Orientation of the α-Helices of Apocytochrome *c* and Derived Fragments at Membrane Interfaces, As Studied by Circular Dichroism†

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ABSTRACT: The orientation of the different helical regions of the mitochondrial precursor protein apocytochrome *c* has been studied using circular dichroism on isolated fragments of this protein associated with oriented films composed of various phospholipids [de Jongh, H. H. J., Goormaghtigh, E., & Killian, J. A. (1994) *Biochemistry* (preceding article in this issue)]. Both the N and C terminus adopt helical structures in a membrane environment. The middle region can also be helical, but only in the presence of the N-terminal domain of the protein. In the presence of the unsaturated lipids dioleoylphosphatidylcholine and dioleoylphosphatidylglycerol, all three helices are found to have a preferred orientation perpendicular to the membrane normal, whereas in the presence of the saturated lipids dimyristoylphosphatidylcholine and dimyristoylphosphatidylglycerol, the terminal helices are preferentially oriented parallel to the membrane normal. In films composed of dioleoylphosphatidylserine, it is found that the N-terminal helix is oriented preferentially perpendicular, whereas the C-terminal helix is aligned more parallel to the membrane normal. The differences in preferred orientation between the terminal helices are demonstrated by molecular modeling of the helices at a water−lipid interface. The results are discussed in light of the translocation of apocytochrome *c* over the outer mitochondrial membrane, an important step in the import process of this protein in mitochondria.

Apocytochrome *c* is the cytosolically synthesized precursor of the mitochondrial protein cytochrome *c*, the latter of which functions in the intermembrane space as an electron carrier in the respiratory chain. In order to reach the intermembrane space, apocytochrome *c* has to cross the outer mitochondrial membrane. The import pathway of apocytochrome *c* is unique compared to other mitochondrial precursor proteins [for a review, see Stuart and Neupert (1990)], since the protein does not possess a cleavable amino-terminal extension (Smith et al., 1979; Hennig & Neupert, 1981), and neither a membrane potential nor ATP or cytosolically exposed proteinaceous components are found to be involved in the import process (Nicholson et al., 1988; Stuart et al., 1990). The outer mitochondrial membrane is the only barrier between apocytochrome *c* and the intermembrane space exposed enzyme heme lyase, which covalently couples a heme group to the two cysteines at positions 14 and 17 in the N-terminal domain of apocytochrome *c* (Nicholson et al., 1987). It is therefore possible that the direct interaction of apocytochrome *c* with the phospholipids of the outer mitochondrial membrane plays an important role in translocation over this membrane. This hypothesis is supported by many biophysical studies showing that apocytochrome *c* is able to insert in monolayers (Pilon et al., 1987; Demel et al., 1989) and in model membranes (Rietveld et al., 1985; Görrissen et al., 1986). It can even reach the interior of vesicles preloaded with trypsin (Jordi et al., 1989) and perturbs the lipid acyl chain order upon interaction (Li-Xin et al., 1988; Jordi et al., 1990). From fluorescence measurements of the single tryptophan-59, it has been shown that the protein senses a more hydrophobic environment upon binding to model membranes (Berkhout et al., 1987), and more recently, by spin labeling apocytochrome *c* at the N-terminal cysteines, Snel and co-workers (1994) observed an immobilization of the spin label together with protection of the spin labels from aqueous quenchers, suggesting a penetration of the labels into the membrane.

In order to gain a better understanding of the mechanisms of translocation of apocytochrome *c* over a membrane, insight into the secondary structure and orientation at the interface is required. From circular dichroism (CD)[1] measurements, it has previously been reported that apocytochrome *c* and its derived fragments are random coiled in aqueous solution (Fisher et al., 1973; Toniolo et al., 1975; Jordi et al., 1989; de Jongh & de Kruijff, 1990). However, upon binding to small unilamellar vesicles, secondary structure was found to be induced in the protein in a lipid specific way (Walter

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[1] Abbreviations: CD, circular dichroism; DOPS, dioleoylphosphatidylserine; DOPC, dioleoylphosphatidylcholine; DOPG, dioleoylphosphatidylglycerol; DMPC, dimyristoylphosphatidylcholine; DMPG, dimyristoylphosphatidylglycerol; DEPS, dielaidoylphosphatidylserine; DPPC, dipalmitoylphosphatidylcholine; (ATR-FT)IR, attenuated total reflection fourier transform infrared; LUVET, large unilamellar vesicle by extrusion technique; DSC, differential scanning calorimetry; SDS, sodium dodecyl sulfate.

