Stabilization of Acetylcholine Receptor Secondary Structure by Cholesterol and Negatively Charged Phospholipids in Membranes†

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ABSTRACT: Fourier-transform infrared (FTIR) spectroscopy was used to study the secondary structure of purified *Torpedo californica* nicotinic acetylcholine receptor (AChR) in reconstituted membranes. Functional studies have previously demonstrated that the ion channel activity requires the presence of both sterol and negatively charged phospholipids in membranes. The present studies are designed to test the hypothesis that the α-helical structure of AChR may be stabilized by specific lipid molecules (sterol and/or negatively charged phospholipids) and that these α-helices may be responsible for the formation of a potential ion channel. FTIR data show statistically significant ($p < 0.005$) spectral changes due to cholesterol and negatively charged phospholipids, respectively. On the basis of standard curves describing the relationship between the spectral properties and the secondary structural contents of water-soluble proteins, the observed spectral change at 931 cm^{-1} can be interpreted as an apparent change in the α-helix content from about 17% in the absence of sterols to about 20% in the presence of sterols, suggesting that protein–sterol interactions increase the helical structure of the AChR molecule. Similarly, the spectral change at 988 cm^{-1} can be interpreted as an apparent increase of β-sheet content in the AChR molecule from about 20% to about 24% due to the presence of negatively charged phospholipids. Functional AChR in membranes thus appears to be correlated with higher α-helical and β-sheet contents. It is concluded that one role of specific interactions between membrane protein and lipid molecules may be to maintain specific secondary structures necessary to support the ion channel function of AChR.

The dynamic interactions at the protein–lipid interface of biological membranes have been studied extensively by various methods (Jost & Griffith, 1982). It is generally believed that the lipid molecules in the molecular shell surrounding membrane proteins are perturbed by the protein molecule (Jost et al., 1973; Devaux, 1983; Marsh, 1983). The apparent binding affinities of lipids with specific proteins at the lipid–protein interface have been measured by spin-labeling (Brotherus et al., 1981; Ellena et al., 1983) and fluorescence quenching methods (London & Feigenson, 1981; Simmonds et al., 1984). In general, the molecular properties of membrane proteins can be considered from two different points of view, one involving dynamic conformational changes and the other involving static structure. The dynamic conformational changes of acetylcholine receptor (AChR)[1] have been shown to require an optimal membrane fluidity (Fong & McNamee, 1986), and the catalytic activity of several membrane enzymes [e.g., Ca^{2+}-ATPase (Lippert & Schultz, 1980; Blazyk et al., 1985; Bigelow et al., 1986) and (Na^+–K^+)-ATPase (Harris, 1985)] has been demonstrated to depend on membrane fluidity which was manipulated by changing temperature. A negative conclusion regarding membrane fluidity effects has also been reported for Ca^{2+}-ATPase (East et al., 1984). However, little is known about how the lipid environment affects the protein structure, even though functional studies have resulted in controversial conclusions among various membrane proteins. For example, hexose transport protein (Connolly et al., 1985) and adenylate cyclase (Whetton et al., 1983) require a critical cholesterol concentration for the maximal catalytic activity, and *Escherichia coli* porin requires lipopolysaccharide (Schindler, 1983). In contrast, Ca^{2+}-ATPase (Navarro et al., 1984; Cheng et al., 1986), lactose carrier (Chen & Wilson, 1984), and cytochrome oxidase (Madden et al., 1983) require only phosphatidylcholine plus cone-shaped lipid molecules such as phosphatidylethanolamine. The differences in lipid requirements will only be resolved by studying specific biochemical functions correlated with specific structural changes.

The nicotinic acetylcholine receptor (AChR) has been purified from *Torpedo californica* and reconstituted into defined lipid environments (McNamee & Ochoa, 1982). The biochemical and biophysical functions of AChR have been extensively studied (Barrantes, 1983; Popot & Changeux, 1984; Anholt et al., 1985; McNamee et al., 1986a), and a low-resolution molecular model is emerging (Lindstrom et al., 1984; Young et al., 1985; Brisson & Unwin, 1985). Thus, the AChR provides an excellent model system to study the protein–lipid interactions in detail. AChR is a ligand-gated cation channel found at the synapses of muscle fibers and developmentally similar tissues such as fish electric organ. It has been proposed that the ion channel itself consists of five amphipathic α-helices (Guy, 1984; Finer-Moore & Stroud, 1984). Previous reports have demonstrated that the ion channel function requires the presence of sterols and phosphatidic acid (Ochoa et al., 1983; Schindler, 1983; Criado et al., 1984; Fong & McNamee, 1986) or other negatively charged phospholipids (McNamee et al.,

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[1] Abbreviations: AChR, nicotinic acetylcholine receptor; ATPase, adenosinetriphosphatase; CD, circular dichroism; CH, cholesterol; DEPC, dielaidoylphosphatidylcholine; DOPA, dioleoylphosphatidic acid; DOPC, dioleoylphosphatidylcholine; DOPG, dioleoylphosphatidylglycerol; EDTA, ethylenediaminetetraacetic acid; FTIR, Fourier-transform infrared; MOPS, 3-(*N*-morpholino)propanesulfonic acid; NCPL, negatively charged phospholipids; PA, (egg) phosphatidic acid; PC, phosphatidylcholine; PG, phosphatidylglycerol; Phe, phenylalanine; SDS, sodium dodecyl sulfate.

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1986b) in the membranes. Therefore, we postulated initially that sterol and negatively charged phospholipids may stabilize the α-helical structures in the AChR from which the potential ion channel is formed.

We report here the vibrational spectroscopic studies of AChR secondary structure in various reconstituted membranes containing purified AChR and synthetic lipids. Fourier-transform infrared (FTIR) data indicate that AChR in sterol-containing membranes possesses a higher α-helical content, while AChR acquires a higher β-sheet content in the presence of negatively charged phospholipids. Our results suggest that changes in membrane protein secondary structure can be correlated with specific lipid–protein interactions and biochemical function.

Materials and Methods

Acetylcholine Receptor Purification and Reconstitution. AChR was purified to homogeneity by affinity chromatography and reconstituted into phosphatidylcholine membranes as described (Ochoa et al., 1983; Fong & McNamee, 1986). The AChR purity was monitored by SDS gel electrophoresis and α-bungarotoxin binding assay. In one case, asolectin (Associated Concentrates, Woodside, NY) was used in place of phosphatidylcholine during affinity chromatography to obtain reconstituted asolectin membranes. The lipid:protein molar ratio was usually 100:1 for these reconstituted membranes. All synthetic lipids and egg PA were obtained from Avanti Polar Lipids (Birmingham, AL).

Rereconstitution of AChR in Complex Membranes. To prepare membrane samples containing a mixture of two or three lipid components, the reconstituted PC membranes were solubilized in 0.5% cholate in buffer A (100 mM NaCl–10 mM MOPS–0.1 mM EDTA–0.02% NaN_3, pH 7.4). The solubilized membranes were combined with a minimal volume (about 10% of the volume of the reconstituted membranes) of a solution containing the desired lipids in 0.5% cholate in buffer A to achieve the desired lipid composition. All additional lipid components were dissolved in chloroform and dried in vacuo and lyophilized before addition of cholate solution, and then sonicated until clear before being combined with reconstituted membranes. The combined mixture was equilibrated at 4 °C for 30 min and dialyzed against buffer A for 48 h with four changes of buffer. The final molar lipid:protein ratio ranged from 150:1 to 400:1. Most of the samples had a lipid:protein ratio in the range of 150–200. Native *Torpedo californica* lipids were extracted by the procedure of Bligh and Dyer (1959) with slight modification (Fong & McNamee, 1986). Incorporation of proteins into membranes was complete as monitored by sucrose gradient equilibrium sedimentation (Fong & McNamee, 1986).

