Hydrophobic Oligopeptides in Solution and in Phospholipid Vesicles: Synthetic Fragments of Bacteriorhodopsin†

Toshiharu Sugihara,‡ Elkan R. Blout, and B. A. Wallace*

ABSTRACT: Hydrophobic oligopeptides which form part of the bacteriorhodopsin sequence (residues 48–54) were synthesized, and their conformations in various solvents and in dimyristoylphosphatidylcholine vesicles were studied by circular dichroism (CD) and nuclear magnetic resonance (NMR) spectroscopy. The peptides studied were Boc-Leu-Val-Pro-Ala-Ile-Ala-Phe-OBzl, Boc-Pro-Ala-Ile-Ala-Phe-OBzl, and Boc-Ile-Ala-Phe-OBzl. As the peptide was elongated, different secondary structures dominated in various solvents. In hexafluoroisopropyl alcohol, there was an abrupt increase in the two negative extremes below 220 nm in the CD spectrum of the heptapeptide, reflecting formation of a helical-like structure, most likely α or distorted α helix. In hexane, Boc-Leu-Val-Pro-Ala-Ile-Ala-Phe-OBzl and Boc-Pro-Ala-Ile-Ala-Phe-OBzl assumed a somewhat β-like structure, while Boc-Ile-Ala-Phe-OBzl did not. In dimyristoylphosphatidylcholine vesicles, the conformation of the heptapeptide was nearly identical with that in hexane, a β-like structure, while the CD spectra of the tripeptide and pentapeptide in vesicles were more reminiscent of helical structures. The behavior of two partially water-soluble peptides, HCl·Ile-Ala-Phe-OBzl and $(Sar)_{\sim 14}$-Ile-Ala-Phe-OBzl, in the presence and absence of the phospholipid vesicles was examined by circular dichroism and by proton and carbon-13 NMR. Both peptides showed similar conformational changes upon interaction with vesicles, but the association was stronger for HCl·Ile-Ala-Phe-OBzl than for its sarcosine derivative. Upon interaction, the mobility of the tripeptide benzyl ester hydrochloride was decreased; its aromatic rings were found to be located within the lipid bilayer, while the aromatic rings of the sarcosine derivative were localized more closely to the vesicle surface and the sarcosine chain remained in the aqueous phase.

The complex nature of membrane systems makes examination of protein conformations in membranes more difficult than in aqueous solutions. So that such studies could be simplified, liposomes have been used extensively as simple model systems for the lipid bilayer (Bangham et al., 1974). However, a good model for the protein component of biological membranes has not yet been found. Since membrane proteins are generally highly hydrophobic, with only a small percentage of hydrophilic amino acid residues, one such model might be a hydrophobic peptide. The requirements for such a model are that it can partition strongly into the membrane and be embedded in the lipid bilayer. It is expected that associated properties might be a secondary structure dissimilar to its structure in aqueous solvents but similar to its structure in hydrophobic solvents, and an effect on the local lipid environment structure. A likely choice would be a portion of the sequence of a known membrane protein. The primary structure of bacteriorhodopsin has been determined (Ovchinnikov, 1979; Khorana et al., 1979), its tertiary structure has been examined (Henderson & Unwin, 1975), and the spatial arrangement of the polypeptide chain in the membrane has been proposed (Engelman et al., 1980). In this paper, a hydrophobic portion (^{48}Leu-Val-Pro-Ala-Ile-Ala-^{54}Phe) of this protein has been chemically synthesized and its structure examined. Additionally, the tripeptide Ile-Ala-Phe-OBzl[1] was made water soluble by attachment of sarcosine residues on its N terminus. Since sarcosine peptides are readily soluble in water as well as in organic solvents, a variety of solvent systems could then be used for studying this peptide. The flexible sarcosine chain is expected to impose little constraint on the hydrophobic tripeptide portion, and the lack of asymmetric carbons could ease the interpretation of chirooptical measurements on the optically active hydrophobic tripeptide. A previous report (Wallace & Blout, 1979) demonstrated that a combination of several spectroscopic methods was useful for investigating a model system and that a hydrocarbon solvent could mimic the phospholipid bilayer. The present study uses a combination of circular dichroism (CD) and proton and carbon-13 NMR spectroscopy to investigate structural properties of the hydrophobic peptides both in solvents of differing polarity and in phospholipid vesicles. Hydrophobic peptides of different size (tri-, penta-, and heptapeptide) were examined to determine if specific structures were formed in polar and apolar solvents and in phospholipid vesicles and to determine if there was any structural similarity between the conformations of the peptides in an apolar medium and in vesicles. Moreover, the behavior of a hydrophobic tripeptide and its sarcosine derivative in the presence and absence of vesicles was investigated to determine the manner in which they interact with the phospholipid vesicles.

Experimental Procedures

Synthesis of Peptides. (*1*) *Boc-Ala-Phe-OBzl.* Boc-Ala-OH (5.30 g, 28 mmol) was dissolved in chloroform (40 mL). At

† From the Department of Biological Chemistry, Harvard Medical School, Boston, Massachusetts 02115 (T.S. and E.R.B.), and the Department of Biochemistry, College of Physicians & Surgeons, Columbia University, New York, New York 10032 (B.A.W.). *Received November 10, 1981.* This work was supported by U.S. Public Health Service Grants GM27292 to B.A.W. and AM 07300 and AM 110794 to E.R.B. B.A.W. is the recipient of a Hirschl Career Scientist Award. The high-field NMR experiments were performed at the NMR Facility for Biomolecular Research at the Francis Bitter National Magnet Laboratory, Massachusetts Institute of Technology, which is supported by Grant RR00995 from the Division of Research Resources of the National Institutes of Health and by the National Science Foundation under Contract C-670.

‡ Present address: Department of Home Economics, Gifu University, Gifu, Japan.

[1] Abbreviations: CD, circular dichroism; NMR, nuclear magnetic resonance; OBzl, benzyl ester; OMe, methyl ester; Boc, *tert*-butyloxycarbonyl; Tos, *p*-toluenesulfonyl; OSu, succinimidyl ester; F_3Ac, trifluoroacetyl; DCHA, dicyclohexylamine.

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−20 °C, *N*-methylmorpholine (3.08 mL, 28 mmol) and isobutyl chloroformate (3.6 mL, 28 mmol) were added, and the solution was stirred for 20 min. A sample of TosOH·Phe-OBzl (11.96 g, 28 mmol) was added; the solution was stirred for 1 h at −20 °C and then allowed to warm to room temperature. After 15 h, the reaction mixture was concentrated in vacuo to give a slightly yellow oil. Ethyl acetate (200 mL) was added, the white precipitate was removed by filtration, and the filtrate was washed with 1 N HCl, 5% $NaHCO_3$, and then with a saturated NaCl solution and dried with Na_2SO_4. Evaporation of the solvent gave a white crystalline residue which was recrystallized from ethyl acetate–hexane to give 9.20 g (77%), mp 95–96.5 °C. Anal. Calcd for $C_{24}H_{30}O_5N_2$: C, 67.59; H, 7.08; N, 6.57. Found: C, 67.54; H, 7.08; N, 6.58.

(*2*) *HCl·Ala-Phe-OBzl.* Dry hydrogen chloride was passed through a solution of Boc-Ala-Phe-OBzl (9.20 g, 22 mmol) in ethyl acetate (100 mL), at 0 °C for 10 min. The solution was kept at 0 °C for an additional 30 min and then evaporated to give a white solid. Ether was added, and the solid was collected on a filter to give 7.60 g (97%), mp 190–192 °C. The material was used without purification for a subsequent coupling.

