Electrostatic and Hydrophobic Contributions to the Folding Mechanism of Apocytochrome *c* Driven by the Interaction with Lipid[†]

Saffron E. Rankin,[‡] Anthony Watts,[§] and Teresa J. T. Pinheiro*,[‡]

Department of Biological Sciences, University of Warwick, Gibbet Hill Road, Coventry CV4 7AL, U.K., and Department of Biochemistry, University of Oxford, South Parks Road, Oxford OX1 3QU, U.K.

Received February 20, 1998; Revised Manuscript Received July 17, 1998

ABSTRACT: In aqueous solution, while cytochrome *c* is a stably folded protein with a tightly packed structure at the secondary and tertiary levels, its heme-free precursor, apocytochrome *c*, shows all features of a structureless random coil. However, upon interaction with phospholipid vesicles or lysophospholipid micelles, apocytochrome *c* undergoes a conformational transition from its random coil in solution to an α-helical structure on association with lipid. The driving forces of this lipid-induced folding process of apocytochrome *c* were investigated for the interaction with various phospholipids and lysophospholipids. Binding of apocytochrome *c* to negatively charged phospholipid vesicles induced a partially folded state with ∼85% of the α-helical structure of cytochrome *c* in solution. In contrast, in the presence of zwitterionic phospholipid vesicles, apocytochrome *c* remains a random coil, suggesting that negatively charged phospholipid headgroups play an important role in the mechanism of lipid-induced folding of apocytochrome *c*. However, negatively charged lysophospholipid micelles induce a higher content of α-helical structure than equivalent negatively charged diacylphospholipids in bilayers, reaching 100% of the α-helix content of cytochrome *c* in solution. Furthermore, micelles of lysolipids with the same zwitterionic headgroup of phospholipid bilayer vesicles induce ∼60% of the α-helix content of cytochrome *c* in solution. On the basis of these results, we propose a mechanism for the folding of apocytochrome *c* induced by the interaction with lipid, which accounts for both electrostatic and hydrophobic contributions. Electrostatic lipid−protein interactions appear to direct the polypeptide to the micelle or vesicle surface and to induce an early partially folded state on the membrane surface. Hydrophobic interactions between nonpolar residues in the protein and the hydrophobic core of the lipid bilayer stabilize and extend the secondary structure upon membrane insertion.

Apocytochrome *c* is the precursor of the mitochondrial protein cytochrome *c*, which functions in the intermembrane space of mitochondria to donate electrons to the inner mitochondrial membrane protein cytochrome *c* oxidase. The precursor protein is encoded by nuclear DNA and synthesized on free cytoplasmic ribosomes without the heme group, and unlike most other mitochondrial precursor proteins, it is synthesized without a cleavable amino-terminal presequence. It does not require ATP or a membrane potential for translocation, and no other protein receptor in the outer mitochondria membrane appears to be involved (*1*). Instead, apocytochrome *c* can insert spontaneously, and partially cross the outer mitochondrial membrane (*2*, *3*). After, or at the same time as translocation across the outer membrane, apocytochrome *c* is recognized by the enzyme cytochrome *c* heme lyase (CCHL)[1] and the heme group is covalently attached to cysteines 14 and 17 (*4*−*6*). The import pathway of cytochrome *c* into mitochondria is therefore simpler than those of most other proteins that have to be transported across a lipid barrier, and can be resolved into three main steps: (a) binding of apocytochrome *c* to the outer mitochondria membrane, (b) insertion and translocation across the membrane, and (c) heme attachment by the enzyme cytochrome *c* heme lyase. Steps a and b appear to be reversible, and the unidirectionality of the import process is imposed by step c through the specific interaction with CCHL (*7*). After heme attachment, dissociation of holocytochrome *c* from CCHL is presumed to be triggered by folding of the polypeptide around the heme group into the native cytochrome *c* structure. Once the folded compact structure is formed, cytochrome *c* is confined to the intermembrane space

[†] This work has been supported by The Royal Society (T.J.T.P.). S.E.R. is supported by the EC TMR network on 'Membrane Biogenesis' FMRX-CT96-0004 (A.W.). T.J.T.P. is a Royal Society University Research Fellow. This project has also been supported in part by The Royal Society International Exchange Programme with Russia.

* To whom correspondence should be addressed. Telephone: +44 1203 528 364. Fax: +44 1203 523 568. E-mail: tp@dna.bio.warwick.ac.uk.

[‡] University of Warwick.

[§] University of Oxford.

[1] Abbreviations: CCHL, cytochrome *c* heme lyase; CD, circular dichroism; CMC, critical micelle concentration; DMPC, dimyristoylphosphatidylcholine (1,2-dimyristoyl-*sn*-glycero-3-phosphocholine); DMPG, dimyristoylphosphatidylglycerol (1,2-dimyristoyl-*sn*-glycero-3-phospho-*rac*-1-glycerol); DOPC, dioleoylphosphatidylcholine (1,2-dioleoyl-*sn*-glycero-3-phosphocholine); DOPG, dioleoylphosphatidylglycerol (1,2-dioleoyl-*sn*-glycero-3-phospho-*rac*-1-glycerol); DOPS, dioleoylphosphatidylserine (1,2-dioleoyl-*sn*-glycero-3-phosphoserine); lysoMPC, 1-myristoyl-2-hydroxy-*sn*-glycero-3-phosphocholine; lysoMPG, 1-myristoyl-2-hydroxy-*sn*-glycero-3-phospho-*rac*-1-glycerol; lysoOPC, 1-oleoyl-2-hydroxy-*sn*-glycero-3-phosphocholine; lysoOPG, 1-oleoyl-2-hydroxy-*sn*-glycero-3-phosphoglycerol; NPN, *N*-phenyl-1-naphthylamine.

S0006-2960(98)00408-5 CCC: $15.00
Published on Web 08/19/1998

and cannot cross back to the cytoplasm, unless the cell becomes apoptotic (*8*).

