of the peptide.

Materials and Methods

Preparation of Peptide T-3. Peptide T-3 was prepared by tryptic digestion of myelin, followed by reduction and alkylation of the myelin membrane and extraction with chloroform/methanol (1:1) containing 5% 0.1 N HCl. The chloroform extract was separated on Sephadex LH-60 eluted with the same solvent (Kahan & Moscarello, 1985). The acidified chloroform/methanol solution was then dialyzed against chloroform/methanol (2:1) in order to remove HCl. For the preparation of the water-soluble form of T-3, the peptide in chloroform/methanol was dried down, redissolved in 2-chloroethanol, and then dialyzed against several changes of distilled water (Cockle et al., 1978).

Preparation of T-3 Lipid Vesicles. A small amount of egg PC[1] (0.5–1.2 mg) in chloroform (10 mg/mL) was mixed with 0.1–0.2 mL of a solution of T-3 (containing 75–150 μg of peptide) in chloroform/methanol (2:1). The solvent was evaporated under N_2. Distilled water (2 mL) was added, and the lipid/T-3 mixture was left to hydrate for 1 h at room temperature. The vesicle suspension was sonicated on ice by successive 1-min bursts for 5 min with a probe sonicator. The resulting mixture was then centrifuged in an Eppendorf table-top centrifuge. The final protein concentration was found to be 25–45 μg/mL by amino acid analysis. The final lipid concentration was found to be 0.23–0.5 mg/mL by phosphorus analysis (Bartlett, 1959). The pH of this solution was 6.0.

Fluorometry. Fluorescence spectra were obtained with a Perkin-Elmer Model 650-40 spectrofluorometer. Light scattering (Rayleigh and Raman) was reduced by using polarizing filters in the excitation and the emission beams

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[1] Abbreviations: PC, phosphatidylcholine; LPC, lysophosphatidylcholine; *N*-Ac-Trp-amide, *N*-acetyltryptophanamide; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride.

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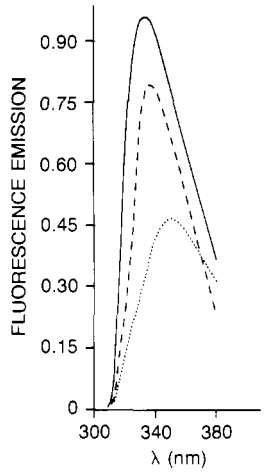

FIGURE 1: Fluorescence emission spectra, normalized to the same amount of protein. Excitation was at 295 nm. T-3 in PC vesicles (—); aqueous T-3 (– –); T-3 in chloroform/methanol (2:1) (···).

(Guilbault, 1973). The sample was contained in a quartz cuvette (1 × 1 cm), and the instrument was operated in the ratio mode. Excitation and emission slits were 4 and 10 nm, respectively. The optical density of all the solutions tested was adjusted, if necessary, by dilution in order to give absorbance of ~0.05 at 295 nm. The fluorescence intensity was found to be directly proportional to concentration up to an absorbance of 0.12. The concentration of protein was determined by amino acid analysis. R_{Trp}, the relative quantum yield of the tryptophan in T-3 compared with that of free tryptophan, was calculated according to the formula:

$$F_1/F_2 \times A_2/A_1$$

where F_1 and F_2 refer to areas under the emission spectra for T-3 and Trp, respectively, and A_2 and A_1 refer to the absorbance at the excitation wavelength for Trp and T3, respectively. The analogous relationship for Tyr was used to determine R_{Tyr}.

For the quenching experiments with acrylamide, small aliquots of an 8 M solution were added to a final concentration of 0.7 M acrylamide. Correction for the attenuation of excitation light intensity by acrylamide was done as described in the text. Correction for the attenuation of fluorescence emission due to dilution was done by titration of another sample of T-3 with the same small aliquots of H_2O instead of acrylamide.