(*3*) *Boc-Ile-Ala-Phe-OBzl.* A sample of Boc-Ile-OSu (Anderson et al., 1964) (2.03 g, 6.1 mmol) in tetrahydrofuran (25 mL) was cooled to 0 °C and treated with HCl·Ala-Phe-OBzl (2.23 g, 6.1 mmol) and triethylamine (0.86 mL, 6.1 mmol). The reaction mixture was stirred at 0 °C for 1 h and then kept at room temperature. After 15 h, the solution (gel) was concentrated in vacuo to a small volume and dissolved in acetone (20 mL). A small amount of insoluble material was filtered off, and 5% $NaHCO_3$ (100 mL) was added to the filtrate. The white precipitate was collected on a filter and washed with water. Precipitation from acetone–water was repeated 5 times. The product was recrystallized from hot acetone: yield 3.07 g (93%); mp 156–157 °C. Anal. Calcd for $C_{30}H_{41}O_6N_3$: C, 66.77, H, 7.66; N, 7.79. Found: C, 66.80; H, 7.63; N, 7.82.

(*4*) *HCl·Ile-Ala-Phe-OBzl.* Dry hydrogen chloride was passed through a solution of Boc-Ile-Ala-Phe-OBzl (1.05 g, 1.95 mmol) in dioxane (30 mL) for 15 min at 0 °C. The solution was then kept at room temperature for 45 min, and the solvent was evaporated to give a white solid, which was recrystallized from dimethylformamide–diethyl ether: yield 0.90 g (97%); mp 190–192 °C. Anal. Calcd for $C_{25}H_{34}O_4N_3Cl$: C, 63.08; H, 7.20; N, 8.83. Found: C, 63.02; H, 7.12; N, 8.70.

(*5*) *Boc-Pro-Ala-Ile-Ala-Phe-OBzl.* Samples of Boc-Pro-Ala-OH (Pease, 1975) (573 mg, 2.0 mmol) and HCl·Ile-Ala-Phe-OBzl (952 mg, 2.0 mmol) were dissolved in dimethylformamide (3 mL). Triethylamine (0.28 mL, 2.0 mmol) was added to solution, followed by *N*-hydroxysuccinimide (230 mg, 4.0 mmol) and *N,N'*-dicyclohexylcarbodiimide (412 mg, 2.0 mmol) in dimethylformamide (1 mL) at 0 °C. The reaction mixture was stirred at 0 °C for 1 h and then allowed to warm at room temperature. After 14 h, the reaction mixture (gel) was diluted with dimethylformamide (3 mL); the precipitated *N,N'*-dicyclohexylurea was filtered off, and 5% $NaHCO_3$ (50 mL) was added to the filtrate. The white precipitate was collected on a filter and washed with water. Precipitation from acetone–water was repeated 5 times. The white solid was dissolved in a small amount of methanol and chromatographed on a column of Sephadex LH-20 (3 × 40 cm) with methanol as solvent. The fast migrating fractions were concentrated to a small volume. A white solid was obtained upon addition of diethyl ether and hexane to the concentrated material. Recrystallization from methanol–diethyl ether–hexane gave 1.13 g (80%): mp 196–197 °C; amino acid analysis for Pro:Ala:Ile:Phe 0.98:1.94:1.00:1.08. Anal. Calcd for $C_{38}H_{53}O_8N_5$: C, 64.48; H, 7.55; N, 9.89. Found: C, 64.34; H, 7.46; N, 9.86.

(*6*) *Boc-Leu-Val-OH·DCHA.* A sample of Boc-Leu-Val-OMe (Laufer & Blout, 1967) (1.17 g, 3.4 mmol) was dissolved in methanol (4 mL) and treated with 1 N NaOH (5.1 mL, 5.1 mmol). The solution was stirred for 2 h at room temperature; the aqueous solution was washed with diethyl ether (5 mL) and then acidified with 10% citric acid. The oil which separated was extracted into ethyl acetate (10 mL × 2), and the organic layer was dried with Na_2SO_4. Evaporation of the ethyl acetate solution gave a colorless oil which was mixed with dicyclohexylamine (0.62 g, 3.4 mmol) in diethyl ether (50 mL) to give a white precipitate. Recrystallization from ethyl acetate–diethyl ether gave 1.51 g (87%), mp 149–151 °C.

(*7*) *F_3Ac·Pro-Ala-Ile-Ala-Phe-OBzl.* A sample of Boc-Pro-Ala-Ile-Ala-Phe-OBzl (644 mg, 0.91 mmol) was dissolved in trifluoroacetic acid (1.5 mL) at room temperature. The solution was evaporated to give an oil which, upon addition of diethyl ether (50 mL), gave a solid. It was filtered and washed with chloroform: 435 mg (66%); mp 227–228 °C. The amorphous solid was used without further purification in the next step.

(*8*) *Boc-Leu-Val-Pro-Ala-Ile-Ala-Phe-OBzl.* Samples of Boc-Leu-Val-OH·DCHA (256 mg, 0.5 mmol) and F_3Ac·Pro-Ala-Ile-Ala-Phe-OBzl (361 mg, 0.5 mmol) were dissolved in dimethylformamide (5 mL) at 0 °C. *N,N'*-Dicyclohexylcarbodiimide (103 mg, 0.5 mmol) and 1-hydroxybenzotriazole (67 mg, 0.6 mmol) were added to the solution. The reaction mixture was stirred for 3 h at 0 °C and then allowed to warm to room temperature. After 15 h, dimethylformamide (5 mL) was added to the solution, and the precipitated *N,N'*-dicyclohexylurea was removed by filtration. A white precipitate formed upon addition of 5% $NaHCO_3$ (100 mL) to the filtrate. The solid was collected on a filter and washed with water. Reprecipitation from methanol–water (3 times) yielded a solid which was dissolved in methanol and chromatographed on a column of Sephadex LH-20 (3 × 40 cm) by using methanol as a mobile phase. A white amorphous powder was obtained: yield 396 mg (86%); mp 132–134 °C; amino acid analysis for Pro:Ala:Val:Ile:Leu:Phe 1.00:2.00:0.95:1.03:1.00:1.06. Anal. Calcd for $C_{49}H_{73}N_7O_{10}$: C, 63.96; H, 8.00; N, 10.66. Found: C, 64.08; H, 8.09; N, 10.62.

(*9*) *$(Sar)_{\sim 14}$-Ile-Ala-Phe-OBzl.* HCl·Ile-Ala-Phe-OBzl (476 mg, 1.0 mmol) was dissolved in water (5 mL), and the solution was adjusted to pH 9 with 5% $NaHCO_3$. The peptide was extracted from the solution (gel) with chloroform (5 mL × 3) and dried with Na_2SO_4. Evaporation of the solvent gave a white solid, which was dried over P_2O_5 in vacuo for 4 h. The white solid (330 mg, 0.75 mmol) was dissolved in nitrobenzene (10 mL), and sarcosine *N*-carboxyanhydride (1.14 g, 9.9 mmol) in nitrobenzene (15 mL) was added to the solution. After 2 days, anhydrous diethyl ether (150 mL) was added to the solution, and the slightly yellow precipitate was collected on a filter. The solid was dissolved in methanol, treated with activated charcoal, and precipitated by diethyl ether. Reprecipitation was done 5 times from methanol–diethyl ether, yield (white powder) 0.80 g. The average number of sarcosine residues attached was found to be 14 from amino acid analysis and proton NMR. The polypeptide was soluble in water, methanol, ethanol, dioxane, acetonitrile, and chloroform, and not soluble in diethyl ether or hexane.

