The Mitochondrial Precursor Protein Apocytochrome *c* Strongly Influences the Order of the Headgroup and Acyl Chains of Phosphatidylserine Dispersions. A ^2H and ^{31}P NMR Study†

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ABSTRACT: Deuterium and phosphorus nuclear magnetic resonance techniques were used to study the interaction of the mitochondrial precursor protein apocytochrome *c* with headgroup-deuterated (dioleoylphosphatidyl-L-[2-$^2H_1$]serine) and acyl chain deuterated (1,2-[11,11-$^2H_2$]dioleoylphosphatidylserine) dispersions. Binding of the protein to dioleoylphosphatidylserine liposomes results in phosphorus nuclear magnetic resonance spectra typical of phospholipids undergoing fast axial rotation in extended liquid-crystalline bilayers with a reduced residual chemical shift anisotropy and an increased line width. ^2H NMR spectra on headgroup-deuterated dioleoylphosphatidylserine dispersions showed a decrease in quadrupolar splitting and a broadening of the signal on interaction with apocytochrome *c*. Addition of increasing amounts of apocytochrome *c* to the acyl chain deuterated dioleoylphosphatidylserine dispersions results in the gradual appearance of a second component in the spectra with a 44% reduced quadrupolar splitting. Such large reduction of the quadrupolar splitting has never been observed for any protein studied yet. The lipid structures corresponding to these two components could be separated by sucrose gradient centrifugation, demonstrating the existence of two macroscopic phases. In mixtures of phosphatidylserine and phosphatidylcholine similar effects are observed. The induction of a new spectral component with a well-defined reduced quadrupolar splitting seems to be confined to the N-terminus since addition of a small hydrophilic amino-terminal peptide (residues 1–38) also induces a second component with a strongly reduced quadrupolar splitting. A chemically synthesized peptide corresponding to amino acid residues 2–17 of the presequence of the mitochondrial protein cytochrome oxidase subunit IV also has a large perturbing effect on the order of the acyl chains, indicating that the observed effects may be a property shared by many mitochondrial precursor proteins. In contrast, binding of the mature protein, cytochrome *c*, to acyl chain deuterated phosphatidylserine dispersions has no effect on the deuterium and phosphorus nuclear magnetic resonance spectra, thereby demonstrating precursor-specific perturbation of the phospholipid order. The inability of holocytochrome *c* to perturb the phospholipid order is due to folding of this protein, since unfolding of cytochrome *c* by heat or urea treatment results in similar effects on dioleoylphosphatidylserine bilayers, as observed for the unfolded precursor. Implications of these data for the import of apocytochrome *c* into mitochondria will be discussed.

In general, cellular proteins both have a unique function and location. Many cellular proteins are synthesized in the cytoplasm and subsequently transported to their final location. For instance, approximately 80% of the mitochondrial proteins are not synthesized in the organelle but are imported from the cytosol [for reviews see Verner and Schatz (1988) and Hartl et al. (1989)]. These precursors have to insert into or translocate across one or two mitochondrial membranes, depending on their final intramitochondrial location. Many mitochondrial proteins are synthesized with cleavable amino-terminal presequences which can contain sufficient information for targeting the protein to mitochondria and for intramitochondrial sorting (Hurt & van Loon, 1986). Despite rapidly accumulating data on how mitochondria import proteins, the actual molecular mechanism of protein translocation across a membrane remains obscure. The possible involvement of lipids in mitochondrial import has been addressed by studying the effect of adriamycin, a drug interacting with acidic phospholipids in mitochondria (Nicolay et al., 1984), on import of precursor proteins into mitochondria (Eilers et al., 1989). Further support for a lipid involvement in import of precursor proteins comes from model membrane experiments with presequences [for review see Roise and Schatz (1988)], in vitro synthesized precursors (Ou et al., 1988), and the purified mitochondrial precursor proteins COX IV-DHFR[1] (Endo & Schatz, 1988; Endo et al., 1989) and apocytochrome *c* [for review see Rietveld and de Kruijff (1986)].

Apocytochrome *c* is an example of a group of mitochondrial precursors that are synthesized without a cleavable amino-terminal presequence (Smith et al., 1979). Members of this group include proteins destined for the outer mitochondrial membrane, intermembrane space, inner mitochondrial membrane, and matrix (Hartl et al., 1989). After synthesis in the cytoplasm, apocytochrome *c* binds to mitochondria without

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[1] Abbreviations: ^2H NMR, deuterium nuclear magnetic resonance; ^{31}P NMR, phosphorus nuclear magnetic resonance; DOPS, 1,2-dioleoyl-*sn*-glycero-3-phosphoserine; DOPC, 1,2-dioleoyl-*sn*-glycero-3-phosphocholine; COX IV, cytochrome oxidase subunit IV; COX IV-DHFR, the presequence of cytochrome oxidase subunit IV fused to mouse dihydrofolate reductase; $\Delta\sigma$, residual chemical shift anisotropy; $\Delta\nu_Q$, quadrupolar splitting; CSA, (residual) chemical shift anisotropy; PIPES, 1,4-piperazinediethanesulfonic acid.