The heme group in cytochrome *c*, which is important both in its function as an electron carrier and in its import pathway into mitochondria, appears to have a crucial role in stabilizing the native fold of cytochrome *c*. Upon removal of the heme, cytochrome *c* loses all the secondary structure characteristic of the native holoprotein and shows the properties typical of a disordered polypeptide in solution, as judged by circular dichroism and intrinsic viscosity (*9*). However, the interaction of the structureless apocytochrome *c* with lipid (in the absence of heme) can originate in a partially folded conformation with a native-like α-helix content of the holoprotein (*10*−*12*). These studies have shown that the interaction of apocytochrome *c* with negatively charged DOPS vesicles or phosphoglycol detergent micelles induces a partially folded α-helical structure. Furthermore, studies with apocytochrome *c* fragments binding to the same lipid systems suggest that the helices are formed within the regions of the sequence where the native α-helices occur in cytochrome *c* (*10*). In contrast, addition of zwitterionic lipid vesicles or neutral detergents did not induce folding of apocytochrome *c*, suggesting an important role of negatively charged lipid headgroups in the folding transition of apocytochrome *c* from its random coil conformation in solution into an α-helical structure when it is associated with lipid. However, small unilamellar vesicles of zwitterionic lipid DOPC were able to induce significant spectral changes in apocytochrome *c* (*11*), while no changes in the protein structure were induced by large unilamellar DOPC vesicles (*10*). These results suggest that the reduced packing of the lipid acyl chains in smaller vesicles, with concomitant greater accessibility of the hydrophobic core of the bilayer, may reveal a "hidden" important hydrophobic component in the folding mechanism of apocytochrome *c* driven by lipid interaction, and this requires further investigation.

It is generally recognized that proteins destined to cross membranes need to be kept in an unfolded or loose conformation. Stabilization of the native structure of proteins and their precursors by specific ligands (*13*, *14*) or by disulfide bonds (*15*, *16*) prevents them from crossing the membrane. In the case of apocytochrome *c*, it appears that nature has devised a mechanism to keep the protein unfolded by placing heme binding only after membrane translocation. However, when the unfolded polypeptide interacts with lipid membranes, the protein folds into an α-helical conformation. Thus, it appears that this membrane-associated partially folded state may play a role in the import mechanism of apocytochrome *c*. It has been suggested that the molten globule state, a compact folded intermediate described in the folding mechanism of many proteins in solution (for a review, see ref *17*), may be involved in membrane insertion and translocation (*18*), and has been described for membrane-bound forms of a few soluble proteins. Such lipid-associated folding intermediates have been reported for some bacterial toxins, including colicin A (*19*) and colicin E1 (*20*), in which formation of a molten globule state appears to be required for insertion into lipid membranes. Studies on the interaction of cytochrome *c* with lipid membranes have shown that a partially denatured state is induced upon binding to negatively charged lipid membranes (*21*), which agrees with earlier suggestions that a molten globule-like intermediate is formed during membrane interaction (*22*−*24*). On the other hand, in contrast with native stably folded proteins, which may become destabilized by the interaction with membranes, many peptides and proteins with little or no structure in solution acquire secondary structure upon binding to lipid membranes. Among those are small toxins (*25*, *26*), antimicrobial peptides (*27*), signal sequences (*28*), and apocytochrome *c* (*10*−*12*). In particular, cytochrome *c* and its heme-free precursor form a unique pair with respect to their interaction with lipid membranes. While the native holoprotein is partially denatured by the interaction with negatively charged membranes, the precursor protein, which is a random coil in aqueous solution, acquires an α-helical structure upon binding to lipid membranes. The partially folded state of apocytochrome *c* and the denatured state of cytochrome *c*, formed upon interaction with negatively charged lipid membranes or micelles, show a high degree of similarity in terms of their native-like secondary structure content and high mobility of the secondary structure elements (*22*), which suggest a common lipid-bound conformation for both precursor and mature proteins (*22*, *29*). Thus, it appears that partially folded states formed in association with lipid membranes might play a role in membrane insertion and/or translocation.

In this work, we have carried out a systematic comparative study of the folding of apocytochrome *c* induced by various types of phospholipids, including diacylphospholipids in bilayer vesicles and lysophospholipids in micelles. We have examined the role of negatively charged and zwitterionic lipid headgroups, both in a tight packing bilayer arrangement of vesicles and in the more open structure of micelles. It was found that binding of apocytochrome *c* to negatively charged phospholipid vesicles induced a partially folded conformation with ∼85% of the α-helical structure of cytochrome *c* in solution. In contrast, in the presence of zwitterionic phospholipid vesicles, apocytochrome *c* remains a random coil, suggesting that electrostatic interactions play an important role in the lipid-induced folding mechanism of apocytochrome *c*. However, negatively charged lysophospholipid micelles induce a higher content of α-helical structure than negatively charged bilayers, and micelles of lysophospholipids with the same zwitterionic headgroup as the phospholipid bilayer vesicles induced ∼60% of the α-helix content of cytochrome *c* in solution. Thus, this study shows that hydrophobic lipid−protein interactions have a dominant role in the lipid-induced folding mechanism of apocytochrome *c*.

EXPERIMENTAL PROCEDURES

Materials. All lysophospholipids (lysoMPC, lysoMPG, lysoOPC, and lysoOPG) and phospholipids (DMPC, DMPG, DOPC, and DOPS) were purchased from Avanti Polar Lipids, Inc. (Birmingham, AL). DOPG was obtained from Sigma Chemical Co. (St. Louis, MO).

Protein Purification. Horse heart cytochrome *c* (type VI) from Sigma, which comes contaminated with a variety of deamidated forms of the protein, was purified by ion exchange chromatography on Whatman CM-32 and eluted with 65−80 mM phosphate buffer at pH 7.0 (*30*). The eluent containing the purified protein was lyophilized, dissolved in cold deionized water and dialyzed against cold deionized

water (4 °C) to remove phosphate, lyophilized, and stored at −20 °C. The cytochrome *c* concentration was measured spectrophotometrically using a molar absorptivity of 29 500 M^{-1} cm^{-1} at 550 nm and pH 7.0 for the protein reduced with sodium dithionite (*31*). Apocytochrome *c* was prepared by chemically removing the heme group of horse heart cytochrome *c* as described by Fisher et al. (*9*). The protein concentration was determined using a molar extinction coefficient of 10 580 M^{-1} cm^{-1} at 277 nm (*32*). Apocytochrome *c* was renatured by a procedure modified from that described by Hennig and Neupert (*33*). Briefly, apocytochrome *c* was dissolved in 8 M urea, 10 mM potassium phosphate (pH 7.0), and 2 mM DTT and the mixture dialyzed against 10 mM phosphate buffer (pH 7.0) containing 2 mM DTT. The renatured protein was stored in aliquots at −20 °C, thawed just before it was used, and kept on ice on the day of measurements at low pH to prevent protein aggregation.