For quenching with I^-, a solution of KI (5 M) was prepared, and a small amount of S_2O^{2-} was added to the iodide solution to prevent I_3^- formation (Lehrer, 1970). Small aliquots of this solution were added up to a concentration of 0.2 M. Another sample of T-3 was titrated in parallel with NaCl solution (5 M) to correct for ionic strength and dilution effects. For the quenching with Cs^+, a CsCl solution (5 M) was prepared, and a similar protocol as for I^- quenching was followed.

Fractional UV absorbances of aromatic residues in T-3 dissolved in chloroform/methanol were calculated by using the extinction coefficients for *N*-acetyl-L-tyrosinamide and *N*-acetyl-L-tryptophanamide derived by Cockle et al. (1978). Thus, $\int_{Tyr}(280)$ was calculated as 0.336 and corresponds to the absorbance of two Tyr residues. Fractional absorbance due to Trp-211 ($\int_{Trp}$) was 0.664. At 295 nm, the absorbance was due 97% to Trp-211.

Results and Discussion

Fluorescence of T-3 in Chloroform/Methanol. The emission characteristics of T-3 in chloroform/methanol (Figure 1) are similar to those of free *N*-Ac-Trp-amide in this solvent (Table I). The quantum yield does not change with λ of excitation, in chloroform/methanol. CD studies showed that T3 exists in this solvent as a extended helix, in analogy with results of intact lipophilin in 2-chloroethanol (Cockle et al., 1978). The peptide T-3, however, exhibited a 3-nm blue shift compared to *N*-Ac-Trp-amide, perhaps because the residues of the peptide were not fully exposed to the solvent. Chloroform is a fluorescence quencher (Eftink, 1976). This explains the lower fluorescence emission intensity of T-3 in chloroform/methanol as opposed to T-3 as an aqueous solution (Figure 1).

Table I: Fluorescence Characteristics of T-3[a]

sample	λ_{max} (nm) (excitation at 295 nm)	$R_{Trp}(295)$	$R_{Trp}(280)$	e^d
T-3 in aqueous solution[b]	335	0.24	0.27	0.25
T-3 in PC vesicles[b]	333	0.28	0.30	0.14
T-3 in LPC micelles[b]	333	0.34	0.36	0.12
T-3 in chloroform/methanol (2:1)[c]	342	0.94	0.94	
N-Ac-Trp-amide in chloroform/methanol (2:1)[c]	345	1	1	

[a] R_{Trp} values represent the mean of three experiments. Standard deviations from the means were within ±5%. [b] R_{Trp} values are expressed relative to tryptophan in water. T-3 in PC vesicles (40 μg/mL) was prepared as described in the text. T-3 in LPC micelle (67 μg/mL) was in a ratio of 1:6 protein:lipid by weight. [c] R_{Trp} values are expressed relative to *N*-acetyltryptophanamide in chloroform/metahnol (2:1). [d] Efficiency of Tyr–Trp singlet resonance energy transfer.

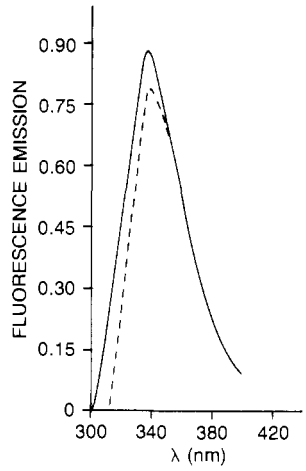

FIGURE 2: Normalized fluorescence emission of aqueous T-3 (67 μg/mL): excitation at 280 nm (—); excitation at 295 nm (– –).

Fluorescence of T-3 in Aqueous Solution. The observed fluorescence of T-3 in aqueous solution is shown in Figure 2. The emission spectrum resulting from excitation at 280 nm showed fluorescence from both Tyr and Trp (Figure 2). Excitation at λ equal to or greater than 295 nm is absorbed mainly by Trp. After these two spectra were normalized in the wavelength range 280–400 nm, the difference between these two fluorescence spectra should correspond to the fluorescence of Tyr. As can be seen from Figure 2, the fluorescence Emission of T-3 with 280-nm excitation is dominated by Trp with a small but significant contribution from Tyr.