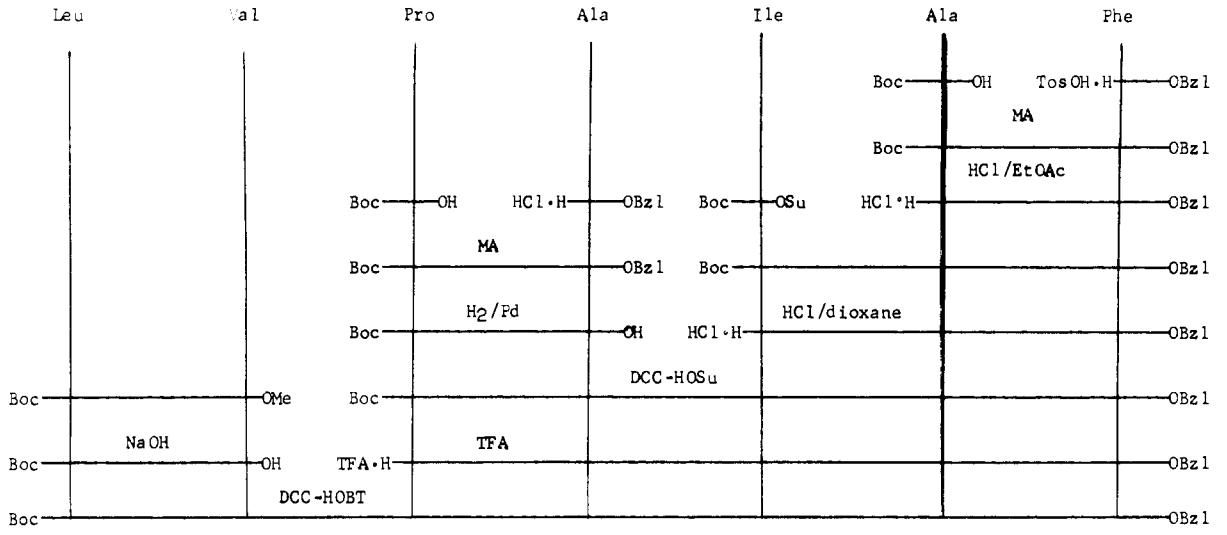

FIGURE 1: Synthetic scheme for Boc-Leu-Val-Pro-Ala-Ile-Ala-Phe-OBzl (48–54 fragment of bacteriorhodopsin). MA, mixed anhydride method; DCC–HOSu, dicyclohexylcarbodiimide–*N*-hydroxysuccinimide method; DCC–HOBT, dicyclohexylcarbodiimide–1-hydroxybenzotriazole method.

Vesicle Preparation. For water-insoluble peptides, vesicles were prepared as previously described (Wallace & Blout, 1979) from dried down chloroform solutions of 4–6 μmol of peptide and 170 μmol of dimyristoylphosphatidylcholine. For removal of any peptide that was not incorporated into the lipid bilayer and for estimation of the minimum partition coefficient, 100-μL samples of vesicles were dialyzed in Spectrum cellulose dialysis tubing with a molecular weight cutoff at 25 000 for 20 h at 21 °C vs. 1 L of deionized water.

For water-soluble peptides, peptide and dimyristoylphosphatidylcholine were dispersed in H_2O or D_2O by shaking with a vortex mixer for 10 min. The dispersion was sonicated and centrifuged in the same manner as for the water-insoluble peptides. The pH of the sample was adjusted with concentrated HCl (DCl in D_2O) or NaOH (NaOD in D_2O). In the titration experiments in which the peptide:vesicle ratio was changed, the vesicle solution was mixed with peptide solution.

Lipid concentration was measured by a modified Fiske–Subbarow method (Fiske & Subbarow, 1925). Peptide concentration was determined on a reversed-phase column (Du Pont ZORBAX-8, 5 μm, 4.6 mm × 25 cm) with a Waters Associate high-performance liquid chromatograph at 2500 psi by using a 75:25 v/v methanol:water mixture as the mobile phase.

Circular Dichroism and UV Spectra. CD measurements were done at 23 °C with a Cary 60 spectropolarimeter fitted with a Model 6001 CD attachment and a variable position detector, operating over a wavelength range of 300–190 nm; 1.0-, 0.5-, and 0.2-mm path-length cells were utilized. For assessment of the light scattering properties of the vesicular samples, the detector position was varied, resulting in different acceptance angles. For most experiments, the cell was located adjacent to the photomultiplier, resulting in maximum light acceptance. The CD data are expressed in terms of molar ellipticity. For $(Sar)_{\sim 14}$-Ile-Ala-Phe-OBzl, calculations are based on the molecular weight of the optically active Ile-Ala-Phe moiety.

NMR Spectra. NMR spectra were obtained on a Brucker HX-270 spectrometer operating in the Fourier-transform mode. Spin–lattice relaxation time (T_1) measurements were done by an inversion–recovery method using a $(180°-\tau-90°-\text{pulse delay})_n$ sequence (Freeman & Hill, 1971). Typically, seven τ values were taken. T_1 values were determined by a nonlinear least-squares program. Lanthanide titration was done by adding a D_2O solution of ytterbium chloride or praseodymium chloride to the peptide–vesicle solution.

Results

Synthesis of Peptides. The heptapeptide Boc-Leu-Val-Pro-Ala-Ile-Ala-Phe-OBzl was synthesized (Figure 1) by coupling the dipeptide Boc-Leu-Val-OH to the pentapeptide Pro-Ala-Ile-Ala-Phe-OBzl. An alternative scheme initially attempted was to couple the tetrapeptide Boc-Leu-Val-Pro-Ala-OH to the tripeptide Ile-Ala-Phe-OBzl. However, synthesis of the tetrapeptide via acylation of Pro-Ala-OBzl with Boc-Val-OH and various attempts to couple Boc-Val-OH with Pro-Ala-OBzl did not give satisfactory results. This might be due to an instability of Pro-Ala-OBzl in the reaction medium and a poor reactivity of Boc-Val-OH with Pro-Ala-OH.

During the course of the syntheses, the peptides were effectively purified by the addition of water. This simple procedure eliminated unreacted starting materials and some of the byproducts, giving a precipitate of hydrophobic peptides.

Ile-Ala-Phe-OBzl was made water soluble by attachment of a sarcosine chain (via polymerization of the *N*-carboxyanhydride of sarcosine with the free amino component as initiator) to the N-terminal amino acid. Since the polymerization was done in an aprotic solvent (nitrobenzene), the molecular weight distribution is expected to be small, although a statistical distribution in the number of sarcosine residues will result. This was confirmed by reversed-phase high-performance liquid chromatography.

CD Spectra. CD spectra of Boc-Leu-Val-Pro-Ala-Ile-Ala-Phe-OBzl (BocLVPAIAF), Boc-Pro-Ala-Ile-Ala-Phe (BocPAIAF), and Boc-Ile-Ala-Phe-OBZl (BocIAF) were obtained in hexafluoroisopropyl alcohol, hexane, and dimyristoylphosphatidylcholine vesicles (Figures 2–4). The CD spectra of all three peptides in trifluoroethanol were similar to those in hexafluoroisopropyl alcohol. In hexafluoroisopropyl alcohol, the ellipticity values for the tripeptide are very low, suggesting the presence of either a multiple conformation or structures with low asymmetry (Figure 2). As the peptide chain was elongated, the magnitude of the two negative extremes increased, perhaps due to the increased formation of a stable secondary structure. There is a dramatic increase in the Cotton effect for the heptapeptide.