Micelles and Vesicles. Aqueous solutions of lysophospholipid micelles were prepared by dissolving the desired amount of dried lipid into 10 mM phosphate buffer (pH 7.0), followed by sonication for 30 min in a water bath sonicator (Ultrawave U-400). Phospholipids in chloroform solutions were dried under a rotary evaporator and left under vacuum for a minimum of 8 h to remove all traces of organic solvent. Vesicles were prepared by hydration of dry lipid with 10 mM phosphate buffer (pH 7.0). The buffer was deoxygenated with nitrogen gas, and hydration of unsaturated lipids was carried out under a nitrogen atmosphere. The resulting multilamellar liposome suspension was sonicated for several hours (on average for about 3 h) in a water bath sonicator (Ultrawave U-400) until a clear suspension of small unilamellar vesicles was obtained. A few representative lipid samples of sonicated vesicles and micelles were checked by thin layer chromatography, after extraction into organic solvent. No degradation products were detected when the samples were compared to the original source lipid. The vesicle and micelle sizes of a few representative samples were determined by dynamic light scattering on a Dyna-Pro 801 dynamic light scattering instrument. Lipid vesicles had diameters ranging from 300 to 600 Å, and micelles show a diameter range of 60−120 Å.

Critical Micelle Concentration. The critical micelle concentration (CMC) of a detergent/lipid mixture depends on the buffer conditions and temperature. For lysoMPG, CMC values of 3 mM and 160 μM have been reported in pure water and in 0.1 M Tris-HCl, respectively, at 30 °C (*34*). Zwitterionic lysoPCs with acyl chains of the same length, and under identical buffer conditions, have lower CMC values then their negatively charged counterparts (lysoPGs). This reflects the higher polarity and hydration of lysoPGs relative to those of lysoPCs. As expected, the CMC decreases with increasing length of the acyl chain for a given lipid headgroup. Typically, the CMC decreases by approximately a factor of 10 for each addition of two methylene groups to the acyl chain (*34*). We have determined the CMC for lysoMPG and lysoMPC under the same experimental conditions used for the studies with apocytochrome *c*, i.e., in 10 mM phosphate buffer (pH 7) at 10 °C, using the fluorescence probe *N*-phenyl-1-naphthylamine (NPN) (*35*). Under these conditions, the CMC values for lysoMPG and lysoMPC were found to be 288 and 40 μM, respectively.

Circular Dichroism Measurements. Far-UV (190−260 nm) circular dichroism spectra were acquired for apocytochrome *c* in solution and in mixtures with sonicated lysophospholipid micelles or phospholipid vesicles. The protein concentration was 10 μM, unless stated otherwise. Lipid concentrations varied from 10 μM to 10 mM or above. For lysolipids, these values cover the concentration range below, through, and above the CMC. Lipid/protein samples were prepared by adding 1 part of a stock solution of 60 μM apocytochrome *c* in 10 mM HCl (pH 2.0) to 5 parts of lipid micelles or vesicles in 10 mM phosphate buffer (pH 7.0) (1:5, v/v). The resulting lipid/protein sample had a final pH of 6.8. Circular dichroism spectra were recorded on a Jasco 715 spectropolarimeter, using quartz cells with a 1 mm path length. Typically, a scanning rate of 100 nm min^{-1}, a time constant of 1 s, and a bandwidth of 1.0 nm were used. The spectral resolution was 0.5 nm, and eight scans were averaged per spectrum. All spectra were corrected for the appropriate background. Spectra of the protein in solution had the background obtained with the corresponding buffer alone subtracted from them. Spectra of apocytochrome *c* in the presence of lipids were similarly corrected for the background arising from the corresponding lipid vesicles or micelles in the absence of protein. All spectra were recorded at controlled temperatures using a cell holder thermostated with a circulating water bath, and the sample temperature was measured to an accuracy of ±0.2 °C. Most spectra were recorded above the lipid phase transition, i.e., in the liquid crystalline L_α phase. For all micelles and vesicles of unsaturated lipids (oleoyl chains), measurements were performed at 10 °C, while for vesicles of saturated lipids (DMPC and DMPG), spectra were recorded both below (10 °C) and above (30 °C) the lipid phase transition [the phase transition temperature for DMPG is ∼23 °C and for DMPC 22−24 °C (*36*)].

Fluorescence Spectroscopy. Equilibrium fluorescence emission spectra were obtained for apocytochrome *c* alone and in mixtures with sonicated lysolipid micelles or phospholipid vesicles. The protein concentration was 10 μM, and lipid/protein samples were prepared as described above for the CD measurements. Experiments were performed on a Perkin-Elmer LS50 luminescence spectrometer. All spectra were recorded using a thermostatically controlled cell holder at the corresponding temperatures used for the CD measurements (see above). The sample temperature was controlled within an accuracy of ±0.2 °C. Tryptophan fluorescence was measured using an excitation wavelength of 295 nm (2 nm bandwidth), and emission spectra were recorded using a 4 nm bandwidth. Typically, eight scans were averaged for each spectrum. All spectra were corrected with the corresponding background spectrum without protein.

RESULTS

Secondary Structural Changes of Apocytochrome c upon Interaction with Lipid. Circular dichroism (CD) was used to monitor structural changes in apocytochrome *c* induced by the interaction with lysophospholipid micelles and phospholipid vesicles. While cytochrome *c* in solution shows the typical CD spectral characteristics of a stably folded protein containing mainly α-helical structure, its heme-free precursor, apocytochrome *c*, exhibits a far-UV CD spectrum typical of proteins with a random coil conformation (Figure

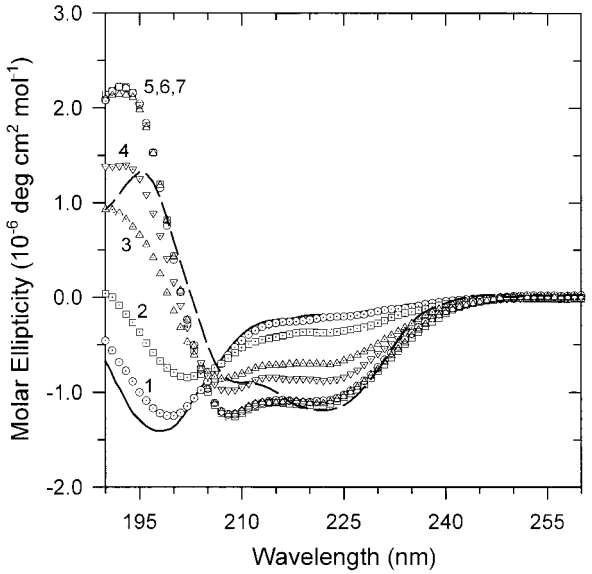

FIGURE 1: Far-UV CD spectra of apocytochrome *c* in solution (solid line) and in the presence of increasing concentrations of lysoMPG (symbols), in comparison with the far-UV CD spectrum of cytochrome *c* in solution (dashed line). The concentration of apo and holo proteins was 10 μM; lysoMPG concentrations were 10 μM (1), 50 μM (2), 180 μM (3), 0.5 mM (4), 1 mM (5), 5 mM (6), and 10 mM (7). Measurements were carried out at 10 ± 0.2 °C, using cells with a 1 mm path length, and each spectrum is an average of eight scans. All spectra were acquired with a resolution of 0.5 nm, but for simplicity, symbols are only shown every nanometer.