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et al., 1986; de Jongh & de Kruijff, 1990). Analysis of the CD spectra of fragments of apocytochrome *c* associated to micelles (Jordi et al., 1989; de Jongh & de Kruijff, 1990), supported by Chou−Fasman predictions (Rietveld et al., 1985; de Jongh & de Kruijff, 1990) and comparison with the known X-ray structure of cytochrome *c* (Bushnell et al., 1990), suggested that the helices mainly reside in the N- and C-terminal regions. It has also been proposed that the middle region of the protein could adopt a helical conformation (Jordi et al., 1989; de Jongh & de Kruijff, 1990).

In this study, we investigated the orientations of the different helices of apocytochrome *c* with respect to large unilamellar vesicles composed of various phospholipids using the recently developed application of CD, which allows a quantitative determination of both the secondary structure and the orientation of the secondary structure elements in films of protein−lipid complexes (see the preceding paper in this issue: de Jongh et al., 1994). In order to distinguish the different contributions of the various helices within the membrane-associated proteins, we studied, in addition to apocytochrome *c*, a number of derived fragments, including a fragment not investigated previously that does not enclose the N- or C-terminal helix. The results obtained are discussed in the light of a mechanism for the spontaneous translocation of apocytochrome *c* over the outer mitochondrial membrane.

MATERIALS AND METHODS

Materials

Dioleoylphosphatidylcholine (DOPC) was synthesized and purified according to established methods (van Deenen & de Haas, 1964). DOPS, DOPG, DMPC, and DMPG were obtained from Avanti Polar Lipids (Pelham, AL) and were used without further purification.

Apocytochrome *c* was prepared by the removal of the heme group of horse heart cytochrome *c* (type VI, Sigma), as described by Fisher et al. (1973). Cyanogen bromide cleavage of cytochrome *c*, as described by Corradin and Harbury (1970), resulted in the carboxy-terminal fragment 81−104 and the heme-containing fragments 1−65 and 1−80. All products were purified on a Bio-Gel P-10 (Bio-Rad, Richmond, VA) column eluted with 10% formic acid. The heme-free fragment 1−80 was prepared by removal of the heme group as described for apocytochrome *c*.

The amino-terminal fragment (residue 1−38) was obtained either by proteolytic cleavage of trifluoroacetamide-labeled cytochrome *c* by trypsin as described by Fanger and Harbury (1965) or by treatment of the heme-containing fragment 1−65 with clostripain (Boehringer Mannheim) as described by Snel et al. (1991) and subsequent removal of the heme as described for apocytochrome *c*. The latter procedure revealed generally higher yields (approximately 25% yield on weight basis) than the first method (approximately 10% yield). The peptides obtained by both procedures had similar elution behavior on both Biogel-P10 (size exclusion) and DEAE-Sephadex A25 (anion-exchange) columns.

Fragment 39−80 was obtained by treatment of the heme-free fragment 1−80 with clostripain as described by Snel et al. (1991), followed by a purification step on a Biogel-P10 column eluted with 10% formic acid in order to remove the clostripain. Subsequently, separation of fragment 39−80 from fragment 1−38 was obtained by incubation of the mixture with thiol-activated Sepharose 4B, as described for the purification of melittin from phospholipase A_2 by Batenburg et al. (1987). The quality of the separation of the two fragments was tested using Ellman's reagent (Ellman, 1959) in an experimental approach similar to that described by Pilon et al. (1992). In the purified fragment 39−80, no SH groups could be identified, whereas the mixture before treatment with thiol-activated Sepharose 4B did contain 1.9 μmol of SH groups/mg of protein (theoretically 2.2 μmol/mg of fragment 1−80 is expected).

Fragment 39−104 was prepared in a way similar to that described by Parr et al. (1978) by treatment of apocytochrome *c* with clostripain. We deviated, however, on two points from the described procedure: (1) we quenched the proteolytic digestion of the protein with ethylenediaminetetraacetic acid after 9 min for optimal production of fragment 39−104; (2) the products were all separated on a Biogel P-10 column eluted with 10% formic acid.