The spectrum of BocIAF in hexane was similar to that in hexafluoroisopropyl alcohol, although the magnitudes of the two peaks were greater in hexane. In contrast, the spectra of the pentapeptide BocPAIAF and the heptapeptide Boc-

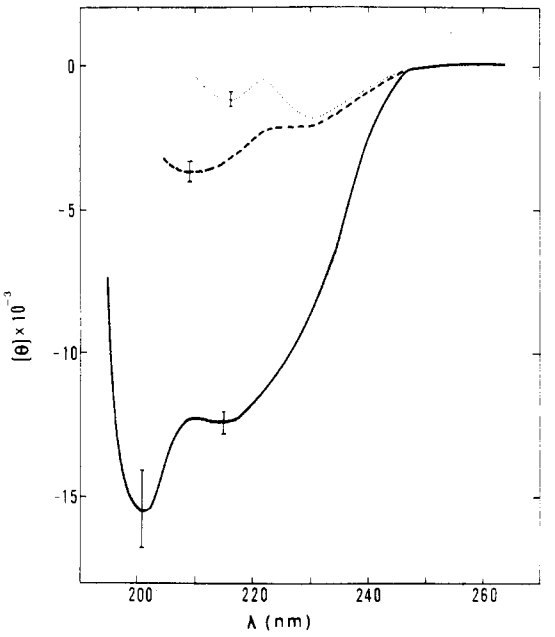

FIGURE 2: Circular dichroism spectra of hydrophobic oligopeptides in hexafluoroisopropyl alcohol: (···) BocIAF, peptide concentration 1.5 mM; (---) BocPAIAF, peptide concentration 1.26 mM; (—) BocLVPAIAF, peptide concentration 3.26 mM.

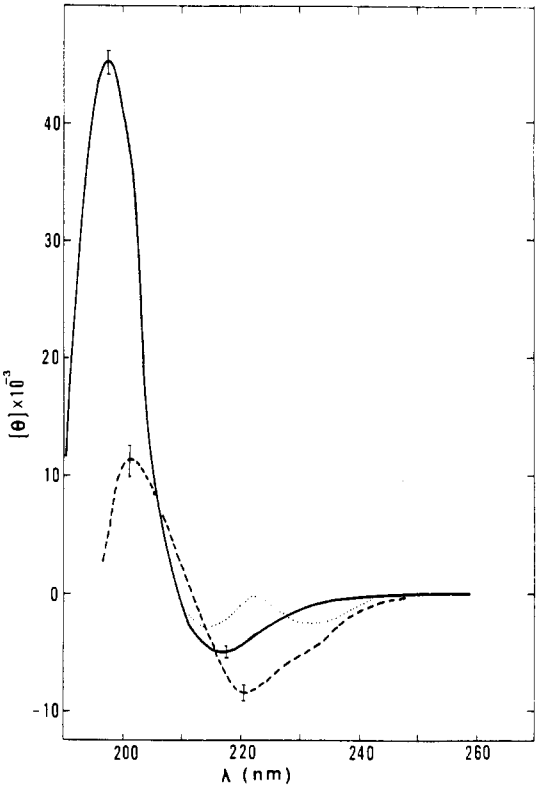

FIGURE 3: Circular dichroism spectra of hydrophobic oligopeptides in hydrophobic solvents: (···) BocIAF, peptide concentration 0.39 mM in 2% ethanol–hexane; (---) BocPAIAF, peptide concentration 0.40 mM in 2% ethanol–hexane; (—) BocLVPAIAF, peptide concentration 0.069 mM in hexane.

LVPAIAF were markedly different in the two solvents (Figure 3). In hexane, or hexane plus 2% ethanol (necessary for solubilization), negative ellipticities at 216–219 nm and positive ellipticities at 198–200 nm are seen which may be reminiscent of β-like structures (Quadrifoglio & Urry, 1968).

Particulate samples (such as vesicles) tend to scatter light and thus have distorted optical spectra. However, studies on

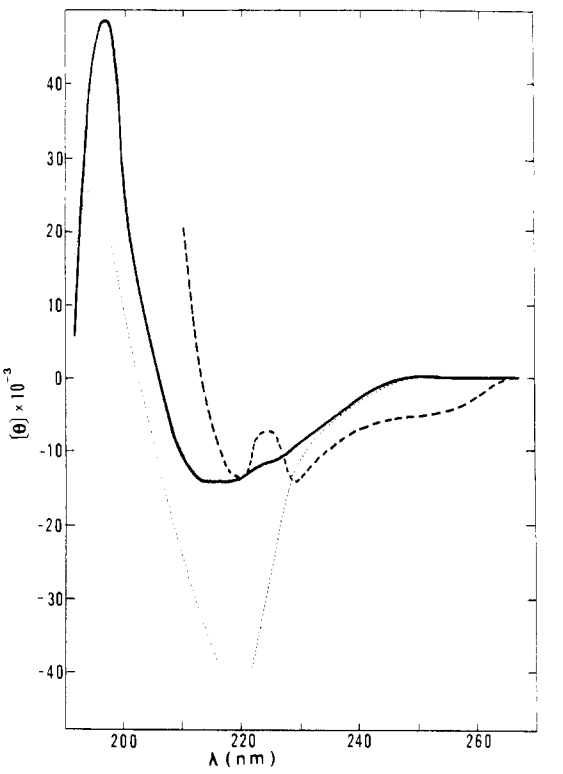

FIGURE 4: Circular dichroism spectra of hydrophobic oligopeptides in dimyristoylphosphatidylcholine vesicles. (···) BocIAF, peptide concentration 0.13 mM, peptide:lipid = 1:130. (---) BocPAIAF, peptide concentration 0.26 mM, peptide:lipid = 1:80. (—) BocLVPAIAF, peptide concentration 0.85 mM, peptide:lipid = 1:40.

proteins (D. Mao and B. A. Wallace, unpublished results) and peptides (Wallace & Blout, 1979) incorporated into small sonicated vesicles of ~300-Å diameter have shown both experimentally and computationally that absorption flattening, absorption obscuring, and differential scattering effects amount to less than 1% of the total ellipticities for these samples, and so can be considered negligible.

In dimyristoylphosphatidylcholine vesicles (Figure 4), both BocIAF and BocPAIAF have relatively large negative peaks and are generally dissimilar to both hexane and hexafluoroisopropyl alcohol spectra. After removal of any aqueous-soluble peptide by dialysis, no change was detected in the CD spectra of BocIAF so the differences detected between this peptide in hexane and the phospholipid vesicles are real and not due to a combination of hydrocarbon- and aqueous-soluble conformations. On the other hand, the CD spectrum of the heptapeptide in vesicles was very similar to that in hexane and is somewhat like the spectrum of a β-sheet structure. In this case, the hydrocarbon solvent was a reasonably good model for the bilayer interior as was seen previously for a peptide dimer with one well-ordered conformation (Wallace & Blout, 1979); the peptide also did not appreciably partition into the aqueous phase.

The CD spectra of HCl·Ile-Ala-Phe-OBzl (IAF) and $(Sar)_{\sim 14}$-Ile-Ala-Phe-OBzl (SIAF) (Figures 5 and 6) in water are affected by the presence of dimyristoylphosphatidylcholine vesicles. IAF in water exhibits a positive peak at 222 nm and a small negative peak at higher wavelengths. When the vesicle solution was added to the peptide solution, the positive peak was increased according to the amount of vesicles added. The intensity of the small, negative peaks in the near-UV region also increased. SIAF assumed a different conformation in the absence of vesicles, but when the vesicle solution was added, a positive peak appeared at 222–224 nm, and this peak in-