1). However, the addition of increasing amounts of lipid to apocytochrome *c* induces the typical ellipticity bands of an α-helical structure. A representative series of these spectral changes induced in apocytochrome *c* by increasing the concentration of the micelle-forming lipid lysoMPG are shown in Figure 1. Similar behavior was found for all other lysolipids and phospholipids studied here. The spectra cross over each other at an isodichroic point of 204 nm, which is consistent with the transition from a random coil conformation to α-helical structure (*37*). These spectral changes indicate that upon interaction with lipid micelles or vesicles apocytochrome *c* undergoes a conformational transition from a random coil to α-helical structure, as observed for other lipid systems (*11*, *12*). In this study, we have investigated the role of electrostatic and hydrophobic lipid−protein interactions in the folding of apocytochrome *c* induced by lipid. In particular, we have examined the role of negatively charged and zwitterionic lipid headgroups, and the nature of the hydrophobic domain, i.e., saturated or unsaturated acyl chains, in the formation of α-helical structure in apocytochrome *c*. In addition, these studies were carried out in two types of lipid systems, (a) phospholipid vesicles and (b) lysophospholipid micelles, and their effects on the secondary structure of apocytochrome *c* were analyzed.

It is recognized that large membrane vesicles give rise to differential light scattering and absorption flattening effects, which may result in significant distortions of the CD spectra of membrane-associated proteins. However, it has been shown that these effects can be minimized by using sonicated vesicles (*38*). CD measurements were performed using sonicated vesicles (diameter range of 300−600 Å), and the appearance of an isodichroic point in a series of CD spectra in the presence of increasing lipid concentrations (Figure 1) was taken as an indication that no significant spectral distortions arose from lipid scattering contributions. The amount of α-helix induced in apoctochrome *c* by various lipid vesicles, relative to the α-helix content of cytochrome *c* in solution, was analyzed by monitoring the relative intensity of the dichroic band at 222 nm as a function of lipid concentration. The 222 nm band is a suitable probe for α-helical structure in proteins, as interference from other secondary structure elements is relatively weak at this wavelength (*39*). The differences in the spectral region around the minimum at 209 nm and maximum around 195 nm between apocytochrome *c* in the presence of lipids and cytochrome *c* in solution (Figure 1) may arise from changes in other secondary structure elements in the protein, or may be due to the presence of optically active heme transitions in the holoprotein which are not present in apocytochrome *c* (*21*, *22*). Therefore, an unambiguous interpretation of the spectral changes for this region of the CD spectrum is not possible.

Binding of apocytochrome *c* to negatively charged phospholipid vesicles induces about 85% of the α-helix content of cytochrome *c* (Figure 2A). Similar behavior was observed for all the negatively charged vesicles studied here above the phase transition, i.e., in the liquid crystalline L_α phase, independent of the chemical structure of the lipid headgroup or acyl chains. However, binding of apocytochrome *c* to DMPG vesicles below the phase transition, i.e., in the gel crystalline L_β phase, induced a higher level of secondary structure which approached 100% of the α-helix content of cytochrome *c* (Figure 2A). With all lipids, the maximum induced α-helical structure reaches a constant value for lipid concentrations equal to or above 2 mM, corresponding to lipid-to-protein ratios of ≥200. Zwitterionic phospholipid vesicles of DOPC and DMPC, both in the liquid crystalline L_α phase, did not induce α-helical structure (Figure 2A), and the far-UV CD spectra of apocytochrome *c* obtained in the presence of these lipids were identical to that of the random coil conformation of apocytochrome *c* in solution (Figure 1).

The interactions of apocytochrome *c* with negatively charged micelles of lysoMPG and lysoOPG show an increase in the 222 nm dichroic band of the protein with increasing lipid concentrations. For lipid concentrations below 100 μM, no significant α-helical structure was formed, whereas above 100 μM, the α-helical structure increases and reaches a maximum value for concentrations of 1 mM lipid and above. Negatively charged lysolipid micelles induced the most α-helical structure with close to 100% of the α-helix content of cytochrome *c* (Figure 2B). In contrast with zwitterionic phospholipid vesicles, a significant amount of α-helix (∼60%) was formed when apocytochrome *c* was mixed with zwitterionic micelles of lysoMPC or lysoOPC. The various folded conformations of apocytochrome *c* induced by the different classes of lipids studied here are summarized in Figure 3, in comparison with the unfolded state of apocytochrome *c* and native cytochrome *c* in solution. Three main folded conformations of apocytochrome *c* induced by lipid interactions were found: (a) a highly helical state of apocytochrome *c* with close to 100% of the α-helix content of the holoprotein formed upon binding to negatively charged micelles or negatively charged lipid vesicles in the L_β phase, (b) a folded conformation with ∼85% of the cytochrome *c*