The fluorescence of T-3 in aqueous solution has an emission maximum at 335 nm, which was independent of the λ of excitation. This indicated that the Trp residue of T-3 was buried in a hydrophobic environment since the emission maximum of free Trp or simple peptides of Trp is near 350

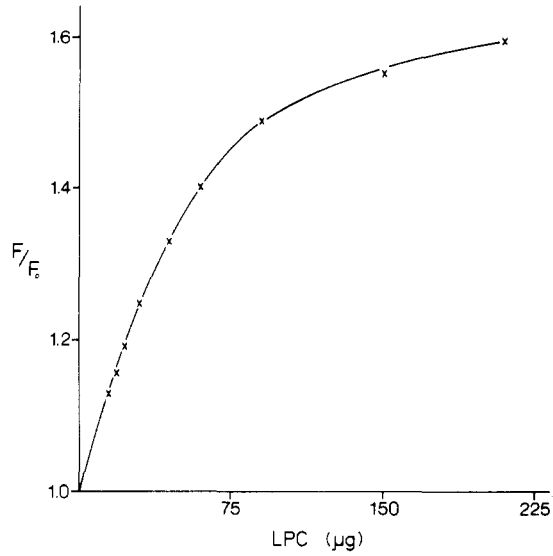

FIGURE 3: Fluorescence titration of T-3 (24 μg/mL) with lysolecithin. The points represent fluorescence intensities at 335 nm with excitation at 295 nm. The solid line is the theoretical curve obtained with the values of K and n given in the text.

nm (Teale, 1960; Lehrer, 1976). The fluorescence quantum yield for a buried but nonquenched Trp is 2.5–3.0 times that for free Trp (Cogwill, 1968). T-3 in aqueous solution has a quantum yield of only 0.24 that of free Trp (Table I), indicating that its fluorescence was quenched. When excitation at 280 nm was used, there was a small increase in quantum yield (0.27). The dependence of quantum yield on excitation wavelength can be interpreted as evidence of resonance energy transfer from Tyr to Trp, assuming that the shape of the Trp spectrum was the same for Trp in T-3 as for free Trp. The efficiency of energy transfer was determined by the distance between Tyr and Trp as well as by the relative orientation of their transition dipoles. The efficiency of energy transfer, e, can be estimated from the following expression (Cockle, 1978):

$$f_{Trp}(280)[R_{Trp}(280) - R_{Trp}(295)] = ef_{Tyr}(280)R_{Trp}(295)$$

For aqueous T-3, e had a value of 0.25 (Table I). It is conceivable that energy transfer in T-3 occurs between Tyr-206 and Trp-211. These two residues are situated close in the sequence (5.4 Å for 3.6 amino acids per turn of α-helix), within the Förster distance (~15 Å) necessary for 50% efficiency of energy transfer to occur (Steinberg, 1971). The other Tyr, at position 262 in the sequence of T-3, may not participate at all in energy transfer with Trp-211. In this case, the value of $e = 0.25$ may represent 50% efficiency of energy transfer only from Tyr-206 to Trp-211.