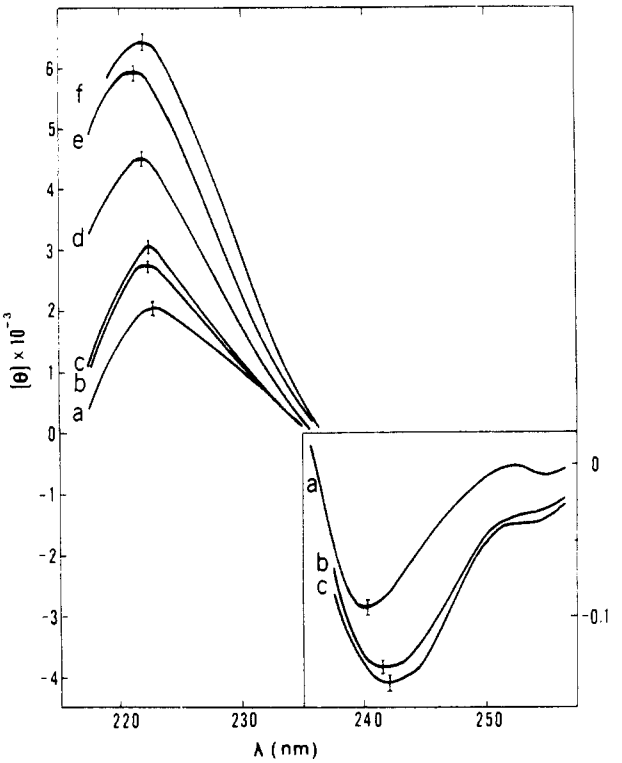

FIGURE 5: Circular dichroism spectra of IAF in the presence of dimyristoylphosphatidylcholine vesicles: pH 4.4; peptide concentration 1.01 mM. Peptide:lipid ratios are (a) 1:0, (b) 1:1.5, (c) 1:2.2, (d) 1:4.5, (e) 1:9.0, and (f) 1:15.

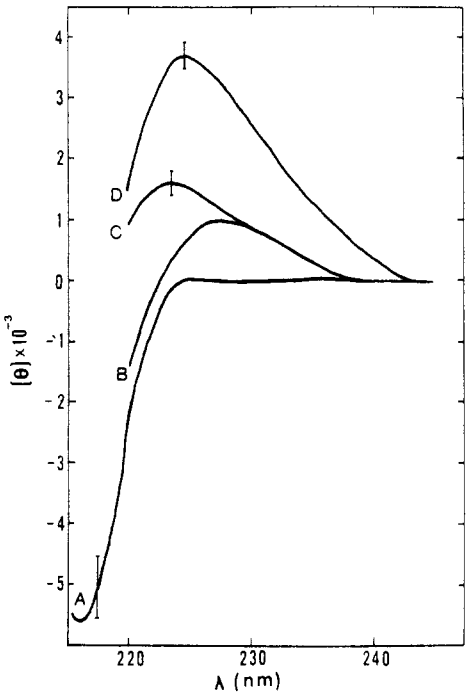

FIGURE 6: Circular dichroism spectra of SIAF in the presence of dimyristoylphosphatidylcholine vesicles: pH 7.4; peptide concentration 0.93 mM. Peptide:lipid ratios are (A) 1:0, (B) 1:7.9, (C) 1:15.5, and (D) 1:30.7.

creased according to the amount of vesicles added. These differences are not due to vesicle spectra, which have negligible ellipticity at these wavelengths. Sonication of the peptide–vesicle solutions did not change the CD spectra.

NMR Spectra. Proton NMR spectra of the water-soluble peptides (IAF and SIAF) were recorded (Figure 7) in the absence and in the presence of dimyristoylphosphatidylcholine

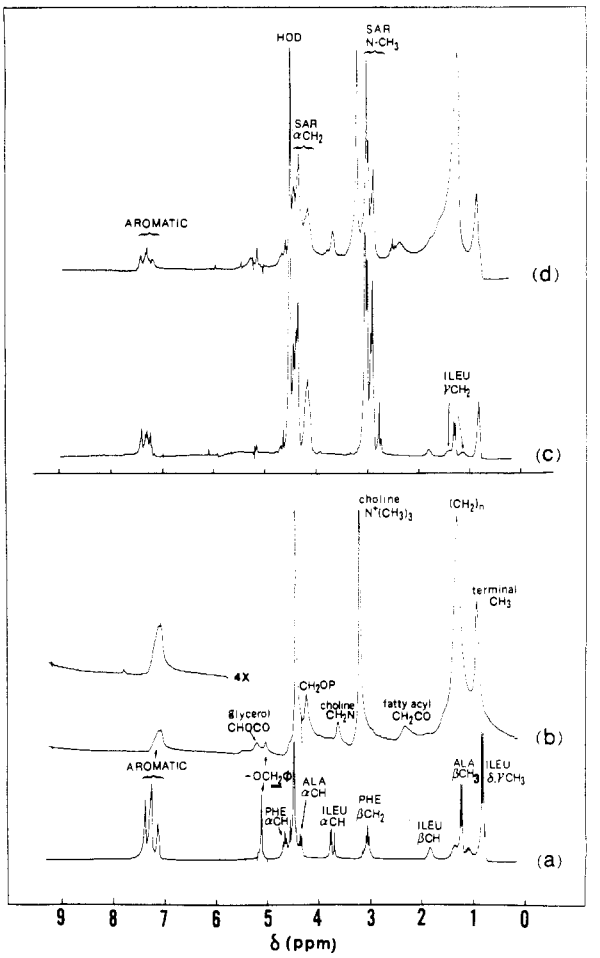

FIGURE 7: Proton NMR spectra at 270 MHz of IAF without (a) and with vesicles (b) and of SIAF without (c) and with vesicles (d) at 47 °C. IAF:lipid = 1:3.3. SIAF:lipid = 1:76. The chemical shifts are relative to external sodium 4,4-dimethyl-4-silapentane-1-sulfonate (DSS) in D_2O.

vesicles. As the vesicle solution was added to the peptide solution, the peptide resonances broadened, and some seemed to disappear or were obscured by the larger resonances due to the more abundant lipid components. The broadening was more pronounced in the spectrum of IAF, and all resonances experienced slight shifts in their positions. These effects are a result of the association with vesicles, which decreases the mobility of the peptides and changes the magnetic environment of the peptide.

Carbon-13 NMR spectra of IAF and SIAF were also recorded in water and in the presence of vesicles. The peak intensities of the peptide resonances in the vesicle system were low; however, in contrast to the proton spectra, almost all peptide resonances were observable (Figure 8). The vesicles also affect the chemical shifts and T_1 values of the peptides (Tables I and II). The values for IAF were perturbed more strongly than those for SIAF. On the other hand, T_1 values of lipid carbons were hardly affected by the peptide (Table III).

Discussion

Hydrophobic Water-Insoluble Peptides. Studies of oligopeptides in organic solvents have been done to determine the critical size for the formation of secondary structures (Goodman et al., 1974; Mutter et al., 1976; Toniolo et al., 1979). The three hydrophobic peptides examined in this work also indicate the tendency for increased secondary structure formation in hexafluoroisopropyl alcohol and hexane as the

Table I: ^{13}C Chemical Shifts of IAF and SIAF in the Absence and Presence of Dimyristoylphosphatidylcholine Vesicles[a]

	IAF[b]		SIAF[c]	
carbon	D_2O	vesicles	D_2O	vesicles
Ile δ	181.8	181.4	182.2	182.1
Ile γ_1	178.4	178.4	177.5	177.6
Ala β	175.5	175.0	175.5	175.6
Ile γ_2	168.3	168.0	168.0	
Ile β	156.1	156.1	156.1	
Phe β	155.6	155.1	154.7	
Ala α	143.1	143.1		
Phe α	138.3		136.2	
Ile α	134.8	135.5	134.2	
CH_2 φ	125.0	125.7	124.8	125.3
aromatic ζ	65.3	66.0	65.7	65.7
aromatic ζ			65.0	65.2
aromatic δ, ε	64.1	64.2	63.9	64.0
aromatic δ, ε	63.8	63.1	63.6	63.1
aromatic δ, ε	63.3		63.2	
aromatic δ, ε			63.0	
aromatic γ	57.3	56.7	54.7	
aromatic γ	56.5	55.7		
Ala C=O	23.9	27.4		
Phe C=O	20.2	24.2		
Ile C=O	18.9	20.6		
Sar C=O			22.5	22.5
Sar C=O			22.1	22.2
Sar C=O			21.9	21.9
Sar C=O			21.6	21.6
Sar N-CH_3			156.7	156.7
Sar α			143.0	143.0
Sar α			142.8	142.8
Sar α			142.5	142.5
Sar α			141.9	141.9

[a] In parts per million from external CS_2. [b] 47 °C, pD 4.4, peptide:lipid = 1:3.3. [c] 47 °C, pD 7.4, peptide:lipid = 1:7.0.