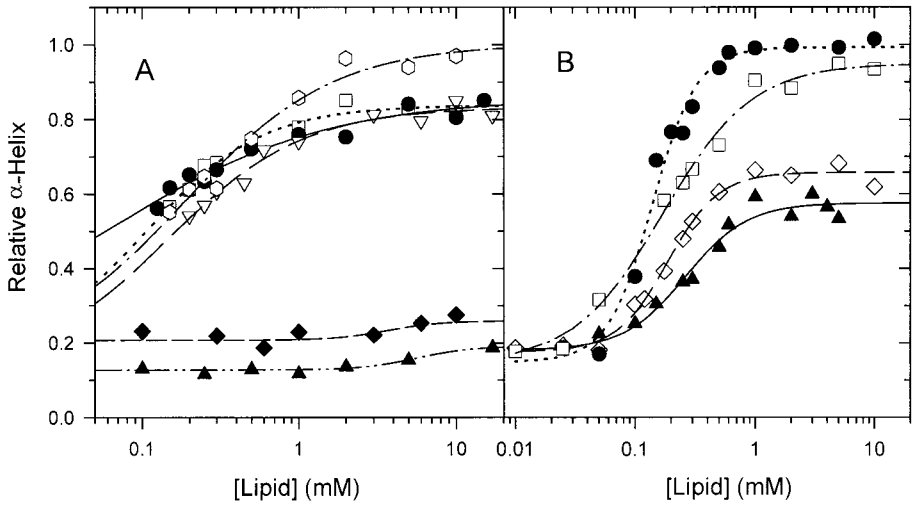

FIGURE 2: Relative α-helix level induced in apocytochrome *c* as a function of increasing concentrations of (A) various diacylphospholipids such as DOPC (▲), DMPC at 30 °C (◆), DOPG (●), DMPG below (⬡) and above the phase transition at 30 °C (□), and DOPS (▽) and (B) various lysolipids such as lysoOPG (●), lysoMPG (□), lysoOPC (▲), and lysoMPC (◇). The α-helix level was monitored at 222 nm relative to the α-helix level of cytochrome *c* in solution. Spectra were recorded at 10 °C, unless stated otherwise, using a 1 mm path length cell. The protein concentration was 10 μM. Lines are for guidance only and have no theoretical significance.

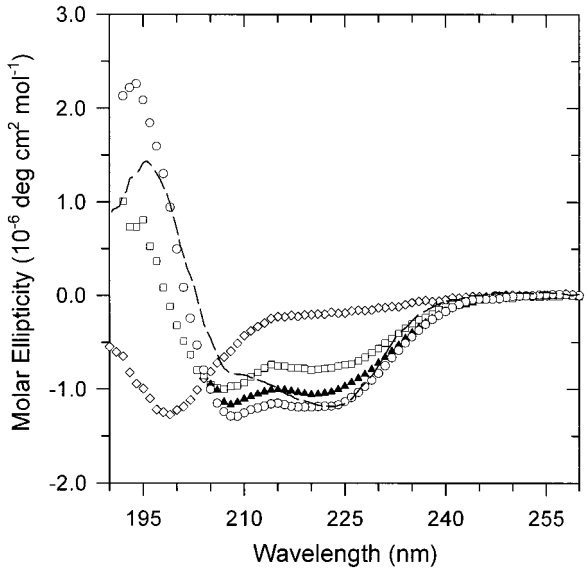

FIGURE 3: Typical far-UV CD spectra of apocytochrome *c* induced by the three classes of lipids studied here for lipid concentrations above 2 mM for negatively charged micelles (○), negatively charged vesicles in the L_α phase (▲), and zwitterionic micelles (□), in contrast with the random coil spectrum for apocytochrome *c* in solution (◇). For comparison, the far-UV CD spectrum of cytochrome *c* in solution is also shown (dashed line). Protein concentrations were 10 μM. Measurements were carried out at 10 ± 0.2 °C, using a 1 mm path length cell, and each spectrum is an average of eight scans. All spectra were acquired with a resolution of 0.5 nm, but for simplicity, symbols are only shown every nanometer.

α-helix formed by the interaction with negatively charged lipid vesicles in the L_α phase, and (c) a third helical state showing about 60% of the intensity of the 222 nm dichroic band of native cytochrome *c* obtained with zwitterionic micelles. All these lipid-associated helical states show no signature on the near-UV region of the CD spectrum (data not shown), indicating that the aromatic side chains are in a highly dynamic state with no restricted orientation.

Trp59 Fluorescence Changes upon Folding and Lipid Interaction. Intrinsic protein fluorescence is a very sensitive probe for monitoring conformational changes in proteins. Apocytochrome *c* has one single tryptophan at position 59 (Trp59), which dominates the fluorescence spectrum. In this study, we have used the fluorescence emission of Trp59 to describe the interaction of apocytochrome *c* with various lysophospholipid micelles and phospholipid vesicles. Large spectral changes were observed upon interaction with lysolipid micelles (Figure 4A). The binding of apocytochrome *c* to zwitterionic micelles of lysoMPC and lysoOPC induces a blue shift of about 10 nm and an increase in the Trp59 fluorescence yield of about 85 and 95%, respectively, relative to the fluorescence emission of unfolded apocytochrome *c* in buffer solution. A large blue shift of about 15 nm was observed for all lipids with the phosphoglycerol (PG) headgroup, for both lysolipid micelles and lipid vesicles. However, the increase in the Trp59 fluorescence yield was only up to 43% of the fluorescence emission of apocytochrome *c* in solution (Figure 4A,B). The blue shifts in the wavelength of maximum fluorescence (λ_{max}) are consistent with the transfer of Trp59 from the hydrophilic environment in unfolded apocytochrome *c* in aqueous solution to a hydrophobic environment upon folding and insertion into the lipid phase. When apocytochrome *c* was mixed with zwitterionic phospholipid vesicles of DOPC and DMPC, both in the liquid crystalline L_α phase, no significant shifts in the λ_{max} of Trp59 fluorescence emission were observed, and only small changes in the fluorescence intensity were detected (Figure 4C). These changes are consistent with the lack of binding of apocytochrome *c* to large DOPC vesicles (*40*). However, in the presence of DMPC vesicles in the gel crystalline L_β phase, a significant λ_{max} blue shift (~8 nm) and a 50% increase in the fluorescence yield were observed for apocytochrome *c*, compared to the unfolded protein in solution (Figure 4C). The blue shift of λ_{max} and the increase in fluorescence observed with apocytochrome *c* in the presence DMPG vesicles below the phase transition temperature were also larger than the changes observed above the phase transition (Figure 4B). These results suggest that apocytochrome *c* interacts more favorably with the gel crystalline L_β phase than with the liquid crystalline L_α phase, and appears to induce further stabilization of the secondary structure, as revealed by the higher α-helix content induced