Fluorescence of T-3 in Lysolecithin (LPC). Lysolecithin (LPC) was added as small aliquots from a 2.5 mg/mL stock solution to the aqueous form of T-3 (Figure 3). This gave rise to enhanced fluorescence intensity, saturable at an LPC/protein weight ratio of ~10 by weight. Near the saturation point, the fluorescence intensity was increased by ~60% relative to that of the aqueous T-3. There was also a 2-nm blue shift in the emission maximum (Table I). The peptide–LPC interaction was analyzed according to the binding equation derived for a single independent binding site composed of n molecules of LPC. The dissociation constant and number of lipid molecules per binding site, as well as the maximum fluorescence changes, were calculated by a nonlinear least-squares curve-fitting program. This analysis of the fluorescence binding curve gave an affinity constant of (1.2 ± 0.2) × 10^6 M^{-1}, with 36 ± 1 lipid molecules/T-3 molecule or 5.1 T-3 molecules/lipid micelle and a maximum fluorescence change of $F/F_0 = 1.73 \pm 0.02$. The theoretical curve constructed with these values agrees well with the experimental data (Figure 3). The affinity constant for the binding of T-3 to LPC is only slightly less than that for lipophilin (~4 × 10^6 M^{-1}), and the number of "binding sites" for T-3 on LPC is within the range found for lipophilin (Cockle et al., 1978). The blue shift in fluorescence emission, the increase in fluorescence quantum yield, and the enhancement in the far-UV circular dichroism of the T-3/LPC complex (unpublished results), relative to aqueous T-3, indicate that lysolecithin produces structural changes in this peptide. This change provides a more hydrophobic environment for the Trp-211 of peptide T-3. The quantum yield changes only slightly with excitation at 280 nm. Therefore, the efficiency of energy transfer from Tyr to Trp is even lower in the presence of LPC than it is for the aqueous solution of T-3.

Fluorescence of T-3 Reconstituted into Phosphatidylcholine (PC) Vesicles. PC vesicles prepared as described under Materials and Methods contained protein and lipid in a weight ratio of 1:9–11, depending on the starting lipid and protein concentrations. Electron microscopy of phosphotungstic acid stained vesicles indicated that the diameter of sonicated vesicles was between 300 and 800 Å. The solution appeared optically clear as judged by the low absorbance at 330 nm.

Peptide T-3 in PC liposomes yielded emission spectra similar to the ones observed for aqueous solutions of T-3 (Figure 1). The emission maximum (333 nm) was identical with that obtained for the LPC/T-3 solution (Table I), indicating a slightly more hydrophobic environment for Trp in liposomes than in aqueous solution. The quantum yield in liposomes was higher than the quantum yield in water by ~17%. Therefore, PC induces similar conformational changes in T-3 as does LPC. This results in a decreased quenching of Trp. As found with LPC, T-3 in liposomes has a higher content of α-helix than its water-soluble form (I. Kahan, R. M. Epand, and M. A. Moscarello, unpublished results). Cogwill (1968) showed that an increase in the helical content of a polypeptide results in an increase in tryptophan fluorescence due to greater hydrogen bonding of the peptide carbonyl, which otherwise is a fluorescence quencher. Other residues such as unreduced disulfide groups (Cogwill, 1967), basic amino acid residues (Shinitzky & Goldman, 1967; Lowey, 1965), or ionized Tyr residues (Steinberg, 1971) also contribute to the quenching of Trp. T-3 may have a small amount of unreduced disulfide or unalkylated sulfhydride group, even though it was reduced and alkylated (Kahan & Moscarello, 1985). Also, the number of moles of T-3 incorporated in lysolecithin micelles (~5) may be different than that in PC vesicles. The extent of protein–protein interaction may influence Trp emission also. In some experiments, the pH was varied between 5 and 12 to determine whether Trp fluorescence was quenched by amino acids with a pK in this range. Between pH 5 and pH 11, the fluorescence remained constant. Increasing the pH above 11 caused precipitation of the vesicle solution.