Preparations of Liposome Samples. Lipid solutions in chloroform were dried in vacuo and lyophilized before addition of buffer A. Liposome samples were obtained by sonication until a homogeneous solution was observed. All liposome samples were prepared in this way, except that the cholate dialysis method was used to prepare the three liposome samples shown in Figure 5B to confirm that liposome samples prepared by sonication have the same FTIR spectra as the samples prepared by dialysis.

Preparation of Membrane Samples for FTIR Spectroscopy. Rereconstituted complex membranes, reconstituted PC membranes, and liposome samples were freeze–thawed in liquid nitrogen at least 4 times, followed by centrifugation at 50 000 rpm for 60 min in a Beckman SW 60 rotor (300000g). The aqueous pellet was used for FTIR measurements. The protein concentration in the pellet was estimated to be 100–200 mg/mL, and the lipid concentration in the pellet was about 0.1–0.3 M.

FTIR Spectroscopy. A 5-μL aliquot of sample was transferred to a sealed demountable cell with BaF_2 windows and a 15-μm spacer. The temperature of the sample cell was controlled by placing the cell onto an aluminum jacket which was equipped with a circulating water bath. The samples were allowed to equilibrate at each temperature for 10 min before being scanned. FTIR measurements were performed with a Perkin-Elmer 1750 FTIR spectrometer, a 7500 laboratory computer, and CDS-3 application software using a 2 cm^{-1} nominal resolution in the ratio mode. Usually, 32 scans were collected and averaged for each measurement in the range of 1800–850 cm^{-1}. Atmospheric background was recorded by using the same windows without spacer before sample scanning. Water absorption in all spectra was removed by subtracting incremental amounts of a pure water spectrum at the same temperature as the sample spectrum in three separate regions, respectively. The water subtraction end point was determined by obtaining a flat base line for the 1734 cm^{-1} peak in the 1800–1480 cm^{-1} region, a flat base line for the 1480–1140 cm^{-1} region, and a flat base line for the 930 cm^{-1} peak in the 1140–890 cm^{-1} region (Lee et al., 1985). The three regions can be merged together after subtraction.

Other Procedures. Protein concentration was determined by the Lowry method (Lowry et al., 1951). Phospholipid concentration was determined by organic phosphorus content (McClare, 1971). Cholesterol concentration was determined by a colorimetric assay kit (Boehringer Mannheim). Thin-layer chromatography was performed in $CHCl_3$–MeOH–H_2O–NH_3 (65:25:4:1) using a silica gel plate (Baker-flex silica gel IB-F from Baker Chemical Co., Phillipsburg, NJ). All soluble proteins were purchased from Sigma (St. Louis, MO) or Worthington (Freehold, NJ).

Results

Chemical Characterization of Rereconstituted Membranes Containing AChR. The molar lipid:protein ratio of rereconstituted membranes containing AChR was determined after rereconstitution or after concentration by ultracentrifugation. The calculated lipid:protein ratios in Figure 1A were based on the amounts of AChR and lipids added during purification and rereconstitution. As shown in Figure 1A, the actually measured lipid:protein ratio was essentially the same as expected.

The cholesterol mole fraction in the rereconstituted membranes was also determined. As shown in Figure 1B, the actually measured cholesterol mole fraction was linearly correlated with the calculated mole fraction which was based on the amounts of lipids added during rereconstitution. To qualitatively determine the mole fraction of other negatively charged phospholipids, lipids were extracted from rereconstituted membranes (Bligh & Dyer, 1959) and analyzed by silica gel thin-layer chromatography in $CHCl_3$–MeOH–H_2O–NH_3 (65:25:4:1) along with a standard solution of lipid mixture having the same composition in chloroform. These experiments indicated that the DOPA:DOPC ratio was qualitatively the same before and after rereconstitution (data not shown). Since the rereconstitution procedure reliably produced membrane samples having the desired membrane composition, the calculated values of lipid:protein ratio and lipid composition are presented in all following experiments.

Assignment of α-Helix and β-Sheet Bands in the Skeletal Vibration Region. The FTIR spectra of DOPC–PA–CH liposomes and AChR in DOPC–PA–CH (56:19:25) membranes are shown in Figure 2. In order to quantitate the amount of

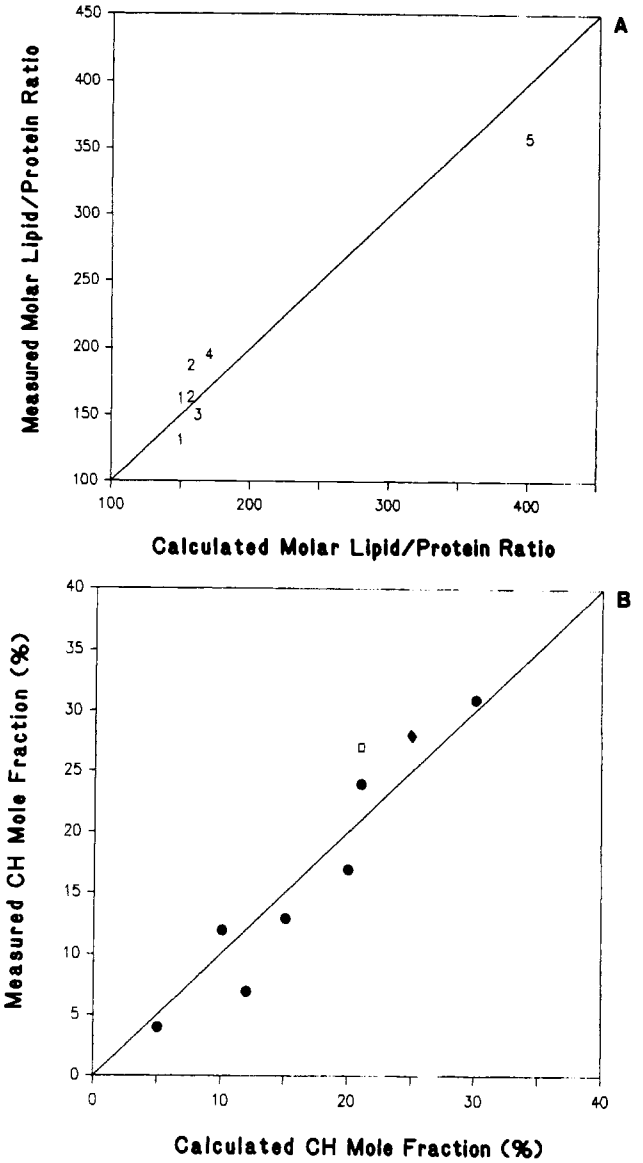

FIGURE 1: (A) Determination of molar lipid:protein ratio in reconstituted membranes containing AChR and different lipid compositions. The lipid composition was measured as described under Materials and Methods. The lipid compositions are (1) DOPC–CH (88:12) membranes before (lower data point) and after (upper data point) ultracentrifugation, (2) DOPC–CH (79:21) membranes before (lower data point) and after (upper data point) ultracentrifugation, (3) DOPC–CH (60:40) membranes after ultracentrifugation, (4) DOPC–PA–CH (56:19:25) membranes after ultracentrifugation, and (5) DOPC–CH (95:5) membranes before ultracentrifugation. (B) Determination of cholesterol mole fraction in reconstituted membranes or concentrated membrane samples: (●) reconstituted AChR–DOPC–CH membranes; (□) reconstituted AChR–DOPC–CH membranes after ultracentrifugation; (♦) DOPC–DOPC–CH (56:19:25) liposomes after ultracentrifugation. The lipid:protein ratios of these membranes range from 100 to 400.