All products, purified on a Bio-Gel P-10 column using 10% formic acid as eluent, were subsequently dialyzed extensively against 10 mM phosphate buffer (pH 7.0) containing 2.5 mM dithiothreitol, with final dialysis against demineralized water. After determination of the protein concentration according to Lowry (1951) and subsequent adjustment of the volume with demineralized water to final concentrations of 1 mg/mL, the proteins were stored at −20 °C. Apocytochrome *c* and the fragments 1−80, 39−104, 39−80, 1−38, and 81−104 migrated as single bands on 15% or 11% tricine SDS−polyacrylamide gels under reducing conditions, as described by Schägger and von Jagow (1987), with apparent molecular weights of 11 500, 8900, 7400, 4700, 4300, and 2900, respectively, correlating well with the weights expected on basis of their amino acid compositions. Under nonreducing conditions, a contamination (less than 5%) of the N-terminal fragment 1−38 could be detected, with an apparent molecular weight comparable to the size of a dimer. During the experiments the extent of this contamination did not change.

Methods

Sample Preparation. Stock solutions of DOPC, DOPG, DOPS, DMPG, and DMPC were prepared by dissolving appropriate amounts of lipid in chloroform. After evaporation of the solvent under reduced pressure, the film was suspended, if not mentioned otherwise, in demineralized water. Large unilamellar vesicles (LUVETs) were obtained by applying the extrusion technique at room temperature using polycarbonate filters of 400 nm, as described by Hope et al. (1985). After the lipid concentration was determined according to Rouser et al. (1970), the volume was adjusted with demineralized water to a phospholipid concentration of 24 mM. The pH values of these stock solutions varied between 6.1 and 6.8. Unless noted otherwise, samples were prepared by the addition of 10 μL of a lipid stock solution to 14 μg of polypeptide (for apocytochrome *c*, this resulted in a lipid to protein molar ratio of 200) and incubation for 10 min at room temperature.

CD Measurements. CD spectra were recorded for peptides in vesicle solution and oriented bilayers, as described by de Jongh et al. (1994). Briefly, one-half of the sample was used, after adjustment of the volume to 40 μL with demineralized

water, to record a spectrum in a cuvette with a 0.02 cm path length. The other half was spread on a quartz plate and dried, resulting in an oriented sample, which was assembled into a closed cell. These latter samples were characterized to contain 2−4 water molecules per lipid. Attempts to hydrate the films by varying the humidity of the cell resulted in extensive β-strand formation as observed by CD, possibly as a result of aggregation of the polypeptides. These samples were not analyzed further. The oriented sample was measured at 16 different angles rotated perpendicular to the plane of the incident light beam in order to eliminate possible linear dichroism artifacts.

CD Spectral Analysis. The analysis of the CD spectra was performed, after subtraction of the corresponding protein-free sample, by a nonlinear regression curve-fitting procedure presented previously (de Jongh et al., 1994). In short, by deconvoluting the CD spectra of the α-helix, β-strand, and random coil of polylysine and the β-turn obtained from an average over 24 proteins with known X-ray structure, in terms of Gaussian absorption bands, a new set of reference spectra has been generated in which every CD band is correlated to a particular direction of absorption within the molecular axis system. This allows us to determine the contribution of every secondary structure element to a recorded experimental spectrum, as well as the different orientations of the α-helices with respect to the incident light beam. For helices there are two directions of absorption within the molecular frame: one absorbing light propagating parallel to the helix axis and one absorbing the light traveling perpendicular to this axis. The CD spectrum of the perpendicular component is most easily recognized by a large intensity at 207 nm, whereas almost no intensity is present at 228 nm. The opposite is observed for the parallel component [see Figure 1 of de Jongh et al. (1994)]. The ratio of the perpendicular and parallel components, denoted as the orientation parameter, ξ, is a measure for the orientation of the helices in the sample with respect to the incident light beam. When the helices have no preferred orientation, the perpendicular contribution will be twice as large as that of the parallel contribution, resulting in $\xi = 2$. The orientation parameter can directly be related to the ensemble-averaged angle, φ, of the helix axis with the incident light, according to $\xi = \tan^2 \varphi$. All fits presented had root-mean-square (RMS) values, as defined by Brahms and Brahms (1980), smaller than 10 and generally ranging from 5 to 7.