Table II: Spin-Lattice Relaxation Times (T_1) of ^{13}C of IAF and SIAF in the Absence and Presence of Dimyristoylphosphatidylcholine Vesicles[a]

	IAF[b]		SIAF[c]	
carbon	D_2O	vesicles	D_2O	vesicles
Ile δ	1.22 ± 0.42		0.55 ± 0.06	
Ile γ_1	0.66 ± 0.08		0.32 ± 0.05	
Ala β	0.32 ± 0.05	0.17 ± 0.01	0.14 ± 0.03	
Ile γ_2	0.36 ± 0.04		0.12 ± 0.02	
Ile β	0.40 ± 0.05	(0.12 ± 0.01)	0.20 ± 0.04	
Phe β	0.17 ± 0.01			
Ala α	0.31 ± 0.03			
Phe α	0.33 ± 0.03		0.30 ± 0.03	
Ile α	0.34 ± 0.03		0.23 ± 0.03	
CH_2 φ	0.33 ± 0.02	0.25 ± 0.04	0.42 ± 0.04	
aromatic ζ	0.37 ± 0.03	(0.12 ± 0.03)	0.42 ± 0.03	
aromatic ζ			0.51 ± 0.15	
aromatic δ, ε	1.55 ± 0.12	0.47 ± 0.03	0.70 ± 0.03	0.80 ± 0.06
aromatic δ, ε	0.90 ± 0.06	0.27 ± 0.03	0.94 ± 0.04	0.66 ± 0.03
aromatic δ, ε	0.60 ± 0.05		0.52 ± 0.04	0.44 ± 0.03
aromatic δ, ε			0.63 ± 0.11	(0.53 ± 0.05)
aromatic γ	2.57 ± 0.36			
aromatic γ	2.29 ± 0.24			
Sar N-CH_3			0.60 ± 0.08	0.60 ± 0.06
Sar α			0.16 ± 0.04	0.14 ± 0.01
Sar α			0.12 ± 0.01	0.12 ± 0.01
Sar α			0.11 ± 0.01	0.12 ± 0.01
Sar α			0.11 ± 0.01	0.11 ± 0.01

[a] T_1 values are in seconds. Values in parentheses were determined from resonances of low intensity and are tentative. [b] 47 °C, pD 4.4, peptide:lipid = 1:3.3. [c] 47 °C, pD 7.4, peptide:lipid = 1:7.0.

peptide chain is lengthened. In dimyristoylphosphatidylcholine vesicles, dominant (stable) structures are seen for all chain lengths although they differ distinctly for each compound. The

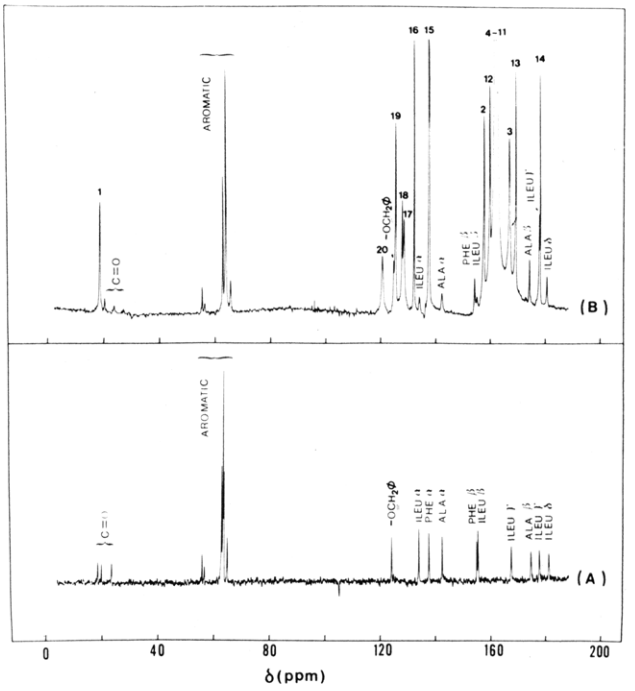

FIGURE 8: Carbon-13 NMR spectra at 67.89 MHz of IAF in the absence (A) and in the presence (B) of dimyristoylphosphatidylcholine vesicles at 47 °C, pD 4.4. (A) 1000 transients, pulse delay 5 s; (B) 71 500 transients, pulse delay 0.5 s. Myristic acid resonances are numbered carbonyl carbon 1 through methyl carbon 14. The resonance of the choline methyl is numbered 15, the CH_2OP is numbered 16, and the CH_2N^+ is numbered 19. The resonance of the glycerol 1-carbon is numbered 17, that of the 2-carbon is numbered 20, and that of the 3-carbon is numbered 18. The chemical shifts are relative to external CS_2.

Table III: Spin-Lattice Relaxation Times of ^{13}C Resonances of Lipids upon Addition of IAF to Vesicles[a]

carbon	with IAF	without IAF
C14	>3.0	>3.0
C13	1.34 ± 0.13	1.28 ± 0.14
C3	0.28 ± 0.03	0.27 ± 0.02
C4–C11	0.52 ± 0.02	0.53 ± 0.03
C12	1.02 ± 0.08	1.01 ± 0.07
C2	0.23 ± 0.01	0.22 ± 0.01
C15 $N^+(CH_3)_3$	0.64 ± 0.01	0.59 ± 0.03
C16 CH_2OP	0.36 ± 0.02	0.31 ± 0.02
C17 CH_2OCOR	0.11 ± 0.01	0.11 ± 0.01
C18 CH_2OP (glycerol)	0.11 ± 0.01	0.12 ± 0.01
C19 CH_2N	0.42 ± 0.03	0.41 ± 0.03
C1 (C=O)	1.35 ± 0.14	1.36 ± 0.11
C20 CHOCOP	0.17 ± 0.01	0.12 ± 0.01

[a] Times are in seconds: 47 °C; pD = 4.4. Numbering of carbons is the same as in Figure 8.

heptapeptide CD spectrum in hexafluoroisopropyl alcohol is quite similar to that of Z-$(Ala)_8$-OMe in trifluoroisopropyl alcohol–1% H_2SO_4 (Goodman et al., 1974) and of polyalanine in hexafluoroisopropyl alcohol (Parrish & Blout, 1972), which have been described as variants of α helices. Thus, though the two negative extremes exhibit blue shifts compared with those of typical α-helical polypeptides, the heptapeptide likely adopts a distorted helical-type conformation in this strongly solvating solvent.

The CD spectra of the hydrophobic peptides showed dramatic differences between strongly solvating systems (hexafluoroisopropyl alcohol or trifluoroethanol) and poorly solvating systems (hexane or 2% ethanol–hexane). β-Like structures were favored in the poorly solvating system; this tendency was more pronounced with longer peptide chain lengths. The

presence of a proline residue in the two longer peptides could result in a β-turn-like conformation (Pease et al., 1973) which gives rise to CD spectra similar to those for β sheets (Bush et al., 1978). The CD spectra of the three peptides in other solvents, i.e., methanol, ethanol, dioxane, and acetonitrile, also reflect strong solvent dependency, but none of the peptides give typical α- or β-like structures in these solvents. In summary, the peptides adopt very different conformations in hydrophobic and hydrophilic solvents.