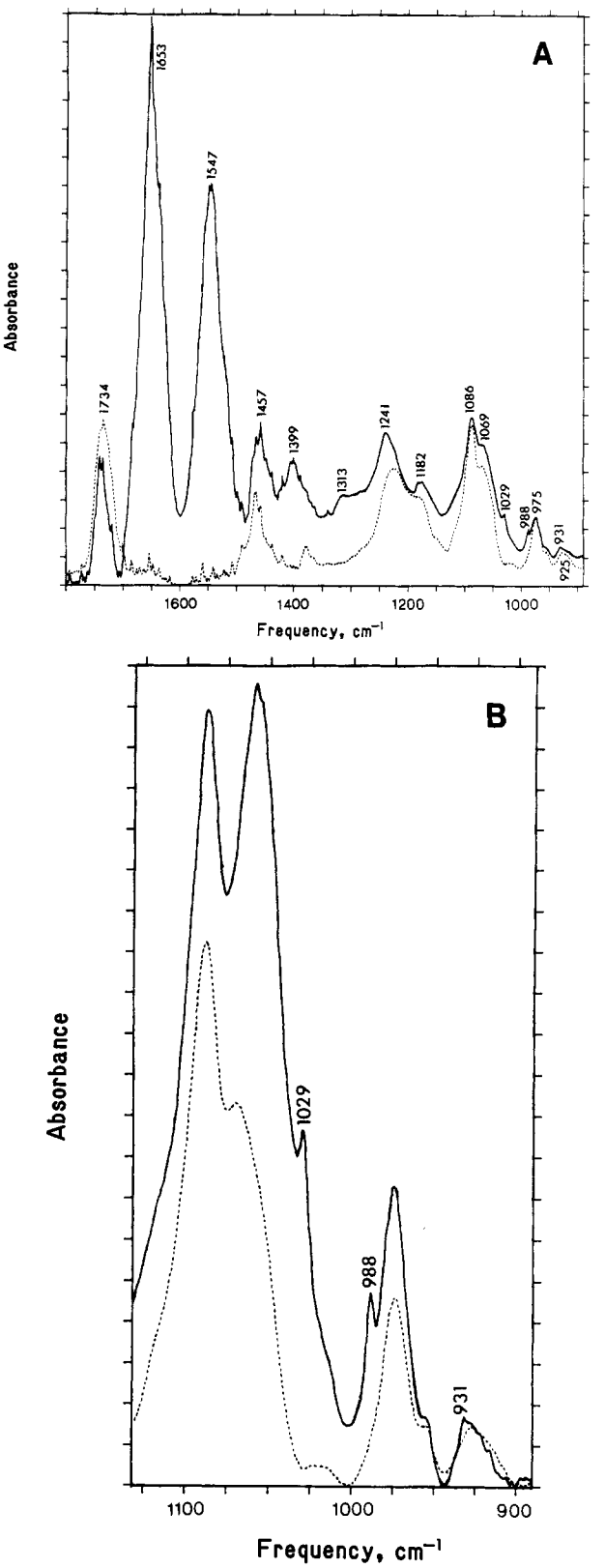

FIGURE 2: (A) FTIR spectra of reconstituted AChR–DOPC–PA–CH (1:112:38:50) membranes (—) and DOPC–PA–CH (56:19:25) liposomes (···) at 4 °C. The spectra were plotted after subtraction of the appropriate amount of water spectrum in three consecutive regions, respectively, and merging the resultant difference spectra. (B) Enlarged region.

secondary structure in AChR, we have focused on the peptide backbone skeletal stretching vibration region near 1000–900 cm^{-1} (Figure 2B). Three peaks can be identified which are not present in liposome spectra, and they are assigned to AChR. These three peaks are not due to residual cholate in the reconstituted membranes since little cholate was detected after dialysis (Ellena, 1982), and liposome samples prepared by either dialysis (spectrum a of Figure 5B) or sonication (dotted line of Figure 2) showed the same FTIR spectra.

The peak at 1029 cm^{-1} is assigned to phenylalanine ring bending according to both literature values (Carew et al., 1975; Parker, 1983a) and the free phenylalanine spectrum (data not shown). The 1029 cm^{-1} peak was used as a conformation-insensitive internal reference for protein concentration. The peak at 988 cm^{-1} was assigned to β-sheet structure since a similar Raman band at 991 cm^{-1} has been observed in IgG

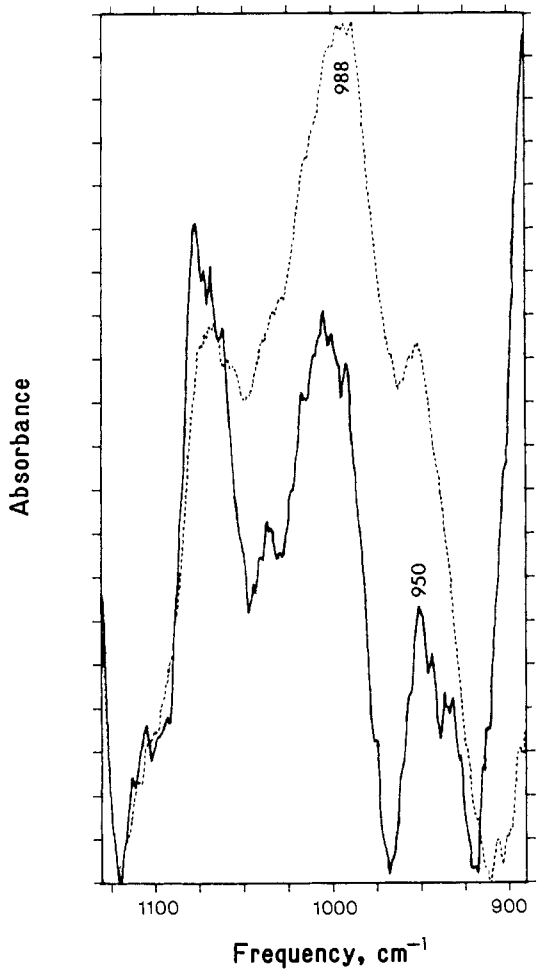

FIGURE 3: FTIR spectra of poly(L-lysine) in pH 10 aqueous solution at 4 °C (—) and at 55 °C (···) obtained after water subtraction.

which is a protein with predominantly β-structure (Pezolet et al., 1976) and in poly(L-lysine) at 1002 cm^{-1} (Yu et al., 1973). Poly(L-lysine) can be used as a model compound for both α-helix and β-sheet because it undergoes a thermally induced α to β transition at high pH (Yu et al., 1973). The FTIR spectra of poly(L-lysine) at pH 10 at two different temperatures are shown in Figure 3. At 4 °C, poly(L-lysine) adopts a predominantly α-helical structure, and a band at 930–950 cm^{-1} is observed. At 55 °C, however, poly(L-lysine) adopts a predominantly β-sheet structure, and the band at 988 cm^{-1} increases dramatically. These data indicate that FTIR measurements give results from secondary structure studies comparable to Raman spectroscopy.