Binding Experiments. In a total volume of 150 μL, 35 μg of apocytochrome *c* or derived fragment was incubated with 3 mM DOPS LUVETs in 10 mM Tris-HCl buffer (pH 7.0) containing 50 mM sodium chloride for 15 min at room temperature. Next, the vesicles were pelleted in a Beckman TL-100 ultracentrifuge (30 min at 20 °C, 110000*g*). The percentages of bound protein were calculated by determining the amounts of protein [according to Lowry (1951)] and phosphate [according to Rouser (1970)] in the supernatant. The estimated error in the percentage is 3%. Determinations on the pellet revealed identical results.

Computer Modeling. The structure, mode of insertion, and orientation of the helices were studied using computer modeling of fragments 1−21, 57−69, and 80−104 of apocytochrome *c* simulated in a DPPC monomolecular layer, where the contribution of the lipid−water interface, the concomitant variation of the dielectric constant, and the transfer energy of atoms from a hydrophobic to hydrophilic environment are taken into account, as described by Brasseur (1990, 1991). In this model, the interaction energy (sum of contributions from van der Waals energy interactions, torsional potential energy, electrostatic interactions, and transfer energy between peptide and the lipid in the monolayer) is minimized until the lowest energy state of the entire aggregate is reached (Brasseur & Ruysschaert, 1986). All calculations were made on an Olivetti CP486 using PC-TAMMO+ (theoretical analysis of molecular membrane organization) and PC-PROT+ (protein plus analysis) software. Graphs were drawn with the PC-MGM+ (molecular graphics manipulation) program.

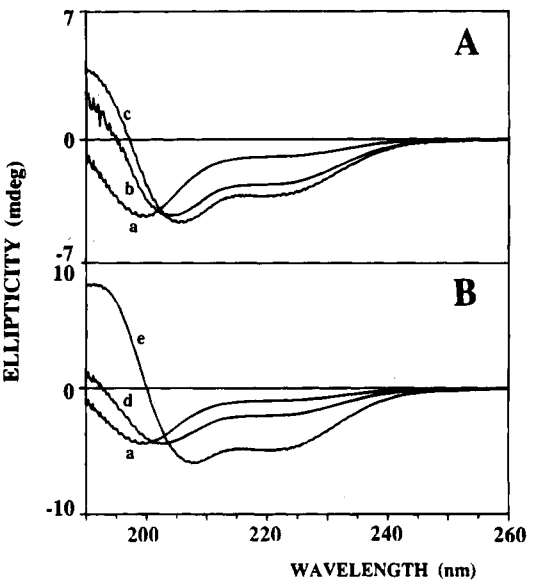

FIGURE 1: CD spectra of various fragments of apocytochrome *c* in the presence of DOPS vesicles in aqueous solution. Besides fragment 39−80 (spectra a), (A) the N-terminal fragments 1−80 (b) and 1−38 (c) and (B) the C-terminal fragments 39−104 (d) and 81−104 (e) are shown at a concentration of 5 μg of protein in 40 μL of 3 mM DOPS in 10 mM phosphate buffer (pH 7.0) containing 50 mM sodium chloride, measured at room temperature in a cuvette with a 0.02 cm path length.

RESULTS

Identification of the Helical Regions of Apocytochrome c in DOPS Model Membranes. In Figure 1A, the CD spectra of fragments 1−80, 1−38, and 39−80 are shown in the presence of vesicles composed of DOPS in 10 mM phosphate buffer (pH 7.0) in the presence of 50 mM sodium chloride. The spectrum of fragment 39−80 shows a negative extreme at 198 nm, which is characteristic for a random-coiled polypeptide (Chang et al., 1978). Comparable spectra were found for all fragments in the absence of lipid (data not shown). However, for fragments 1−80 and 1−38, the presence of phospholipids results in a spectral change, revealing negative extremes at 207 and 222 nm indicative of the presence of α-helices in these peptides (Chang et al., 1978). For the membrane-associated C-terminal fragment 81−104, the presence of helices is also suggested from the CD spectrum (Figure 1B, spectrum e), whereas this is less obvious for fragment 39−104 (spectrum d). In Table 1, the helical contents of the various fragments and of the whole protein (spectrum not shown) are presented, obtained from a curve-fitting procedure described in the Methods section. In contrast to expectations raised previously (Jordi et al.,