Fluorescence Quenching by Acrylamide. Another method of investigating the degree of exposure of Trp-211 in peptide T-3 is to add an external quencher like acrylamide, a polar uncharged compound, to the system (Cockle et al., 1978). Samples of aqueous peptide T-3 or peptide/lipid complexes were titrated with small aliquots of 8 M acrylamide to a final acrylamide concentration of 0.7 M (Figure 4A). The fluorescence was measured at 333 nm except for the water-soluble form, for which it was measured at 335 nm. Since acrylamide absorbs light at the excitation wavelength (295

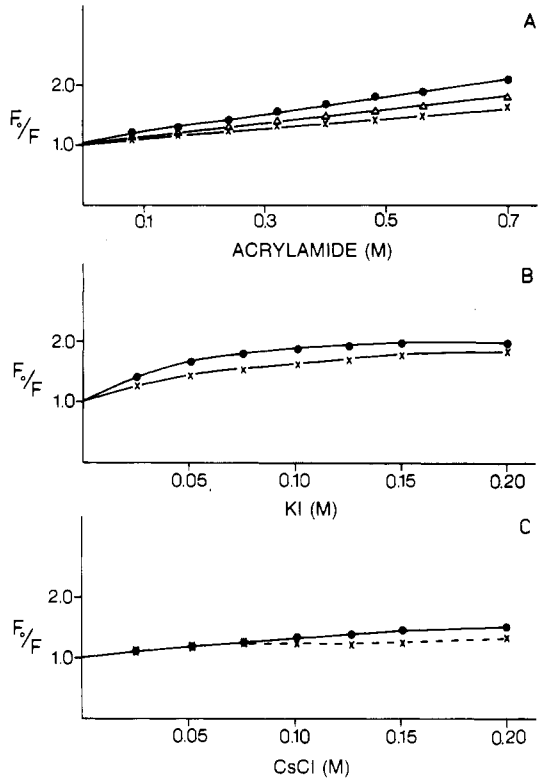

FIGURE 4: Stern–Volmer plots for quenching with acrylamide (A), KI (B), and CsCl (C). The points are fluorescence intensities measured at the emission maximum with excitation at 295 nm. Aqueous T-3 (●); T-3 in LPC micelle (Δ); T-3 in PC vesicles (×).

Table II: Acrylamide Quenching Parameters for T-3

sample	K_q (M^{-1})	$K_q/R_{Trp}(295)$
T-3 in aqueous solution	1.6	8.9
T-3 in PC liposomes	1.2	4.3
T-3 in LPC micelles	0.9	5.1

nm), an inner filter correction factor of antilog $\Delta A/2$ was applied (where ΔA is the incremental increase in the absorbance at the excitation wavelength). The molar extinction coefficient of acrylamide at 295 nm was ~0.23 M. Acrylamide did not induce a conformational change in T-3 since no appreciable protein difference spectrum was observed in the presence of the highest concentration of the quencher. No change in the emission maximum took place during titrations, suggesting that there was no change in the polarity of Trp. Also, the fluorescence emission difference spectrum of acrylamide quenching was constant, suggesting a uniform population of Trp. The quenching of the fluorescence by acrylamide follows the Stern–Volmer equation $F_0/F = 1 + K_q[Q]$. K_q equals $k\Gamma$, where k is the bimolecular collisional rate constant and Γ is the fluorescence lifetime of the unquenched species. There is little quenching by acrylamide of Trp-211 either in aqueous solution or in the presence of the lipids LPC or PC (Figure 4A). As shown in Table II, the most quenched sample is the aqueous T-3 with ~50% quenching at the highest acrylamide concentration used. The least quenched is T-3 in liposomes with ~40% quenching at 0.7 M acrylamide. The frequency of collision for Trp-211 in T-3 is very low and is similar to the buried single Trp of RNase T1, but it is not completely shielded as is the Trp of azurin (Eftink & Ghiron, 1976). The fact that acrylamide has some access to Trp-211 is not unexpected since this polar, uncharged compound can penetrate into the membrane. In the membrane, Trp-211 showed a slightly higher degree of shielding. The acrylamide quenching parameters are listed in Table II. The rate constant (k) was approximated by the ratio $K_q/R_{Trp}(295)$ since the value of Γ has not been determined. This assumes that Γ is proportional to the observed relative quantum yield $R_{Trp}(295)$ as has been shown (Weinryb & Steiner, 1968) for a large number of Trp derivatives under a wide variety of conditions. The values for K_q/R_{Trp} show that upon interaction with lipid or detergent there is an increase in the shielding of tryptophan from acrylamide. This effect is slightly more marked in PC vesicles than in lipophilin micelles, the reverse of the order found with lipophilin.