A third assignment was made for α-helix structure at 931 cm^{-1} (Frushour & Koenig, 1975). As shown in Figure 2B, phospholipid C–C stretching has an absorption band at 925 cm^{-1} (Wallach, 1972; Cameron & Mantsch, 1978), and this overlaps with the α-helical skeletal band at about 935 cm^{-1} assigned to many soluble and membrane proteins and model peptides (Small et al., 1970; Koenig & Frushour, 1972; Frushouer & Koenig, 1974, 1975; Lin & Koenig, 1976; Aoki et al., 1980; Rooney et al., 1984). However, the 931 cm^{-1} peak can be assigned to regular secondary protein structure for the following reasons. First, thermal denaturation caused this composite peak at 931 cm^{-1} to shift to a position approaching the phospholipid peak at 925 cm^{-1} due to the decrease in protein intensity (Figure 4), while liposome spectra do not have such a frequency shift. Functional studies also demonstrated that the temperature dependence of ion-gating activity has the same relationship as the thermal denaturation curve in Figure

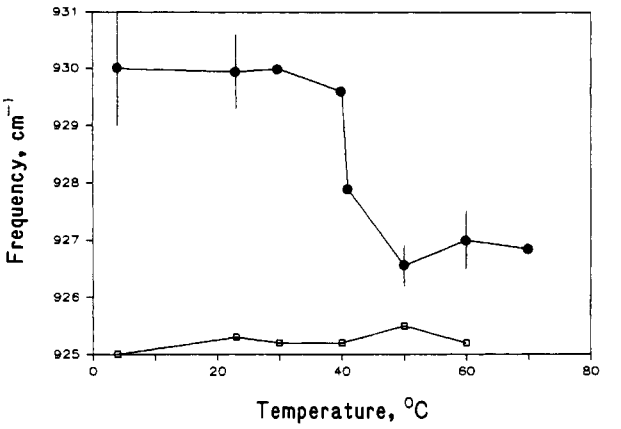

FIGURE 4: Temperature dependence of FTIR absorption frequency for the AChR α-helix skeletal vibration (●) in reconstituted DOPC–PA–CH (56:19:25) membranes and the phospholipid acyl chain (□) in DOPC–PA–CH liposomes.

4 (Soler et al., 1984). Second, spectra of liposomes containing different lipid composition all had the same absorption frequencies as the one shown in Figure 5B, and all AChR-containing membranes had the same absorption frequencies as the one in Figure 5A. Therefore, the 931 cm^{-1} peak is not arising from CH or negatively charged phospholipids. Third, as shown later in Figure 6, the integrated intensity of the 931 cm^{-1} peak also decreased as a function of temperature, which is consistent with the thermal denaturation effect on regular secondary structure. Fourth, the possibility that the 931 cm^{-1} peak might arise from protein-perturbed lipid acyl chains was ruled out since DEPC liposome spectra at 4 °C (gel phase, motionally restricted) and 23 °C (liquid-crystalline phase) had the same frequency at 925 cm^{-1}. Fifth, the possibility that the 931 cm^{-1} peak might arise from fatty acyl chains adjacent to AChR whose dielectric constant may be different from that of lipids was ruled out since this peak does not decrease (instead it increases) when there is CH present in membranes, which will displace some of the protein-adjacent phospholipids (Figure 5). Therefore, the 931 cm^{-1} band can be unambiguously assigned to the α-helix skeletal vibration.

Subtraction of Lipid Absorption from Spectra of Reconstituted Membranes. Since the protein α-helix peak at 931 cm^{-1} overlaps with the lipid peak at 925 cm^{-1}, it is necessary to establish a method of quantitating the protein absorption of this composite band. After water background was removed from both the lipid spectrum and the reconstituted membrane spectrum, a difference spectrum was obtained by subtracting incremental amounts of the lipid spectrum from the reconstituted membrane spectrum in the 1140–890 cm^{-1} region. The lipid spectrum was recorded from liposome samples having the same composition and at the same temperature as the reconstituted membrane spectrum. The end point of subtraction was determined by two criteria: (1) the lipid component has been completely removed at 925 cm^{-1} so that a symmetric peak at 931 cm^{-1} was obtained, and (2) there was no oversubtraction at all other regions (no negative base lines). An example of spectral subtraction is shown in Figure 5. Undersubtraction in the difference spectra was always observed at regions near the phenylalanine and β-sheet bands due to nonliner absorption as a function of concentration in different IR regions. However, they did not interfere with peak integration since these two peaks are well separated from lipid absorption peaks.

After the lipid component was subtracted from the protein-containing membrane spectra, the integrated intensity was then calculated from the difference spectrum. The integration

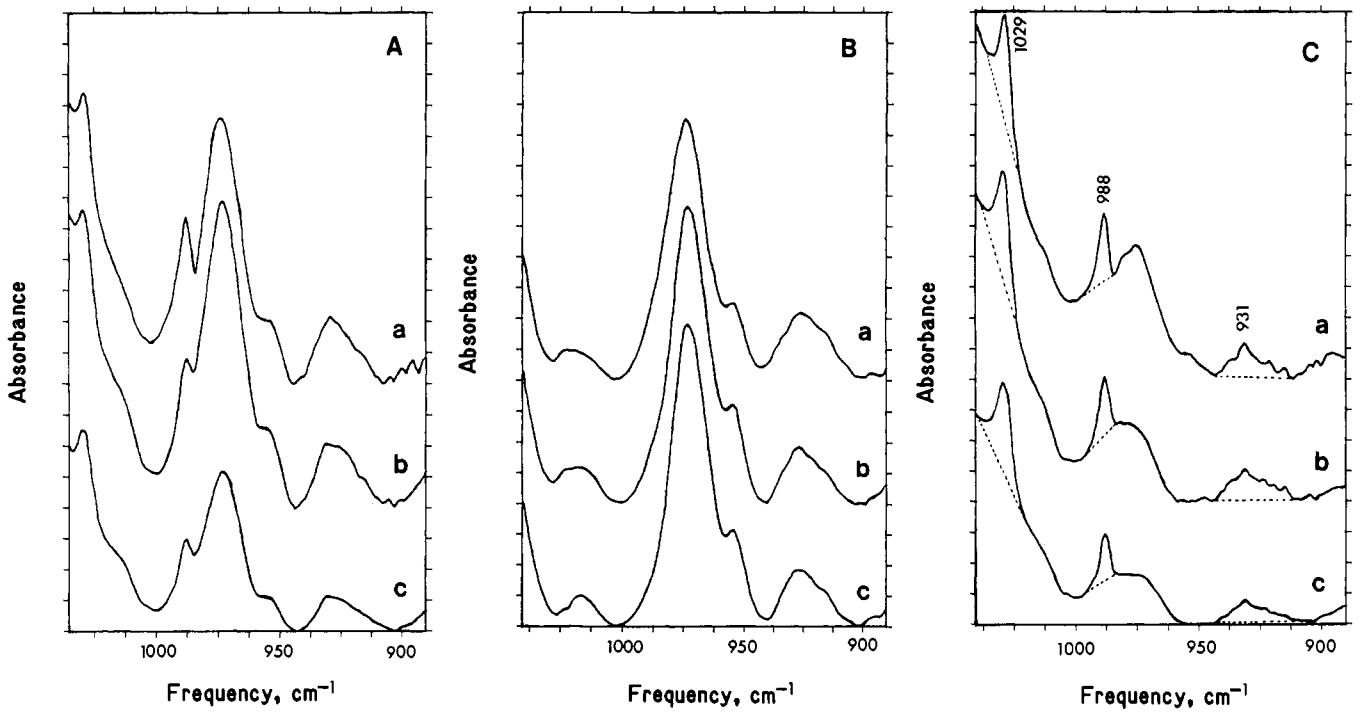

FIGURE 5: (A) FTIR spectra of reconstituted AChR membranes after water subtraction. (B) FTIR spectra of liposomes prepared by cholate dialysis after water subtraction. (C) Difference spectra generated from spectra of AChR-containing membranes and AChR-free liposomes. Integrated intensities were calculated as shown. The lipid compositions are (a) DOPC–PA–CH (56:19:25), (b) DOPC–CH (75:25), and (c) DOPC. All spectra were measured at 4 °C.