Table 1: Percentages of α-Helix (Corresponding Number of Residues) of Various Fragments of Apocytochrome *c* upon Binding to DOPS Vesicles in 10 mM Phosphate Buffer (pH 7.0) in the Presence of 50 mM Sodium Chloride at Room Temperature and the Percentage of Protein Bound to the Vesicles under These Conditions

fragment	% helical	% binding
1−80	28.3 (22.6)	92
1−38	38.1 (14.5)	97
39−80	6.1 (2.6)	88
39−104	19.8 (13.1)	94
81−104	62.2 (14.9)	94
1−104	31.4 (32.7)	91

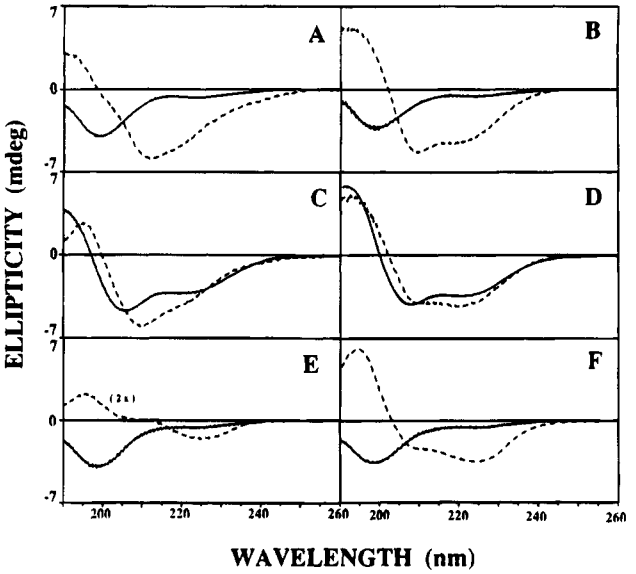

FIGURE 2: CD spectra of complexes of the N-terminal fragment 1−38 (A, C, E) and the C-terminal fragment 81−104 (B, D, F) in the presence of membranes of DOPC (A, B), DOPS (C, D), and DMPC (E, F) in aqueous solution (solid lines) and oriented on quartz plates (dashed lines), recorded at room temperature in the absence of salt.

1989; de Jongh & de Kruijff, 1990), fragment 39−80 contains no helical segments. Since the total number of helical residues in fragment 1−80 is larger than the number in fragment 1−38, it can be suggested either that the presence of the residues 39−80 induces a larger helical stretch at the N-terminus or that the induction of a helix in fragment 39−80 depends on the presence of residues 1−38. In contrast, the helical content of the C-terminal fragment remains approximately 14 amino acids, irrespective of the presence of residues 39−80. On the basis of Chou−Fasman predictions, we suggest that the middle region of the protein also contains a helix. The different fragments and the entire protein all bind to a comparable extent to DOPS vesicles (Table 1), indicating that the differences in helicity are not a result of variations in binding. The helicity of apocytochrome *c* is slightly smaller than the summation of the individual fragments due to a larger content of β-structures.

Circular Dichroism of Apocytochrome c in Aqueous Solution and in Oriented Films. In Figure 2, the CD spectra of the N- and C-terminal fragments are shown in the presence of various phospholipids in the absence of salt in aqueous solution and when oriented on quartz plates. The analysis of the spectra of the samples in aqueous solution is presented in Table 2. From comparison with Table 1, it can be concluded that the presence of 50 mM salt, needed to pellet the vesicles properly in the binding experiments, does not affect the α-helical content of the polypeptides in the presence of DOPS. The presence of the zwitterionic lipids DOPC and DMPC does not result in the formation of secondary structure in the peptides, whereas the negatively charged lipids DOPS, DOPG, and DMPG induce α-helical structures to a comparable extent, varying for the different fragments (Table 2). In all cases, the orientation parameters for the helices obtained are close to 2. This value corresponds to an isotropic distribution of the helices, as expected in aqueous solution where the particles have free rotational tumbling. The largest deviations in this value are found for the samples where the α-helical contribution was low.

For all systems, large spectral changes are observed upon drying the sample on quartz plates. For example, in the presence of the zwitterionic lipids DOPC and DMPC, secondary structure is induced for both the N and C termini. In films of DOPC, a large intensity around 207 nm can be observed (Figure 2A,B, dashed lines), which is characteristic of helical polypeptides having their helix axes perpendicular to the incident light beam. In contrast, in films of DMPC, for both terminal fragments a reduced intensity around 207 nm and a strong intensity around 228 nm can be observed (Figure 2E,F). These spectra are typical for helical polypeptides having their helix axes parallel to the incident light beam. In films of DOPS, for the N-terminal fragment a spectrum is found that resembles that of the peptide in a film of DOPC, which is suggestive of a perpendicular orientation, whereas for the C-terminal fragment a spectrum is obtained where the intensity around 207 nm is slightly reduced, indicative of a more parallel orientation.