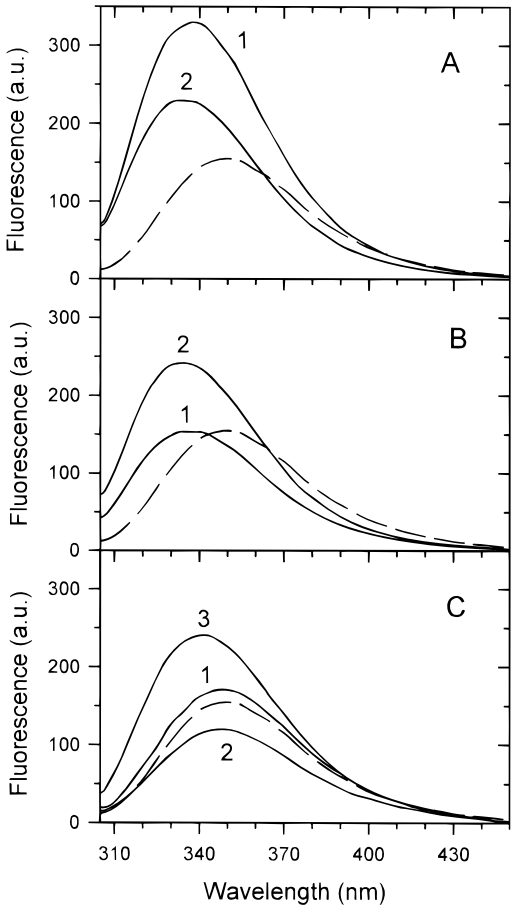

FIGURE 4: Equilibrium fluorescence emission spectra of apocytochrome *c* in solution (dashed line in panels A−C), and in the presence of (A) micelles of lysoMPC (1) and lysoMPG (2), (B) negatively charged vesicles of DMPG at 30 (1) and 10 °C (2), and (C) zwitterionic vesicles of DOPC (1) and DMPC at 30 (2) and 10 °C (3). Spectra of apocytochrome *c* in solution and with lipid were recorded at 10 °C, if not stated otherwise. The protein concentration was 10 μM, and lipids were all at a concentration of 2 mM.

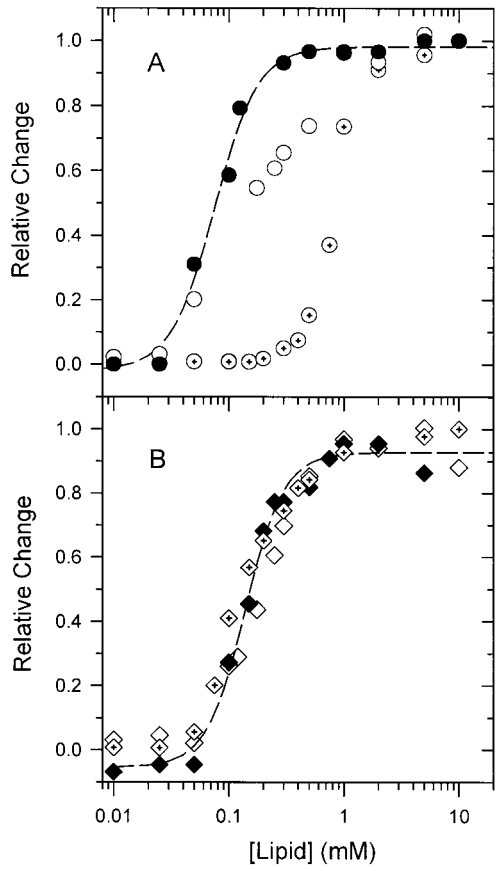

FIGURE 5: Normalized blue shifts of tryptophan fluorescence λ_{max} of apocytochrome *c* (filled symbols) and the relative changes in α-helical structure (open symbols) induced with increasing concentrations of lipid are compared to their CMC profiles (center-crossed symbols) for (A) lysoMPG and (B) lysoMPC. Blue shifts were measured relative to the unfolded apocytochrome *c* in solution and normalized to the average value of the maximum blue shifts obtained above 500 μM lipid. The relative α-helix level was measured as described in Figure 2 and the overall change normalized to 1. Both fluorescence and CD measurements were performed at 10 ± 0.2 °C, and the protein concentration was 10 μM. The CMC profiles were monitored by the relative fluorescence emission intensity changes of NPN as a function of lipid concentration (see Experimental Procedures). Lines are for guidance only and have no theoretical significance.

by DMPG (Figure 2A) and DMPC (data not shown) below the phase transition.

We have monitored the Trp59 fluorescence of apocytochrome *c* as a function of increasing concentrations of lysoMPG and lysoMPC. The changes observed for the blue shift of λ_{max} with increasing lipid concentrations have a lipid dependence similar to that found for the changes in α-helical structure. However, while for the negatively charged lipid lysoMPG the transition (monitored by either far-UV CD or tryptophan fluorescence) occurs at concentrations below the CMC (Figure 5A), for the zwitterionic lipid lysoMPC the changes are associated with micelle formation (Figure 5B). These results suggest that apocytochrome *c* interacts with monomeric molecules of lysoMPG, but not with lysoMPC monomers.

DISCUSSION

Electrostatic and Hydrophobic Components in the Lipid-Induced Folding of Apocytochrome c. Previous studies on the interaction of apocytochrome *c* with lipid vesicles and detergent micelles suggested that negative charges in lipid headgroups play an important role in the process of lipid-induced folding of apocytochrome *c* (*10*, *11*). However, in this study, we found that large conformational changes in apocytochrome *c* are obtained not only upon interaction with negatively charged lipid micelles and vesicles but also with zwitterionic lysolipid micelles (Figures 2B). Our results can be summarized in four main points. (a) Negatively charged phospholipid vesicles in the L_α phase induce a partially folded state with ∼85% of the α-helix content of cytochrome *c*. (b) Apocytochrome *c* remains a random coil in the presence of zwitterionic phospholipid vesicles. (c) Negatively charged lysophospholipid micelles lead to a folded state containing about 100% of the α-helix content of cytochrome *c*. (d) In contrast with zwitterionic vesicles, micelles of zwitterionic lysophospholipid were able to induce a folded conformation of apocytochrome *c* with ∼60% of the α-helix content of cytochrome *c*. All negatively charged phospholipid vesicles studied here induced similar levels of secondary structure (Figure 2A), regardless of the chemical structure of the lipid headgroup or variations in the acyl chain between saturated or unsaturated hydrocarbon chains. These vesicles have in common a charged membrane surface with a net negative charge per lipid molecule of −1 and were all

in the liquid crystalline L_α phase. In contrast, the fact that apocytochrome *c* remains a random coil in the presence of zwitterionic phospholipid vesicles supports the idea of an essential role of lipid−protein electrostatic interactions in the folding mechanism of apocytochrome *c* induced by lipid bilayers (*10*, *11*). However, this work reveals two new findings: (a) negatively charged lysophospholipid micelles induced more α-helical structure in apocytochrome *c* than equivalent vesicles, and (b) apocytochrome *c* acquired an α-helical conformation with zwitterionic micelles. The folding effects of lysolipids in micelles are compared with those of equivalent diacylphospholipids in vesicles; i.e., these lipids have identical headgroup moieties. However, micelles have a more accessible hydrophobic core than vesicles, due to the reduced packing of the lysolipid acyl chain in the micelle compared to the tighter packing of the phospholipid acyl chains in the bilayers of vesicles. Thus, the additional folding effects induced in apocytochrome *c* by micelles appear to reveal important hydrophobic contributions to the mechanism of lipid-induced folding of apocytochrome *c*. Our results are consistent with previous reports that small zwitterionic unilamellar vesicles induced secondary structure in apocytochrome *c* (*11*), whereas large zwitterionic unilamellar vesicles had no effect (*10*).