Table I: FTIR Frequencies of α-Helix and β-Sheet Skeletal Vibration Bands from the Present Studies and Literature Values of Secondary Structures for Several Soluble Proteins[a]

protein	cm^{-1} (α)	% α-helix	ref	cm^{-1} (β)	% β-sheet	ref	% Phe	ref
bovine pancreas insulin	957	52	*b*				5.88	*c*
chicken egg ovalbumin	933	25	*b*	983	25	*b*	5.4	*d*
chicken egg lysozyme	934	29–42	*b*	988	10, 16	*b, e*	2.33	*c*
bovine serum albumin	936	55, 58	*b, f*				4.59	*c*
bovine pancreas α-chymotrypsinogen A	934	10–20	*b*	983	34	*e, g*	2.44	*h*
human IgG				992	37, 50–60	*e, i, j*	3.49	*c, k*
concanavalin A				990	52, 57	*l, m*	4.5	*c*

[a] All spectra were recorded at 23 °C at pH 7.4 (except insulin in pH 4 acetate buffer) with a protein concentration of about 100–200 mg/mL. Water absorption was removed by incremental subtraction in all spectra. [b] Carey (1982). [c] Fasman (1976). [d] Lewis et al. (1950). [e] Pezolet et al. (1976). [f] Rooney et al. (1984). [g] Kraut (1971). [h] Hartley (1964). [i] Poljak et al. (1974). [j] Deisenhofer (1981). [k] Cunningham et al. (1970) and Rutishauser et al. (1970). [l] Hardman & Ainsworth (1972). [m] Feeke et al. (1975).

was performed according to two rules (Figure 5C): (1) if the peak was symmetrical, the minimum and maximum frequencies were identified, and the area above the base line was calculated; (2) if the peak was not symmetrical, the base line was interpolated, and the area above this base line was calculated.

As shown in Figure 6, thermal denaturation caused a continuous decrease in both α-helix (931 cm^{-1}) and β-sheet (988 cm^{-1}) absorption, which provides consistent evidence for the assignment of α-helical and β-sheet bands.

Establishing a Correlation between Skeletal Absorption and Structural Content. Protein helical content has been correlated with the relative intensity of the skeletal vibration band at 940 cm^{-1} by measuring I_{940}/I_{1004} in which I_{1004} is the intensity of the Raman active phenylalanine band (Lin & Koenig, 1976; Aoki et al., 1982; Rooney et al., 1984). We repeated such measurements using FTIR and extended them to β-sheet content measurements with several soluble proteins having known three-dimensional structure (Table I). We used the Phe band at 1029 cm^{-1}, the β-sheet band at 992–980 cm^{-1}, and the α-helix band at 957–930 cm^{-1} (Table I). The Phe absorption band at 1029 cm^{-1} was used as an internal reference of protein concentration. As shown in Figure 7, the normalized integrated intensity for both α-helix and β-sheet correlates well with the secondary structure content. Therefore, the spectral indexes of relative peak area $A_{skeletal(\alpha)}/A_{Phe}$ and $A_{skeletal(\beta)}/A_{Phe}$, can be used to estimate the secondary structural contents of unknown proteins.

Secondary Structures of AChR in Various Reconstituted Membranes. In order to test the hypothesis that sterol and/or negatively charged phospholipids may stabilize AChR structures responsible for ion channel function through specific lipid–protein interactions, reconstituted membranes containing purified AChR and various synthetic lipids were prepared for FTIR spectroscopy. Summarized in Table II are spectral indexes of relative peak area obtained from difference spectra such as the ones shown in Figure 5C. These results clearly indicated that cholesterol or sterols in membranes caused an increase in the α-helical spectral index, while negatively charged phospholipids in membranes caused an increase in the β-sheet spectral index. Combined with the data shown in Figure 7, we concluded that AChR α-helices are stabilized by neutral rigid molecules like cholesterol and β-sheet structure in AChR is stabilized by negatively charged phospholipid head groups. This conclusion is schematically presented in Figure 8, in which the secondary structural contents were calculated

Table II: Spectral Indexes of Relative Peak Area for Reconstituted Membranes Containing AChR[a]

membrane composition	no. of preparations	$A_{\alpha,931}/A_{Phe,1029}$[b]	$A_{\beta,988}/A_{Phe,1029}$[b]	ion channel function[c]
(i) PC only				
DOPC	2	0.43 ± 0.09 (4)	0.46 ± 0.05 (4)	-
DEPC (4 °C)	2	0.47 ± 0.08 (6)	0.29 ± 0.15 (6)	-
DEPC (23 °C)		0.39 ± 0.11 (4)	0.28 ± 0.12 (4)	-
(ii) PC and CH				
DOPC-CH (9:1)	3	0.65 ± 0.17 (10)	0.38 ± 0.14 (10)	- predicted
DOPC-CH (3:1)	3	0.84 ± 0.24 (6)	0.39 ± 0.07 (6)	-
DOPC-CH (6:4)	2	0.70 ± 0.14 (10)	0.42 ± 0.06 (7)	-
(iii) PC and PA				
DOPC-DOPA (9:1)	3	0.41 ± 0.20 (11)	0.68 ± 0.13 (11)	- predicted
DOPC-DOPA (3:1)	3	0.30 ± 0.07 (10)	0.58 ± 0.08 (10)	-
DOPC-DOPA (6:4)	2	0.34 ± 0.04 (8)	0.41 ± 0.06 (8)	- predicted
(iv) PC, sterol, and negatively charged phospholipids				
DOPC-PA-CH (56:19:25)	3	0.96 ± 0.32 (7)	0.71 ± 0.15 (7)	+
DOPC-DOPG-CH (56:19:25)	2	0.61 ± 0.09 (4)	0.67 ± 0.08 (4)	+
DOPC-asolectin (4:6)	2	0.96 ± 0.22 (2)	0.69 ± 0.13 (2)	+ predicted
asolectin	2	0.91 ± 0.24 (6)	0.89 ± 0.08 (6)	+
PC-native lipids (4:6)	2	0.72 ± 0.07 (6)	0.70 ± 0.07 (6)	+ predicted

[a] All spectra were obtained at 4 °C unless indicated otherwise in parentheses. [b] The number of measurements is listed in parentheses. Each measurement was the average of at least 32 scans. [c] Data from Fong and McNamee (1986).