The results of the analysis of the spectra of the oriented samples are presented in Table 3, including data for DOPG and DMPG and the entire protein (spectra not shown). The N-terminal fragment in films of the unsaturated lipid DOPC is approximately 45% helical with an orientation parameter for this helix of much larger than 2, indicating a preferred orientation of the helix axis: perpendicular with respect to the incident light. Translating the orientation parameter to the angle with the incident light, we obtain a value of 65°. This could indicate that all helices in the film have precisely this angle with the incident light. Alternatively, it is also possible that a population of 56% of those helices is aligned perpendicular to the incident light, with the remaining percentage of helices having an isotropic distribution, or even that 86% is oriented perpendicular and 14% parallel. All intermediate situations are, of course, also possible.

For simplicity, we will interpret the data in terms of the averaged preferred angle only. In films of DOPS an angle of 67° is found for the N-terminal which is very similar to the angle in films of DOPC. In the presence of DOPG, where only a small helical content is found, it is possible that the interpretation of the data is complicated due to aggregation upon drying of the film. The spectra of the N-terminal fragment in the presence of DMPC (Figure 2E) and DMPG (not shown) were found to be comparable and qualitatively suggest that the helices have a preferred orientation parallel to the incident light beam. Unfortunately, we were not able to analyze these spectra with RMS values smaller than 10. The absolute intensity of both spectra is strongly reduced, suggesting that they are distorted due to absorption flattening, as has been observed for films of polypeptides with a strong tendency to form large aggregated

Table 2: Contribution of α-Helices and Their Orientation Parameter, ξ, for Several Fragments of Apocytochrome *c* in the Presence of Various Phospholipids in Aqueous Solution

	DOPC		DOPS		DOPG		DMPC		DMPG	
fragment	helix %	ξ	helix %	ξ	helix %	ξ	helix %	ξ	helix %	ξ
1−38	6.2	1.91	36.8	1.92	20.8	2.04	5.9	1.79	24.9	2.00
81−104	8.1	2.12	59.4	1.96	50.1	2.04	3.9	2.23	58.9	1.98
1−104	6.2	1.89	30.9	1.90	31.6	2.03	7.4	2.16	24.0	2.01

Table 3: Contribution of α-Helices and Their Orientation Parameter, ξ, for Several Fragments of Apocytochrome *c* in the Presence of Various Phospholipids in Oriented Systems

	DOPC		DOPS		DOPG		DMPC		DMPG	
fragment	helix %	ξ	helix %	ξ	helix %	ξ	helix %	ξ	helix %	ξ
1−38	44.2	4.52 (64.8°)	45.0	5.34 (66.6°)	16.2	2.86 (59.4°)				
81−104	30.7	4.06 (63.6°)	62.1	0.82 (42.2°)	32.7	2.01 (54.8°)	68.2	0.48 (34.6°)	67.2	0.38 (31.6°)
1−104	32.7	5.19 (66.3°)	38.7	1.88 (53.9°)	29.8	2.25 (56.3°)	31.7	0.70 (39.9°)	27.6	0.41 (32.6°)
difference[a]	9.4	9.82 (72.3°)	9.1	2.54 (57.9°)	17.0	2.20 (56.0°)				

[a] Calculated from the comparison of the contribution from the N- and C-terminal fragments to the entire protein with the data obtained for apocytochrome *c*.

complexes in membranes like bacteriorhodopsin or alamethicin (de Jongh et al., 1994).

The helices in the C-terminal fragment in the presence of DOPC have an orientation parameter comparable to that of the N-terminal helix in this lipid system, corresponding to an angle of 64°. In the presence of DOPG, no preferred orientation of this fragment can be detected. In the presence of DOPS, the peptide has a higher helical content, with yet a different orientation parameter now corresponding to an angle of 42°, indicating a parallel orientation of the peptide. For DMPC and DMPG small orientation parameters are also obtained, correlating to angles of 35° and 32°, respectively.