Fluorescence Quenching by I^- and Cs^+. As opposed to acrylamide, which penetrates the membrane, ionic quenchers like I^- and Cs^+ should quench only surface Trp residues (Lehrer, 1971). Figure 4B shows the Stern–Volmer plots for the fluorescence quenching of aqueous T-3 or T-3 reconstituted in PC with I^-. No change in the emission maximum was observed during these titrations, suggesting no change in the polarity of Trp. Figure 4C shows the curve for Cs^+ quenching. The same results were obtained when the titration was performed in distilled water at pH 6.0 or in 10 mM Tris-HCl, pH 8.4. The level of quenching with the highest concentration of I^- was similar to the corresponding quenching with acrylamide. However, the shape of the titration curve in the case of I^- showed deviation from the Stern–Volmer equation. The extent of quenching appeared to reach a maximum value at low I^- concentrations, suggesting that I^- binds to the charges exposed to the aqueous environment. Such an effect was reported by Lehrer (1971) for the quenching of human serum albumin fluorescence by I^-. Even so, the quenching of Trp-211 by I^- is quite small. Feinstein & Felsenfeld (1975a) also found a very weak quenching constant of Folch Pi protein for I^- (k = 0.2). Similar results were obtained with Cs^+ although the quenching in this case was even smaller than that with I^-, implying that T-3 has fewer negative charges exposed. The possible source of positive charges on T-3 would be Lys-217, Lys-228, and His-237. There may also be one negative charge on Gly-231 at the pH's used for this titration. PC is a zwitterionic lipid with zero net charge at neutral pH, although Cs^+ could bind to the negatively charged phosphate group of PC. However, the titration of the water-soluble T-3 was very similar to the titration of T-3 in liposomes, suggesting that Cs^+ binds directly to the protein. These binding sites are likely to be located on the surface of the membrane. Ion binding to these sites causes the local concentration of Cs^+ or I^- to be very high, resulting in the quenching of "buried" Trp.

Conclusions

(1) The fluorescence properties of T-3 are indicative of a tryptophan residue in a hydrophobic and/or constrained environment in the aqueous form or when complexed with lipid.

(2) Emission spectra in the presence and absence of lipid were similar, indicating that the protein folds and/or self-associates in aqueous solution in such a way as to sequester hydrophobic residues.

(3) In the presence of LPC or PC liposomes, Trp-211 of T-3 had a more hydrophobic environment, and the fluorophore was less quenched and was more shielded from acrylamide than in the water-soluble peptide. The lipid further decreases the efficiency of energy transfer between Tyr and Trp.

(4) The fluorescence intensity of the LPC/T-3 complex was increased compared to PC/T-3, implying less quenching in the presence of LPC. However, acrylamide quenching is slightly higher than that in PC; therefore, T-3 in lysolecithin micelles exists in a slightly different conformation than when incorporated in PC vesicles.

(5) These data show that Trp-211 was located in the sequence embedded in the membrane or in a hydrophobic segment of T-3 and possibly close to Tyr-206. This embedded sequence must be quite short since charged residues nearby in the sequence are exposed on the surface of the membrane. The data presented here agree well with the known properties of Trp in lipophilin (Cockle et al., 1978) and also with more recent experiments designed to establish the sites of intramembranous labeling of T-3 by the carbene generated from 3-(trifluoromethyl)-3-(*m*-[^{125}I]iodophenyl)diazirine (Kahan & Moscarello, 1985).

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Registry No. Trp, 73-22-3; *N*-Ac-Trp-amide, 2382-79-8.

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