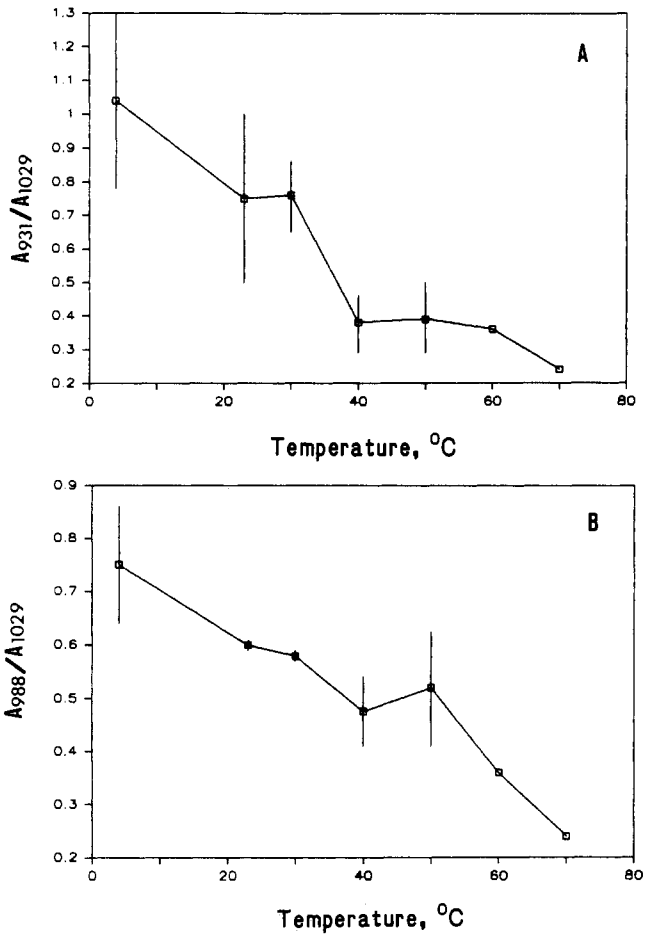

FIGURE 6: Temperature dependence of FTIR integrated intensity (relative to the Phe integrated intensity A_{1029}) for α-helices (A) and β-sheets (B) in reconstituted AChR-DOPC-PA-CH (1:112:38:50) membranes.

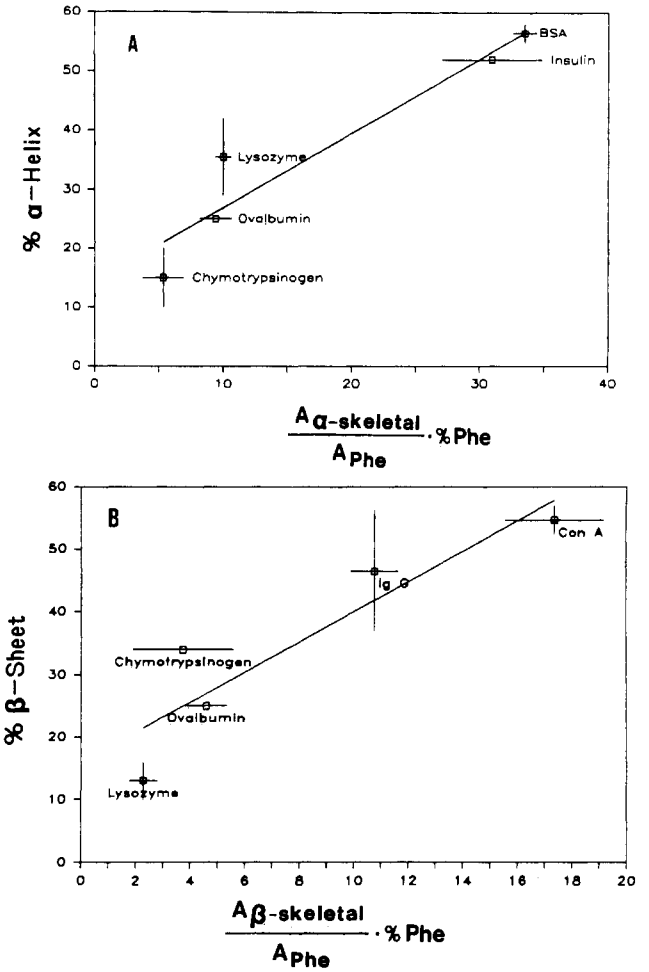

FIGURE 7: (A) Correlation between α-helical content and the spectral indexes $A_{\alpha\text{-skeletal}}/A_{Phe,1029}$ multiplied by the percentage of Phe in each protein. (B) Correlation between β-sheet content and the spectral indexes $A_{\beta\text{-skeletal}}/A_{Phe,1029}$ multiplied by the percentage of Phe in each protein. The x-axis variations are the mean ± SD from the present studies while the y-axis variations are the range of data obtained by different authors. Water subtraction and intensity integration were performed as in AChR membrane spectra.

by multiplying the total Phe content of 5.1% in AChR (Popot & Changeux, 1984) with the spectral indexes listed in Table II and using the linear regression lines in Figure 7. Student's t tests were performed between sterol-containing membranes and membranes without sterols, and between membranes with and without negatively charged phospholipids. The mean α-helical spectral index of AChR in all the six sterol-free membranes was 0.39 while the mean α-helical spectral index in all the eight sterol-containing membranes was 0.79. The pooled standard deviation was ±0.17, and the difference between the two groups of membranes was significant ($p < 0.005$). As predicted from Figure 7, AChR in membranes

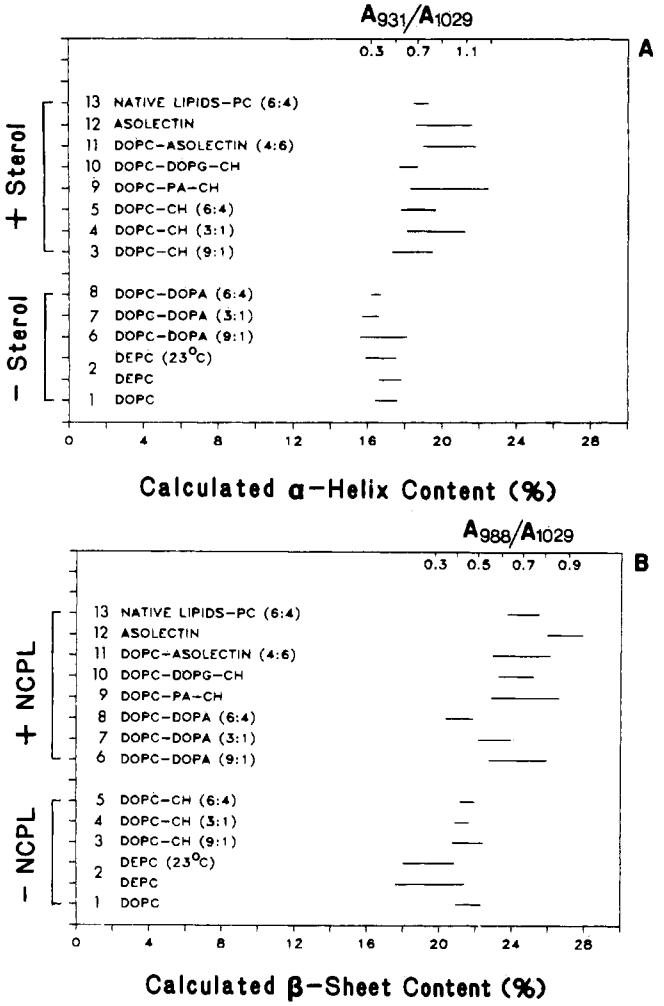

FIGURE 8: Calculated secondary structural content of AChR at 4 °C in various reconstituted membranes using the data in Table II and Figure 7. Each bar represents the mean ± SD. The lipid composition is as follows: (1) DOPC; (2) DEPC; (3) DOPC–CH (9:1); (4) DOPC–CH (3:1); (5) DOPC–CH (6:4); (6) DOPC–DOPA (9:1); (7) DOPC–DOPA (3:1); (8) DOPC–DOPA (6:4); (9) DOPC–PA–CH (56:19:25); (10) DOPC–DOPG–CH (56:19:25); (11) DOPC–asolectin (4:6); (12) asolectin; (13) native lipids–PC (6:4).