For apocytochrome *c* itself, it was found that in the presence of DOPC the helices are preferentially aligned perpendicular to the incident light, just like the isolated N- and C-terminal fragments under these conditions (Table 3). For films of apocytochrome *c* with DOPG, no preferred orientation of the helices can be detected. The observation that for apocytochrome *c* also in the presence of DOPS, no preferred orientation is observed for the helices can be explained by a canceling of the ensemble-averaged orientation by the two different orientations for the termini, as observed for the terminal fragments separately (Table 3). Preferred orientations parallel to the light beam are found in the presence of DMPC and DMPG.

From a comparison of the summation of the N- and C-terminal contributions relative to the entire protein with the results obtained for apocytochrome *c* for the various lipids, one can extract information on the suggested helix in the middle region of the protein (Table 3). In all cases for which this could be calculated, an additional 10−18 residues are found to be helical in apocytochrome *c*, compared to the two individual termini. This number agrees well with that obtained from the measurements with DOPS vesicles in aqueous solution (Table 1). The orientation of these helical residues in the presence of the unsaturated lipids appears to have a slight preference for orientation perpendicular to the incident light beam. The fact that, in the presence of DOPG, a much larger helical content is attributed to this middle region could be explained by smaller β-strand formation of apocytochrome *c* in the presence of DOPG than in the individual fragments.

Computer Modeling. In order to obtain thermodynamic insight into the orientation of the different helices at a polar−apolar interface, the suggested helical parts of apocytochrome *c* have been simulated at a lipid−water interface (Figure 3). The hydrophobic residues Ile-9 and Phe-10 of the N-terminal region and Ile-57, Leu-64, and Leu-68 of the helix proposed in the middle region all face the hydrophobic domain. Both the N-terminal and the helix in the middle region of the protein are found to be aligned parallel to the interface. The C-terminal helix appears to orient differently at this interface, with the N-terminal part situated in the hydrophobic phase and the C-terminus in the hydrophilic phase. His-18, Trp-59, Tyr-67, and Tyr-97 are, according to this computer modeling, all situated at the interface, which is in good agreement with the suggested localization of these aromatic groups in SDS micelles (Snel et al., 1991).

DISCUSSION

Binding of (a fragment of) apocytochrome *c* to phospholipid membranes is essential for the induction of α-helical structures in this protein. This is based on the observations that (1) in the absence of lipids the protein is random coiled (de Jongh & de Kruijff, 1990), (2) in the presence of zwitterionic lipids no secondary structure is induced (Table 2), probably as a lack of binding as described for apocytochrome *c* and membranes of egg PC (Rietveld et al., 1983), and (3) when the polypeptide is oriented in the presence of zwitterionic lipids on a quartz plate, and hence is forced to interact, secondary structure is induced (Table 3), while in the absence of lipids no α-helix formation was found (not shown). On the basis of this work, previous studies (de Jongh & de Kruijff, 1990), and Chou−Fasman predictions (Rietveld et al., 1985), we suggest that apocytochrome *c* can potentially adopt helical structures in the presence of model membranes at the N-terminus (in the region 1−22) and at the C-terminus (in the region 80−101), both of approximately 15 residues. An α-helix was also predicted in the region

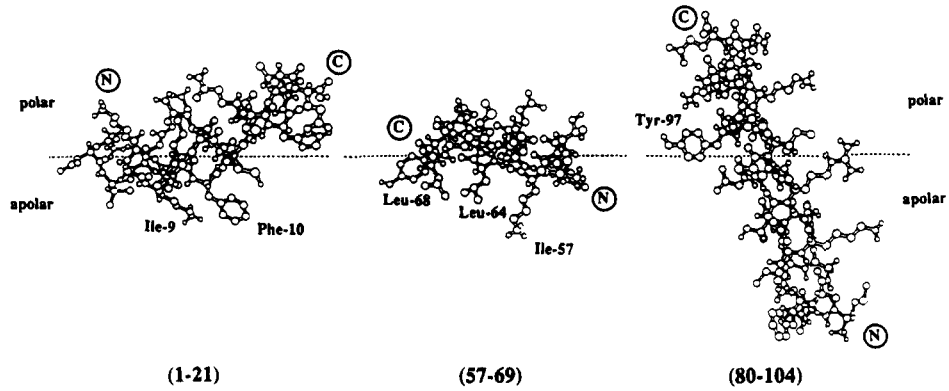

FIGURE 3: Graphs obtained by computer modeling of the fragments 1−21, 57−69, and 80−104 of apocytochrome *c* at a polar−apolar interface, which is represented by the dashed line.