The three hydrophobic peptides were also incorporated into phospholipid vesicles. The large molar ellipticity of BocIAF and BocPAIAF in dimyristoylphosphatidylcholine vesicles suggests that both peptides exist predominantly as ordered conformations. BocLVPAIAF in vesicles appears to be a β-like structure although the CD spectrum is somewhat distorted. This may be due in part to the low dielectric constant of the medium, which causes spectral shifts, or the physical constraints imposed by the lipid bilayer, which tends to distort the peptide. In a previous report (Wallace & Blout, 1979), it was demonstrated that a hydrophobic tripeptide adopts the same conformation in phospholipid vesicles as in a hydrocarbon solvent, and it was suggested that this solvent was a good model for the membrane environment. In this study, the hydrocarbon solvent also clearly mimics the environment of lipid vesicles as seen by the heptapeptide: the CD spectra in vesicles and hexane are very similar, another indication that the peptide is embedded in the lipid bilayer. For the shorter chain length peptides, more significant differences were noted between the hydrocarbon solvent and vesicle spectra. This may be due to the relative flexibility of the molecules in the solvent, which permits adoption of multiple conformations whose summed ellipticity may be closer to zero. In vesicles, a single structure may dominate, resulting in a larger total ellipticity.

The water-insoluble peptides are hydrophobic and can be regarded as models for intrinsic membrane proteins. They are strongly associated with the bilayer and have partition coefficients of at least 3×10^7 favoring the lipid bilayer over water. They adopt different conformations in membranes and in hydrophilic solvents, and thus they may be regarded as fulfilling basic requirements for membrane models. Comparisons of the behavior of hydrophobic peptides of different size in a hydrophobic solvent and in phospholipid vesicles give some information on the effect of the hydrophobic environment of the lipid bilayer which may be applicable to the structure of proteins located in the membrane.

Water-Soluble Peptides. The peptides IAF and SIAF are soluble in water; thus, the vesicle:peptide ratio can be varied significantly, and the resulting vesicle-induced CD changes can be followed systematically. Addition of dimyristoylphosphatidylcholine vesicles to a solution of IAF or SIAF caused changes in their CD spectra. A similar change was induced in both peptides: an increase in the positive peak at 222–224 nm. For IAF, a small increase in the negative band at 230–240 nm was also observed. Both these peptides may have similar structures in the presence of vesicles.

The positive band at 222–224 nm is more likely a contribution from the aromatic rings of the phenylalanine benzyl ester moiety rather than from a peptide backbone transition, or due to aggregation. Phenylalanine derivatives have been found to have positive CD maxima at 222 nm (as do tyrosine and tryptophan); this CD maximum may be due to the interaction of the aromatic ring with the peptide backbone (Sears & Beychok, 1973). The appearance of the positive peak is also correlated with the population of side-chain rotamers as indicated by the coupling constants of the phenylalanine

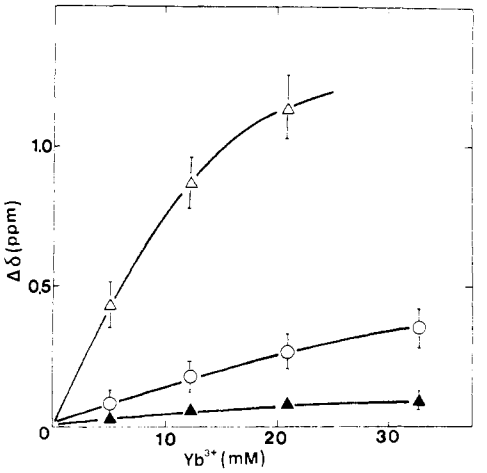

FIGURE 9: Yb^{3+} titrations of IAF and SIAF in the presence of dimyristoylphosphatidylcholine vesicles at 47 °C. (△) Outer $N^+(CH_3)_3$. (○) Aromatics of SIAF, peptide:lipid = 1:29. (▲) Inner $N^+(CH_3)_3$ and aromatics of IAF, peptide:lipid = 1:17.

$C_\alpha H$–$C_\beta H_2$. BocIAF has a positive band in acetonitrile but not in trifluoroethanol. The coupling constants are found to be 7.8 and 6.4 Hz in acetonitrile-d_3 and 6.4 and 5.9 Hz in trifluoroethanol-d_3, corresponding to the population of the classical side-chain rotamer, $P_I(\chi_1 = -60°):P_{II}(\chi_1 = 180°):P_{III}(\chi_1 = 60°) = 0.41:0.50:0.09$ and 0.30:0.37:0.33, respectively (Feeney, 1976). IAF has the positive band in various solvents (methanol, ethanol, trifluoroethanol, and water), and the coupling constants fall in the ranges 7.3–7.8 Hz and 6.1–6.2 Hz, which are close to those of BocIAF in acetonitrile. This peak is unlikely to be a result of aggregation: BocIAF does aggregate in 0.2% ethanol–hexane, but its CD spectrum does not include the positive band, while in acetonitrile it does have the positive peak (at 222 nm), but proton NMR indicates that its amide proton resonances are unaffected over the range $(2–60) \times 10^{-4}$ M, suggesting no aggregation occurs at these concentrations. If the positive peak is attributable to the aromatic ring of the phenylalanine side chain, then the phospholipid vesicles act by changing the conformation of IAF and SIAF such that the phenyl ring interacts more strongly with the peptide backbone. A change in side-chain rotamers is likely since the rotamer population of the phenylalanine side chain is sensitive to solvent polarity (Kobayashi & Nagai, 1977).

The effects of vesicles on the peptides are also observed by NMR spectroscopy. The peptide resonances are broadened, and the ^{13}C peaks experience a chemical shift, reflecting a difference in the peptide environment.

While ^{13}C chemical shift data indicate general information on the environment of IAF in the vesicle system, a more precise localization is provided by paramagnetic ion titration of the IAF–vesicle complex (Figure 9). Lanthanide ions do not penetrate the bilayer but are located in the aqueous medium surrounding the membrane and may coordinate to the vesicle surface, resulting in shifts of NMR resonances of protons or carbons located close to the vesicle surface (Weinstein et al., 1979). In unsonicated vesicles, the choline methyl resonances due to the inner half of the bilayer are shifted only slightly upon addition of either Pr^{3+} or Yb^{3+}; the aromatic resonances of IAF are shifted to approximately the same extent, suggesting that the C-terminal phenylalanine residue lies within the lipid bilayer of the vesicles. This is confirmed by other data: the UV absorption spectrum (240–270 nm) of the IAF aromatic ring in the presence of vesicles was red shifted by 2 nm compared to that in water and was equivalent to that

in 5% ethanol–hexane, a hydrophobic solvent (data not shown). The addition of IAF to phospholipid vesicles caused an upfield shift in the proton resonances of the phospholipid hydrocarbon chains [fatty acyl CH_2CO and $(CH_2)_n$]. This behavior resembles that of melittin for which a tryptophan residue caused an upfield shift of lipid hydrocarbon resonances upon association with phospholipid vesicles (Bony et al., 1979) and indicates that the C-terminal phenylalanine benzyl ester residue is perturbing and thus is located in the bilayer. However, IAF does not substantially affect the mobility of either the lipid hydrocarbon chain or the choline head groups; T_1 values of the vesicle carbon atoms with IAF were practically identical with those without IAF (Table III), which contrasts with the results for another peptide (Nicolau et al., 1974) and protein (Chapman et al., 1979).