The blue shifts observed for the fluorescence emission λ_{max} for apocytochrome *c* upon binding to either negatively charged lysolipid micelles or negatively charged phospholipid vesicles were all very similar (∼15 nm), which suggest a common environment for Trp59. The binding of the tripeptide, Lys-Trp-Lys, to negatively charged lipid vesicles shows a blue shift of about 14 nm (data not shown) when compared with the emission maximum in aqueous solution, and no significant changes in the overall fluorescence intensity. The λ_{max} blue shifts are consistent with a more hydrophobic environment for Trp59 upon protein folding and insertion into the lipid phase. The lipid concentration dependence for the λ_{max} of tryptophan fluorescence emission correlates well with the secondary structural changes in apocytochrome *c* monitored by far-UV circular dichroism, for both zwitterionic and negatively charged micelles (Figure 5). Thus, the tryptophan fluorescence changes can be associated with the protein structural changes induced by the interaction with lipid. The comparison of the blue shift results with the CMC profiles of lysoMPG and lysoMPC, determined under identical experimental conditions (Figure 5), suggests that apocytochrome *c* binds to monomeric molecules of lysoMPG. Binding of just a few lipid molecules appears to be enough to drive the folding of apocytochrome *c*, with the polar lipid headgroup making favorable charge interactions and the hydrocarbon tail providing hydrophobic interactions. In contrast, binding of apocytochrome *c* to lysoMPC requires the lipid to be in micelles (Figure 5B).

Our combined results on various lipids in both micelles and bilayers (vesicles) unravel a strong hydrophobic component in the lipid-induced folding mechanism of apocytochrome *c*. While electrostatic lipid−protein interactions appear to direct the polypeptide to the lipid surface and to induce an early partially folded state on the membrane surface (S. E. Rankin et al., submitted), completion of the folding process involves hydrophobic interactions between nonpolar residues in the protein and the hydrophobic core of the lipid bilayer. The results suggest that upon membrane insertion hydrophobic interactions stabilize and extend the secondary structure. Whether the kinetic folding intermediates and final folded conformations formed in negatively charged micelles or vesicles are distinct or equivalent to those formed in zwitterionic micelles remains to be established, and that is the subject of further investigations. It should be emphasized that in the absence of a net negatively charged lipid surface, folding is observed to a lesser extent, and only with zwitterionic micelles where hydrophobic interactions are more readily available. When electrostatic interactions are present, in both micelles and vesicles, a higher level of folding of apocytochrome *c* is achieved. Therefore, electrostatic lipid−protein interactions play an important role in the mechanism of protein folding in lipid membranes. The electrostatic contribution to the lipid-induced folding mechanism of apocytochrome *c* is independent of the chemical structure of the lipid headgroup and has also been observed with nonbiological detergent micelles of SDS (*10*). These results, taken together with the observation that many unfolded peptides can acquire helical structure in polar lipid membranes (*25*−*28*), suggest that the role of electrostatic lipid−protein interactions in the mechanism of protein folding in lipid membranes may represent a general phenomenon. It is possible that the folding events driven by lipid−protein electrostatic interactions may resemble those of salt-induced protein folding in aqueous solutions.

Lipid-Induced Folding of Apocytochrome c and Implications for Mitochondrial Import. Apocytochrome *c* is synthesized on free cytoplasmic ribosomes, and heme attachment occurs after import into mitochondria, where the polypeptide folds around the heme group into the native cytochrome *c* structure. Unlike the processes for most other mitochondrial precursor proteins, this import process does not require ATP or a membrane potential, or other protein receptors in the outer mitochondrial membrane (*1*). Instead, apocytochrome *c* appears to insert spontaneously and partially cross the outer mitochondrial membrane (*2*, *3*). It has been postulated that the molten globule state might be involved in membrane insertion and translocation (*18*). Molten globule-like states have been described for membrane-bound forms of a few soluble proteins (*19*−*21*, *41*). On the other hand, in contrast with native stably folded proteins, which may become destabilized by the interaction with membranes, many peptides and proteins with little or no structure in solution acquire secondary structure upon binding to lipid membranes (*42*). Among these is apocytochrome *c* which acquires α-helical structure upon interaction with lipid membranes (Figure 3). Whether these membrane-associated α-helical partially folded states are involved in the mechanism of membrane insertion of apocytochrome *c* remains to be established. However, on the basis of energetic considerations, it appears that formation of secondary structure is a prerequisite for spontaneous membrane insertion (*42*; for a review, see ref *43*). The high energy cost associated with the transfer of a free peptide bond into a bilayer interface of ∼1.2 kcal/mol (*44*) suggests that a reduction of this cost, through the formation of hydrogen bonds, strongly favors the formation of secondary structure in peptides bound to lipid interfaces (*45*). Furthermore, studies on the interaction of signal sequence peptides with phospholipid membranes have shown that if the peptide does not have the ability to

form a stable α-helix, it does not insert into the membrane (*46*). Thus, it is plausible that the partially folded α-helical conformation of apocytochrome *c* induced upon interaction with lipid membranes may represent the translocation competent sate of the protein (*22*, *29*). The presence of ~40% negatively charged lipid headgroups in the outer mitochondrial membrane (*47*) may ensure the proper targeting of apocytochrome *c* to the membrane surface driven by electrostatic lipid−protein interactions, and membrane insertion would be driven by folding of the polypeptide through hydrophobic lipid−protein interactions. Finally, this membrane-associated partially folded intermediate state of apocytochrome *c* may serve as a basic recognition mechanism for the enzyme CCHL, in which a folded intermediate state of apocytochrome *c* would be required for heme binding.