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the involvement of a protease-sensitive component on the outside of the outer mitochondrial membrane (Nicholson et al., 1988). Subsequently, the amino terminus of the protein translocates across the outer mitochondrial membrane after which a heme group is covalently attached to two cysteine residues (14 and 17) by the enzyme heme lyase, which has its active site located in the intermembrane space (Nicholson et al., 1987). The attachment of the heme is tightly coupled to the complete translocation of the protein across the outer mitochondrial membrane (Hennig & Neupert, 1981; Nicholson et al., 1988; Dumont et al., 1988). Apocytochrome c has a high affinity for model membranes containing negatively charged phospholipids (Rietveld et al., 1986a), suggesting that the negatively charged lipids that are present at the outside of the outer mitochondrial membrane (Sperka-Gottlieb, 1988) function as the initial high-affinity binding sites. Model membrane experiments also showed that the ability to (partially) translocate across a lipid bilayer (Dumont & Richards, 1984; Rietveld et al., 1986a) is confined to the amino terminus of the protein (Jordi et al., 1989a). Covalent attachment of the heme results in a tightly folded mature protein, cytochrome c, which is no longer able to efficiently insert into or translocate across model membranes (Jordi et al., 1989a; Demel et al., 1989). For the mitochondrial import this would imply that heme coupling traps the mature protein in the intermembrane space.

Recently we have studied the interaction of varioous fragments of apo- and holocytochrome c to obtain information on the relative contribution of the various regions of the protein to the protein–lipid interactions (Zhou Li-Xin et al., 1988; Jordi et al., 1989a; Demel et al., 1989). On the basis of these and previous studies [for review see Rietveld and de Kruijff (1986)] a model was proposed in which various parts of apocytochrome c insert into the membrane and in which the amino terminus at least partially translocates across the bilayer (Jordi et al., 1989a). The prediction of this model is that, as a result of the apocytochrome c–lipid interaction, large changes in the molecular organization of the lipids should take place. In this study we investigate the lipid organization by NMR since this technique is ideally suited to give information on both the macroscopic and local order of the phospholipids. Here we report on the interaction of apo- and holocytochrome c and derived peptide fragments with headgroup-deuterated (dioleoylphosphatidyl-L-[2-$^2H_1$]serine) and acyl chain deuterated (1,2-[11,11-$^2H_2$]dioleoylphosphatidylserine) dispersions. The deuterium nuclear magnetic resonance (^2H NMR) and phosphorus nuclear magnetic resonance (^{31}P NMR) measurements show that apocytochrome c and not cytochrome c has large perturbing effects on the order of both the headgroup and acyl chains of the phospholipids.

Materials and Methods

Polypeptides. Horse heart cytochrome c (type VI) was obtained from Sigma (St. Louis, MO). In unfolding experiments cytochrome c was treated for 1 h at 95 °C or incubated in 8 M urea. Gel filtration experiments using a Sephadex G-25 column eluted with 8 M urea showed that the heme group was still covalently attached to the protein (data not shown) after these treatments.

Apocytochrome c was prepared by removal of the heme group from cytochrome c (Fisher et al., 1973) and was subsequently subjected to a renaturation procedure as described by Hennig and Neupert (1983). The protein was stored at −20 °C in 50 mM NaCl, 10 mM 1,4-piperazinediethanesulfonic acid (PIPES), pH 7.0, and 0.01% β-mercaptoethanol (PIPES buffer) at a concentration of approximately 1.5 mg/mL as determined by using the molar extinction coefficient as previously reported (Stellwagen et al., 1972).

Apocytochrome c derived peptides of sequence positions 1–38, 1–59, 1–65, 39–104, and 81–104 and the cytochrome c derived heme-containing peptide of sequence position 1–65 were prepared and their purity and identity checked via the methods cited and described previously (Jordi et al., 1989a).

A peptide corresponding to amino acid residues (2–17) of the presequence of the mitochondrial protein cytochrome oxidase subunit IV was synthesized by Merrifield solid-phase peptide synthesis and subsequently purified by preparative reverse-phase HPLC using a gradient of acetonitrile in water to which 0.1% trifluoroacetic acid was added. The peptide was pure (>98%) according to analytical HPLC and amino acid analysis. Peptides were lyophilized and weighed, and 0.5 mM solutions were prepared in (deuterium-depleted) PIPES buffer. The concentration was confirmed by a BCA protein assay (Pierce, Rockford, IL) using bovine serum albumin as a standard.