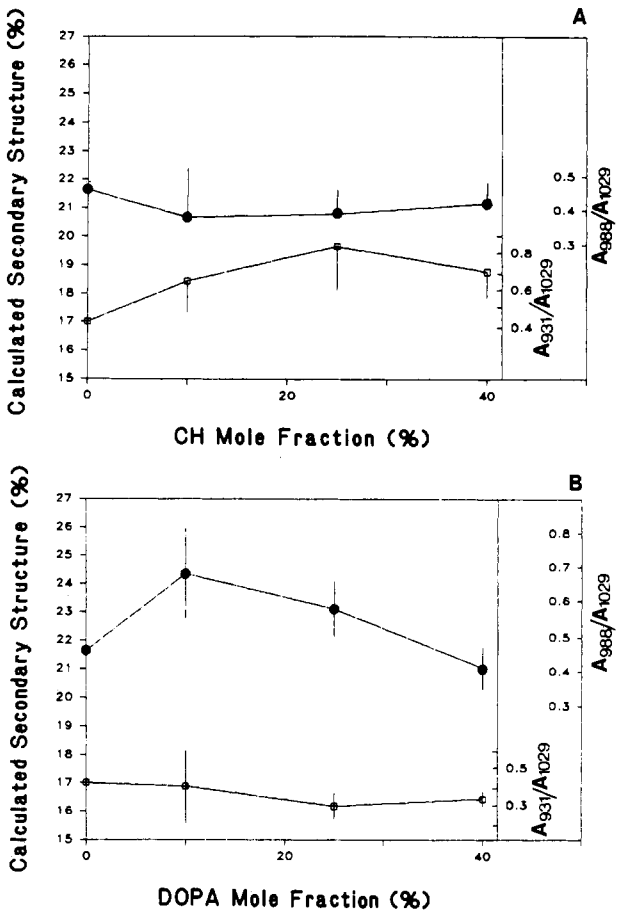

FIGURE 9: Calculated secondary structural contents [α-helix (□); β-sheet (●)] of AChR at 4 °C in reconstituted DOPC–CH membranes (A) and DOPC–DOPA membranes (B) as a function of lipid mole fraction in the membranes.

devoid of sterols contains about 17% α-helices, while AChR in sterol-containing membranes contains about 20% α-helices (Figure 8A).

On the other hand, a significant effect on the AChR β-sheet spectral index was observed in the presence of negatively charged phospholipids. The mean β-sheet spectral index of AChR in all the six membranes devoid of negatively charged phospholipids was 0.37 while the mean β-sheet spectral index in all the eight membranes containing negatively charged phospholipids was 0.67. The pooled standard deviation was ±0.10, and the t test rejected the null hypothesis ($p < 0.005$). As predicted from Figure 7, AChR in membranes devoid of negatively charged phospholipids contains about 20% β-sheets while AChR in membranes with negatively charged phospholipids contains about 24% β-sheets (Figure 8B).

Lipid to protein ratios ranging from 150 to 400 had no effect on the FTIR spectral indexes since preparations with the same lipid composition but slightly different lipid:protein ratio gave rise to similar spectral indexes (Table II). The lipid phase behavior also did not affect the spectral indexes (see DEPC membranes at 4 and 23 °C in Table II).

As shown in Figure 9A, cholesterol content in membranes has little effect on the calculated β-sheet content while AChR manifests more α-helical structure in membranes with 10–40% cholesterol. In contrast, AChR acquires higher β-sheet content in membranes containing 10–25% PA, but its α-helical content is not changed (Figure 9B) as predicted from Figure 7. In any particular membrane sample, the increase in α-helices is not accompanied by a decrease in β-sheet content, and vice versa. Therefore, the increase in secondary structural contents may result from a decrease in either turns, loops, or random coils. When both sterol and negatively charged phospholipids are present, both α-helical and β-sheet contents are increased (Table II and Figure 8). These results suggest that the cholesterol and negatively charged phospholipid effects are basically independent.

Discussion

AChR is one example of membrane proteins that require specific lipid molecules in the membrane to support their biological functions. It has been shown that the activation of the AChR cation channel requires the presence of cholesterol and phosphatidic acid (Ochoa et al., 1983; Criado et al., 1984; Fong & McNamee, 1986) or other negatively charged phospholipids (McNamee et al., 1986b). This lipid requirement is considered to be essential for the static structure of AChR since some aspects of the conformational changes associated with AChR do not require the presence of specific lipid molecules (Fong & McNamee, 1986). Therefore, the studies of lipid requirement for membrane proteins should be directed at two different aspects, the dynamic properties and the static structure of membrane proteins. The present results provide new insights into the structure–function relationship of AChR as affected by the lipid environments. It is demonstrated here

that the functional effects of lipid molecules can be correlated with their structural effects.

Vibrational spectroscopies have been widely used to study protein secondary structures, and it has been shown that spectroscopic data agree with X-ray diffraction results for soluble proteins [see Parker (1983)]. In the case of membrane proteins, however, such a correlation has not been possible since only one membrane protein's high-resolution structure is available (Deisenhofer et al., 1985). In spite of this disadvantage, instructive information can still be obtained from spectroscopic studies. Several methods are available to deduce various secondary structures from vibrational spectra. One of them involves fitting the amide I spectrum of a protein to a linear combination of amide I spectra from a set of reference proteins whose secondary structures have been determined by X-ray crystallography (Williams, 1983). Another method is using deconvolution procedures to obtain each structural component in the amide I band (Thomas & Agard, 1984; Byler & Susi, 1986). A third method is based on the correlation between peptide backbone skeletal vibration intensity or amide III intensity and secondary structural content (Lin & Koenig, 1976; Aoki et al., 1982; Rooney et al., 1984; Pezolet et al., 1976). We used the last method in the present studies in order to avoid the very strong H_2O absorption at the amide I region (Parker, 1983b). We also found here that FTIR measurements can provide the same correlation for α-helical content based on the skeletal vibration intensity at 930–960 cm^{-1} (Figure 7A) as Raman spectroscopy does (Rooney et al., 1984). In addition, it is shown here that β-sheet content can be correlated with the skeletal vibration intensity at 980–990 cm^{-1} (Figure 7B). Thus, measuring the skeletal vibration intensity will provide an estimated value for both α-helical and β-sheet contents.

The first report on the secondary structure of AChR from *Torpedo nobiliana* in detergent was provided by Moore et al. (1974) in which 34% α-helices and 29% β-sheets were found by using far-UV CD spectroscopy. A preliminary Raman spectrum of AChR from *Torpedo marmorata* has also been reported without detailed structural analysis (Aslanian et al., 1983). Recently, Yager et al. (1984) showed that AChR from *Torpedo californica* in reconstituted DEPC membranes contained 34% antiparallel β-sheets and 25% ordered α-helices using Raman amide I spectral analysis, although AChR in DEPC membranes does not have any ion channel activity (Fong & McNamee, 1986). A similarly high β-sheet content in detergent-solubilized AChR membranes was also reported by Cascio et al. (1986) from CD spectroscopic analysis. Using FTIR spectra in the skeletal vibration region, we found that AChR contains about 20% α-helices and 24% β-sheets in functional membranes while AChR contains about 17% α-helices in cholesterol-deficient membranes and 20% β-sheets in membranes without negatively charged phospholipids (Figure 8). The quantitative discrepancy between FTIR and Raman data is likely due to systematic differences since Yager et al. (1984) used the linear combination procedure to solve the secondary structure from the amide I spectrum. Nevertheless, we observed a higher apparent β-sheet content than α-helical content on the basis of FTIR data, which is consistent with the results inferred from Raman data. These experimental results do not agree with a predicted secondary structure deduced from statistical amino acid sequence analysis (Finer-Moore & Stroud, 1984) in which 44% α-helices and 27% β-sheets were predicted. Such inconsistency between prediction and experiment will not be resolved until high-resolution three-dimensional structures or new theories for predicting membrane protein secondary structure are available.