ACKNOWLEDGMENT

We thank Dr. A. Rodger (Department of Chemistry, University of Warwick, Coventry, U.K.) for the use of the fluorimeter, Professors H. Roder (Fox Chase Cancer Center, Philadelphia, PA), O. B. Ptitsyn (Russian Academy of Sciences, Pushchino, Russia), and W. L. C. Vaz (Department of Chemistry, University of Coimbra, Coimbra, Portugal) for stimulating discussions, and Dr. E. A. Bryson for critical reading of the manuscript.

REFERENCES

1. Stuart, R. A., and Neupert, W. (1990) *Biochimie 72*, 115−121.
2. Hartl, F. U., and Neupert, W. (1990) *Science 247*, 930−938.
3. Glick, B., and Schatz, G. (1991) *Annu. Rev. Genet. 25*, 21−44.
4. Smith, M., Leung, D. W., Gillian, S., Astell, C. R., Montgomery, D. L., and Hall, B. D. (1979) *Cell 16*, 753−761.
5. Hennig, B., and Neupert, W. (1981) *Eur. J. Biochem. 121*, 5241−5247.
6. Pfanner, N., Hartl, F.-U., and Neupert, W. (1987) *Eur. J. Biochem. 175*, 205−212.
7. Mayer, A., Neupert, W., and Lill, R. (1995) *J. Biol. Chem. 270*, 12390−12397.
8. Liu, X., Kim, C. N., Yang, J., Jemmerson, R., and Wang, X. (1996) *Cell 86*, 147−157.
9. Fisher, W. R., Taniuchi, H., and Anfinsen, C. B. (1973) *J. Biol. Chem. 248*, 3188−3195.
10. Jordi, W., Li-Xin, Z., Pilon, M., Demel, R. A., and de Kruijff, B. (1989) *J. Biol. Chem. 264*, 2292−2301.
11. De Jongh, H. H. J., and de Kruijff, B. (1990) *Biochim. Biophys. Acta 1029*, 105−112.
12. Pinheiro, T. J. T., Elöve, G. A., Roder, H., de Jongh, H. H. J., de Kruijff, B., and Watts, A. (1995) I=in *Perspectives on Protein Engineering and Complementary Technologies*, Vol. 3, pp 62−65, Mayflower Worldwide, Oxford, U.K.
13. Eilers, M., and Schatz, G. (1986) *Nature 322*, 228−232.
14. Randall, L. L., and Hardy, J. S. (1986) *Cell 46*, 921−928.
15. Vestweber, D., and Shatz, G. J. (1988) *J. Cell Biol. 107*, 2037−2034.
16. Hwang, C., Sinskey, A. J., and Lodish, H. F. (1992) *Science 257*, 1496−1503.
17. Ptitsyn, O. B. (1995) *Adv. Protein Chem. 47*, 83−229.
18. Bychkova, V. E., Pain, R. H., and Ptitsyn, O. B. (1988) *FEBS Lett. 359*, 133−163.
19. Van der Goot, F. G., Goncalez-Manas, J. M., Lakey, J. H., and Pattus, F. (1991) *Nature 354*, 408−410.
20. Lakey, J. H., Gonzalez-Manas, J. M., van der Goot, F. G., and Pattus, F. (1992) *FEBS Lett. 307*, 26−29.
21. Pinheiro, T. J. T., Elöve, G. A., Watts, A., and Roder, H. (1997) *Biochemistry 36*, 13122−13132.
22. De Jongh, H. H. J., Killian, J. A., and de Kruijff, B. (1992) *Biochemistry 31*, 1636−1643.
23. Spooner, P. J. R., and Watts, A. (1992) *Biochemistry 31*, 10129−10138.
24. Pinheiro, T. J. T., and Watts, A. (1994) *Biochemistry 33*, 2451−2458.
25. Thiaudiere, E., Siffert, O., Talbot, J. C., Bolard, J., Alouf, J. E., and Dufourcq, J. (1991) *Eur. J. Biochem. 195*, 203−213.
26. Shai, Y. (1994) *Toxicology 87*, 109−129.
27. Maloy, W. L., and Kari, U. P. (1995) *Biopolymers 37*, 105−122.
28. Gierasch, L. M. (1989) *Biochemistry 28*, 923−929.
29. Pinheiro, T. J. T. (1994) *Biochimie 76*, 489−500.
30. Brautigan, D. L., Ferguson-Miller, S., and Margoliash, E. (1978) *Methods Enzymol. 53*, 128−191.
31. Margoliash, E., and Walasek, O. F. (1967) *Methods Enzymol. 10*, 339−348.
32. Stellwagen, E., Rysavy, R., and Babul, G. (1972) *J. Biol. Chem. 247*, 8074−8077.
33. Hennig, B., and Neupert, W. (1983) *Methods Enzymol. 97*, 261−274.
34. Stafford, R. E., Fanni, T., and Dennis, E. A. (1989) *Biochemistry 28*, 5113−5120.
35. Brito, R. M. M., and Vaz, W. L. C. (1986) *Anal. Biochem. 152*, 250−255.
36. Cevc, G., and Marsh, D. (1987) in *Phospholipid bilayers. Physical principles and models*, Wiley-Interscience, New York.
37. Chou, P. Y., and Fasman, G. D. (1978) *Annu. Rev. Biochem. 47*, 251−276.
38. Mao, D., and Wallace, B. A. (1984) *Biochemistry 23*, 2667−2673.
39. Woody, R. W. (1996) in *Circular dichroism and the conformational analysis of biomolecules* (Fasman, G. D., Ed.) pp 25−67, Plenum Press, New York and London.
40. Rietveld, A., Sijens, P., Verkleij, A. J., and de Kruijff, B. (1983) *EMBO J. 2*, 907−913.
41. Banuelos, S., and Muga, A. (1995) *J. Biol. Chem. 270*, 29910−29915.
42. Wimley, W. C., and White, S. H. (1996) *Nat. Struct. Biol. 3*, 842−848.
43. Watts, A., and Pinheiro, T. J. T. (1998) in *Membrane Protein Insertion and Translocation* (Phoenix, D., Ed.) Portland Press, London (in press).
44. Jacobs, R. E., and White, S. H. (1989) *Biochemistry 28*, 3421−3437.
45. White, S. H., and Wimley, W. C. (1994) *Curr. Opin. Struct. Biol. 4*, 79−86.
46. Wang, Y., and Weiner, H. (1994) *Biochemistry 33*, 12860−12867.
47. Daum, G. (1985) *Biochim. Biophys. Acta 822*, 1−42.

BI980408X