Lipids. 1,2-Dioleoyl-*sn*-glycero-3-phosphatidylcholine (DOPC) and 1,2-dioleoyl-*sn*-glycero-3-phosphatidylserine (DOPS) were synthesized and purified according to established methods (Comfurius & Zwaal, 1977; van Deenen & de Haas, 1964).

Acyl chain deuterated 1,2-[11,11-$^2H_2$]phosphatidylcholine was synthesized essentially as published by Chupin et al. (1987). Acyl chain deuterated 1,2-[11,11-$^2H_2$]phosphatidylserine was prepared from acyl chain deuterated phosphatidylcholine by phospholipase D base exchange as described by Comfurius and Zwaal (1977).

Synthesis and characterization of dioleoylphosphatidyl-L-[2-$^2H_1$]serine will be described elsewhere. The structure of base and location of the single deuteron (boldface) in headgroup-deuterated phosphatidylserine were as follows: $-OCH_2C^2H(^+NH_3)COO^-$.

Sample Preparation. A dry lipid film (5–40 μmol of lipid) was dispersed in deuterium-depleted PIPES buffer (0.5–4 mL), and subsequently the dispersion was subjected to five freeze–thaw cycles, after which minor changes in pH (<0.5) were adjusted with a deuterium-depleted NaOH solution. To this dispersion was added the appropriate amount of apo- or holocytochrome c solution, and the mixture was incubated for 30 min at 30 °C. After each protein addition, the hydrated protein/lipid mixture was centrifuged (4 °C, 27000*g*, 25 min) and the pellet was resuspended in deuterium-depleted PIPES buffer. The binding of the polypeptide to the liposomes was measured by determining the protein content of the supernatant. All other peptide solutions were added directly in the NMR tube which contained 5 μmol of lipid in 0.5 mL of deuterium-depleted PIPES buffer. After the final addition the bound peptide was separated from nonbound peptide by centrifugation (4 °C, 27000*g*, 25 min) and the polypeptide:lipid ratio in the pellet was determined. The binding results for apo- and holocytochrome c and derived peptides were essentially in agreement with the published binding curves (Jordi et al., 1989a). Addition of the presequence (amino acid residues 2–17) or heat-denatured cytochrome c in polypeptide:lipid molar ratios of 1:10 resulted in polypeptide–lipid complexes with a protein:lipid molar ratio of 0.029 and 0.038, respectively (data not shown).

Sucrose Density Centrifugation. Protein/lipid samples, prepared identically with the NMR samples, were loaded on top of a stepwise sucrose gradient (10 μmol of phosphorus per gradient) consisting of 40% (1 mL), 35% (1 mL), 30% (1 mL), 25% (2 mL), 20% (2 mL), 15% (2 mL) 10% (1 mL), and 5%

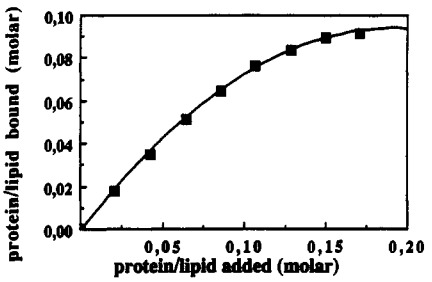

FIGURE 1: Binding of apocytochrome *c* to DOPS dispersions.

(1 mL) sucrose (w/w). Centrifugation was carried out for 14–16 h at 38 000 rpm in a SW 41 Beckman rotor at 4 °C. Isolated fractions were collected and the protein and lipid (Rouser et al., 1970) content was determined.

To obtain information on the lipid organization of the various bands, the separate fractions of six sucrose gradients were pooled and washed with 25 mL of PIPES buffer by centrifugation at 4 °C, 27000*g*, for 25 min, after which ^{31}P and ^{2}H NMR measurements were performed.

NMR. Proton-noise-decoupled ^{31}P NMR spectra were recorded at 121 MHz on a Bruker Instruments, Inc., MSL 300 spectrometer with a 14-μs 90° radiofrequency pulse. The decoupler was gated with an input power of 3 W during 3% of the pulse cycle. To test the efficiency of proton decoupling, the input power was 6 and 10 W in some measurements. No changes in ^{31}P NMR line shapes were observed, indicating full decoupling at 3 W. Typically, 1000–5000 transients were recorded by using a 38.5-kHz sweep width, 4K data points, and a 1-s interpulse time. Free induction decays were exponentially filtered corresponding to a 50-Hz line broadening. The CSA was calculated from the position of the high-field peak.