Although the exact numerical values of secondary structural content need to be refined by X-ray crystallography, vibrational spectroscopic studies can still provide valuable information about the effects of lipid environments on membrane protein structure. Such an approach has been adopted by Dunker et al. (1981) to examine the conformational state of filamentous phage coat protein in phospholipid membranes with different fatty acyl chains. Detailed studies of the effect of cholesterol on membrane protein helicity have been carried out by Rooney et al. (1984) using spectroscopic methods, from which a substantial increase in protein helical structure was inferred in cholesterol-enriched erythrocyte membranes compared to cholesterol-depleted membranes. Since crude plasma membranes were used in those studies, it was not possible to correlate the structure of specific proteins with structural effects of cholesterol.

It is shown here that in a reconstituted membrane system containing purified AChR and synthetic lipids, sterol molecules do indeed increase the FTIR absorption assigned to α-helical structure. Such an increase is statistically significant, and it may reflect large changes in local protein structure. The α-helical content of AChR inferred from spectral analysis is about 17% in membranes without sterols as opposed to about 20% in membranes with sterols. The AChR molecule consists of 2333 amino acids, and 1% of the primary structure corresponds to at least 20 amino acid residues. Therefore, a 3% difference in structure is large enough to affect local domain structure and able to induce further structural changes.

The effects on membrane protein helicity measured here are reversible because all complex membranes in which AChR has higher secondary structural content were obtained by a rereconstitution procedure from PC membranes in which AChR has low secondary structural content. This helical enhancement effect by cholesterol is more likely due to the sterol–helix interaction at the hydrophobic protein–lipid interface rather than interaction at the head-group region involving the hydroxyl group since PA, PC, or PG does not have similar effects, and we consider that it is the rigidity of the neutral sterol molecule that stabilizes helical structures. It has been found that the temperature factors of helices are small, indicating a very rigid structure compared to other kinds of structural features (Frauenfelder et al., 1979; Artymiuk et al., 1979; Sternberg et al., 1979). One possible mechanism of helical enhancement by cholesterol is that the sterol rigid ring structure, whose long axis is parallel to the membrane normal, fits into the grooves of tilted α-helices (mainly class 3 or class 4 grooves, in which the class number represents the separation of amino acid residues from which ridges and grooves are formed). Such a packing model is similar to the ridge-into-groove helix packing model in proteins (Chothia et al., 1981). In this model, the phospholipid acyl chain is not rigid enough to impose any structural constraint. The helix–sterol packing may improve the overall AChR packing which may account for the increase of α-helical content. Another mechanism is also possible. It has been found that cholesterol in phospholipid bilayers can decrease the water permeability of membranes at temperatures higher than T_m (Carruthers & Melchior, 1983). It has been proposed recently that more ordered helix structure in soluble proteins results as the amount of bound water is decreased (Jakobsen et al., 1986). Therefore, the decrease in water permeability of bilayers due to the presence of cholesterol may also account for the increase of helical content. The structural explanation of cholesterol's effect on AChR channel function is consistent with functional

studies from which it was concluded cholesterol is required for the channel formation (Ochoa et al., 1983; Criado et al., 1984; Fong & McNamee, 1986) or cholesterol stabilizes channel conductance and appearance (Schindler, 1983).

In addition, we also found that the functionally necessary negatively charged phospholipids increase the calculated β-sheet content from about 20% to 24% in AChR. Such an effect on protein secondary structure has not been reported before. This effect may arise from the electrostatic interaction between phospholipid head groups and hydrophilic protein domains at the membrane surface region. Fourier-transform analysis of hydrophobicity along the AChR amino acid sequence revealed a strikingly high intensity at the frequency of 150–180° for the amino acid sequence 50–170 in all four subunits (Finer-Moore & Stroud, 1984). Since 180° is the ideal β-sheet period, amphipathic β-sheet structure is predicted to form at this N-terminal domain. By interacting with the lysine, arginine, and histidine residues at this region of each subunit (Noda et al., 1983), the negatively charged phospholipid head groups could pose some stabilizing effects on the formation of β-sheet structure which otherwise may not be as favorable as in the presence of electrostatic interaction. For example, the segment from 53 to 69 (NVRLRQQWIDVRLRWNP) of the α-subunit shows a strong amphipathic periodicity of 180° (data not shown), indicating the potential formation of amphipathic β-structure. This segment has a net charge of 3+ clustered on the hydrophilic side which would make the β-sheet structure unstable. However, this amphipathic structure could be stabilized by interacting with negatively charged phospholipid head groups.

When both sterol and negatively charged phospholipids are present in the membranes, both α-helical and β-sheet contents increase accordingly as in sterol-containing membranes and negatively charged phospholipid-containing membranes. This structural change correlates exactly with the lipid requirements for AChR ion channel activity since only the fourth group in Table II supports AChR channel activity. Therefore, the α-helices stabilized by sterol molecules are likely to be essential for maintaining the ion channel structure, while the β-sheets stabilized by negatively charged phospholipids are considered to be crucial in transmitting conformational changes induced specifically by AChR agonists.

The method used here to estimate the secondary structural contents is an indirect one based on the structure of soluble proteins. Since a correlation between FTIR skeletal absorption and structural contents can be established, it is reasonable to predict that the secondary structure of membrane proteins can also be correlated with the skeletal absorption. Possibilities other than an increase in secondary structural content, which might also explain the skeletal absorption changes due to lipid environments, cannot be ruled out completely at the present time. For example, it has been suggested that nonstandard helices (Krimm & Dwivedi, 1982) with the plane of peptide bond not parallel to the helix axis (α_{II}) may have absorption properties different from that of standard helices (α_I).

We originally expected that the secondary structural content would increase monotonically as a function of the mole fraction of specific lipid. This is only true when the mole fraction of PA is small since the β-sheet spectral index seems to decrease at a mole fraction higher than 30% DOPA (Figure 9B). Such an effect is likely due to high surface charge density at high PA concentration in membranes, which may result in destabilization of membrane proteins.

In summary, it was found that sterols can increase the estimated amount of α-helical structure in AChR, and negatively charged phospholipids increase the estimated amount of the β-sheet structure. The former effect is likely applicable to other membrane proteins, while the latter effect may be more specific to certain types of membrane proteins, such as those containing many positive amino acid residues in the extramembrane domain. At least in the case of AChR, these two effects are correlated with the functional effects of lipid environments. The spatial localization of those segments stabilized by specific lipids is being investigated.

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Registry No. DOPC, 10015-85-7; DEPC, 41688-09-9; DOPA, 14268-17-8; DOPG, 62700-69-0; cholesterol, 57-88-5; poly(L-lysine) (homopolymer), 25104-18-1; poly(L-lysine) (SRU), 38000-06-5.

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