59−70. The CD data indicate, however, that such a helix can only be formed in the presence of the N-terminal domain (Table 1). We will now discuss the results obtained for each of these fragments separately and view their behavior in light of their possible role in the translocation process.

It has been suggested that the N-terminal part of apocytochrome *c* is sufficient for both the binding to and translocation across the mitochondrial outer membrane (Veloso et al., 1984). In a translocation assay, this fragment could be digested by trypsin-enclosed PS/PC vesicles (Jordi et al., 1989). Furthermore, it has been described that the N-terminal fragment 1−38 has a strong influence on the energy content of the phase transition of DEPS membranes (Li-Xin et al., 1988) and that it can penetrate in monolayers composed of DOPS (Jordi et al., 1989). In apparent contrast, we have shown in this work that the helix of the N-terminal fragment (1−38) of apocytochrome *c* in oriented films of the unsaturated lipids DOPC, DOPG, and DOPS has a preferred orientation perpendicular to the incident light beam (i.e., flat on the membrane) (Table 3). Such an orientation was also suggested from the modeling studies. This is not necessarily in conflict with the proposed dynamical role (Jordi et al., 1989) of the N-terminus in the translocation process. Even a preferred orientation of this helix perpendicular to the membrane normal does not exclude a momentary insertion, just sufficient for covalent heme attachment to cysteines 14 and 17 by the enzyme heme lyase, which is located in the intermembrane space. This heme attachment then could trap the N terminus on the inside, initiate folding, and thereby drive the translocation.

That indeed the orientation of the N-terminal helix at the interface is not stable can be suggested from the results in films of DMPC and DMPG, in which this helix has a preferred orientation of its axis parallel to the incident light beam. At present we do not understand this specificity, but we would like to point out that an orientation parallel to the bilayer normal may be more common for amphiphilic helices in DMPC and DMPG, since it was also observed under these conditions for the C-terminal fragment of apocytochrome *c* (Table 3) and for melittin (de Jongh et al., 1994).

The importance of the C-terminal part of the protein for binding to mitochondria has been demonstrated previously by the inhibitive effect of excess C-terminal fragment (residues 66-104) on the import of apocytochrome *c* in mitochondria (Matsuura et al., 1981). It was also this region that was found to be protected from externally added proteases in the presence of PE/PS membranes (Lee & Kim, 1989) or soybean lipid extract (Dumont & Richards, 1984). It has also been shown that this part of the protein not only penetrates in DOPS monolayers but can also penetrate in monolayers composed of DOPC (Jordi et al., 1989). Interestingly, our CD studies indicate that the preferred orientation of the C-terminal helix in the fragment 81−104 has a headgroup specificity in unsaturated lipids. In membranes composed of DOPC, it shows a preferred orientation perpendicular to the incidentlight beam (i.e., flat on the membrane), whereas in films of DOPG there is no preferred orientation, and in DOPS membranes a preferentially parallel orientation is observed. This latter orientation was also suggested by modeling studies. We propose that the C-terminal fragment, upon association with the mitochondrial outer membrane, has some orientational flexibility, which could be of importance for the translocation of the protein across the membrane.

Our CD measurements suggest that the orientation of the helices in apocytochrome *c* is similar to those found for the different fragments of the protein. The middle fragment has a slightly preferred orientation perpendicular to the membrane normal, as was also suggested by the modeling studies.

The observations in this study suggest an additional role of protein−lipid interactions for the import process of apocytochrome *c* across the mitochondrial outer membrane. It is clear that a stable anchoring of the N terminus at the membrane−water interface would be unfavorable for the translocation of this fragment. However, a stable anchoring of any other segment of the protein, either at the interface or in the hydrophobic part of the membrane, would also make it difficult for the protein to dissociate from the membrane after translocation. On the basis of the results of this study, and supported by recent ESR measurements on spin-labeled apocytochrome *c* (Snel et al., 1994), we propose that the C terminus of apocytochrome *c* inserts into the mitochondrial outer membrane as a loose anchor, while the N-terminal fragment preferentially adopts an orientation at the water−lipid interface. However, the presence of the C-terminal anchor could facilitate a momentary insertion of the N terminus into the membrane followed by heme attachment and translocation of the protein, which in turn could be facilitated by the orientational flexibility of the C-terminal fragment.

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