The broadening of the NMR resonances of IAF results from immobilization of the peptide upon association with vesicles. For further examination of the effect on mobility, spin–lattice relaxation times (T_1) of peptide carbons were determined (Table II). The T_1 values of α carbons could not be measured in the presence of vesicles; however, a decrease in T_1 values by $^2/_3$–$^1/_2$ was observed for the Ala β, Ile β, and aromatic carbons. The observed T_1 values contain contributions from peptide both in the aqueous phase and in vesicles, since titration of the IAF solution with vesicles indicated that approximately $^2/_3$ of the peptide remains unbound at lipid:peptide molar ratios of 3.3:1, assuming that the bound peptide is directly proportional to the increase in the positive CD band at 222 nm. Thus, it is likely that IAF is in rapid equilibrium between the aqueous phase and vesicles, since only a single set of resonances was observed for each proton or carbon atom.

In comparison to IAF, the sarcosine derivative of the tripeptide has a low tendency to associate with vesicles. It required considerably larger amounts of lipid for the Ile-Ala-Phe moiety to adopt a similar structure, as detected by CD. ^{13}C chemical shifts of SIAF were not very different in the presence of vesicles from those in water.

Upfield shifts of 0.3–0.4 ppm for $N-CH_3$ and 0.3–0.6 ppm for α carbons are expected when sarcosine peptides are transferred from water to a hydrophobic medium (chloroform) (T. Sugihara and E. R. Blout, unpublished results). Since no shifts in the sarcosine peaks were detected for SIAF, it is likely that the sarcosine chain remains in the aqueous phase surrounding the vesicles. The slight variation observed in the presence of vesicles suggests that this hydrophobic tripeptide lies mostly in a polar environment (Table I). This is supported by UV spectroscopy. The aromatic absorptions at 240–270 nm were not red shifted in the presence of vesicles, in contrast to the case of IAF. Titration with lanthanide ions (Figure 9) suggests that the aromatic rings of the C-terminal phenylalanine benzyl ester residue are located relatively close to the surface of the vesicles. In addition, the proton resonances of the lipid hydrocarbons did not shift upfield upon addition of SIAF, which also contrasts with IAF. Similar T_1 values for the sarcosyl residues were obtained with and without vesicles, although the T_1 values for the aromatic rings of the IAF were possibly slightly altered. There was very little effect of the peptide on the ^{13}C NMR T_1 relaxation values for the lipid resonances (Table III) except perhaps for the head groups. These results, together with the CD experiments, indicate a peculiar structure for the SIAF–vesicle complex in which the hydrophobic portion associates with the phospholipid vesicles, possibly near the vesicle surface, while the hydrophilic portion remains in an aqueous phase. The weaker association of SIAF with vesicles might be attributed to increased hydrophilicity upon attachment of the sarcosine chain.

Conclusions

The hydrophobic heptapeptide BocLVPAIAF adopts different structures according to the environment in which it is found: in a polar solvent (hexafluoroisopropyl alcohol or trifluoroethanol), the heptapeptide assumes a helixlike structure, while in a nonpolar solvent (hexane) or in phospholipid vesicles a somewhat β-like structure predominates. This peptide may be useful as a model for membrane studies, although it adopts a conformation which is different than many other membrane-embedded proteins and peptides, which exhibit increased α-helical content upon association with phospholipids (Hammes & Schullery, 1970; Cockle et al., 1978; Shirahama & Yang, 1979) and are generally characterized by long spans of α helix (Wallace, 1982).

In this study, a hydrophobic peptide with an attached hydrophilic chain was found to interact with vesicles although the association was not strong and occurred at the vesicle surface. The role of hydrophilic amino acid residues in membrane-embedded proteins may be to remain in an aqueous phase and/or associate with lipid polar groups, which would result in locking the peptide position relative to the surface, aiding the hydrophobic portion in spanning the membrane. The results reported here suggest that a useful model peptide for proteins which span the membrane may be one which is largely hydrophobic and is capable of inserting into vesicles effectively with a hydrophilic portion which can remain on the vesicle surface or in the aqueous phase.

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Properties of Conserved Amino Acid Residues in Tandem Homologous Protein Domains. Hydrogen-1 Nuclear Magnetic Resonance Studies of the Histidines of Chicken Ovomucoid†

Takashi Ogino,‡ David H. Croll, Ikunoshin Kato,§ and John L. Markley*

ABSTRACT: Peaks corresponding to the C_ϵ protons of the four histidine residues (positions 58, 111, 123, and 182) of chicken ovomucoid have been assigned in ^1H NMR spectra (360 or 470 MHz) of the native single-chain protein and of fragments of the protein corresponding to its three homologous structural domains. Comparison of the ^1H NMR pH titration behavior of these histidine residues and the deuterium exchange rates of their C_ϵ-H positions show the following: (1) The chemical shift properties of histidine residues 58, 123, and 182 differ despite the fact that the three residues are located in homologous positions in the three tandem domains. (2) The properties of three of the four histidine residues (58, 111, and 123) do not change appreciably when the domains in which they are located are isolated, indicating that their environments are similar in both the fragment and the native protein. (3) The properties of the fourth histidine (182) differ in the isolated domain and in the native protein. (4) The observed properties of the histidine residues stem primarily from intradomain interactions that remain constant in isolated domains rather than from interactions with neighboring domains; an interdomain interaction is required to explain the behavior of only histidine-182. (5) The chemical shift of histidine-111 is affected by the titration of the side chain of aspartate-98 with pH_{mid} 2.6 in native ovomucoid but not in isolated second domain; the chemical shift of histidine-182 is perturbed by the titration of the carboxyl group of the C-terminal cysteine-186 with pH_{mid} 2.4 in native ovomucoid and pH_{mid} 2.6 in isolated third domain.

The avian ovomucoids are glycoproteins whose structure has been shown to be composed of three tandem, homologous, structural domains, each of which is a potential or actual inhibitor of a serine proteinase (Kato et al., 1978). Analysis of the chicken ovomucoid gene has shown that the DNA also is organized into domains. There is one intron within each region of the DNA coding for a structural domain, and one intron is located in DNA that codes for the linkage region between each structural domain (Stein et al., 1980).

As shown in Figure 1, the four histidine residues of chicken egg white ovomucoid are located in positions 58, 111, 123, and 182 (Kato et al., 1978, with revisions). Three of the four histidines occupy homologous positions in the first (His^{58}), second (His^{123}), and third (His^{182}) domains. The second domain contains the additional, nonhomologous, histidine residue (His^{111}). Also indicated in Figure 1 are specific points at which the peptide chain can be cleaved for production of isolated structural domains (Kato et al., 1978; I. Kato et al., unpublished results).

Because of the strong homology among domains, one might predict that the properties of the three conserved histidines would be similar. However, a ^1H NMR study of the histidines of ovomucoid, carried out before the protein was sequenced, demonstrated that this is not the case (Markley, 1973b). The present study was undertaken to determine whether the histidines differ because of intradomain or interdomain interactions. First it was necessary to assign histidine resonances to specific residues in the sequence. Then the properties of each histidine in native ovomucoid were compared with its properties in isolated fragments of the molecule.

Experimental Procedures

Materials. Enzymes and chemicals were from the following sources: CNBr, ^2H_2O (99.7% and 100% isotopic purity), Bio-Gel P-10, and Bio-Gel P-30, Bio-Rad; Sephadex G-25, Pharmacia; Whatman CM-52 carboxymethylcellulose, Reeve

† From the Department of Chemistry, Purdue University, West Lafayette, Indiana 47907. *Received January 20, 1982.* Supported by National Institutes of Health Grants GM 19907, GM 10831, and RR 01077 to the Purdue University Biochemical Magnetic Resonance Laboratory.
‡ Present address: Department of Molecular Biophysics and Biochemistry, Yale University, New Haven, CT 06520.
§ Present address: Albert Einstein College of Medicine, Bronx, NY 10461.

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