^{2}H NMR spectra were recorded on the MSL 300 at 46.1 MHz with a 11-μs 90° radio-frequency pulse. A quadrupolar echo pulse sequence was used as described by Chupin et al. (1987) with a pulse separation of 35 μs, a 71.5-kHz sweep width, 4K data points, and a 0.1–0.2-s interpulse time. An exponential multiplication (corresponding to 200-Hz line broadening) was applied to the accumulated free induction decays (typically 5000–10 000 transients) before Fourier transformation, and the spectra were subsequently symmetrized.

T_1 relaxation times were measured and calculated essentially as described previously (Chupin et al., 1987) with an accuracy of approximately 5%. In all cases the data points could be fitted with a single exponential curve.

^{2}H NMR spectral simulations were performed on a microcomputer (Apple Computer Inc., Cupertino, CA) as previously described (Chupin et al., 1987).

Small-Angle X-ray Diffraction. NMR samples that had been stored at −20 °C were thawed, and subsequently X-ray experiments were performed at 30 °C on a Kratky camera as previously described (Bouwstra et al., 1989).

Results

We have studied the interaction of externally added apocytochrome *c* to dioleoylphosphatidylserine dispersions as a model for the interaction of the protein with the negatively charged lipid component of the outer mitochondrial membrane.

Headgroup Region. Preliminary ^{31}P NMR results on the interaction of apocytochrome *c* with bovine brain phosphatidylserine bilayers have been published (Rietveld et al., 1983). In the present studies we analyzed the apocytochrome *c*–PS interaction by ^{31}P NMR more thoroughly and improved the

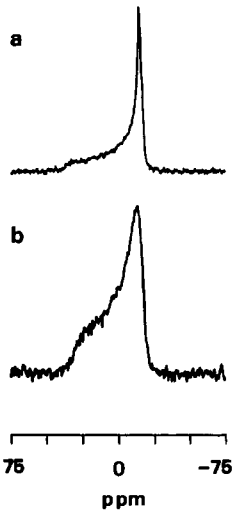

FIGURE 2: 121-MHz ^{31}P NMR spectra of DOPS dispersions in the absence (a) and presence of saturating amounts of apocytochrome *c* (b) at 30 °C. Added protein:lipid (molar) is 1:5.8.

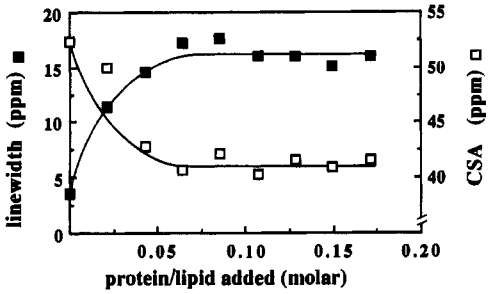

FIGURE 3: Line width at half peak height and residual chemical shift anisotropy (CSA) of 121-MHz ^{31}P NMR spectra of mixtures of DOPS dispersions and apocytochrome *c* at 30 °C.

system in three aspects: the protein concentration was lowered from 50 to approximately 2 mg/mL to reduce aggregation of the protein (Stellwagen et al., 1972), a high-field spectrometer was used to improve the line shapes, and in addition was determined under the NMR conditions the binding of apocytochrome *c* to DOPS bilayers. Figure 1 shows that the binding of apocytochrome *c* to DOPS liposomes saturates at approximately 1 protein/10 negatively charged phospholipids in agreement with previous studies (Rietveld et al., 1986a; Jordi et al., 1989a) and indicating that the protein exhibits normal binding under the conditions used for the NMR experiments. The ^{31}P NMR spectrum of DOPS dispersions has a line shape typical of phospholipids undergoing fast axial rotation in extended liquid-crystalline bilayers (Figure 2a) in agreement with previous studies (Browning & Seelig, 1980). The value of the residual chemical shift anisotropy (CSA or $\Delta\sigma$) of 52 ppm is considerably larger than observed for other phospholipids (Browning & Seelig, 1980). Addition of apocytochrome *c* results in ^{31}P NMR spectra with a reduced $\Delta\sigma$ and an increased line width at half peak height (Figure 2b). These spectral features are expected for an increased isotropic motional averaging of the CSA (Burnell et al., 1980). Additionally, changes in T_2 can contribute to the increased line width. The value of the line width at half peak height and the CSA at different protein:lipid ratios (Figure 3) show that maximum spectral changes are already observed upon addition of apocytochrome *c* in a protein:lipid molar ratio of 0.06 (corresponds to 45% of the saturation binding).

To get more information on the effect of apocytochrome *c* on the order of the headgroup of the phospholipids, we have performed experiments with headgroup-deuterated dioleoyl-

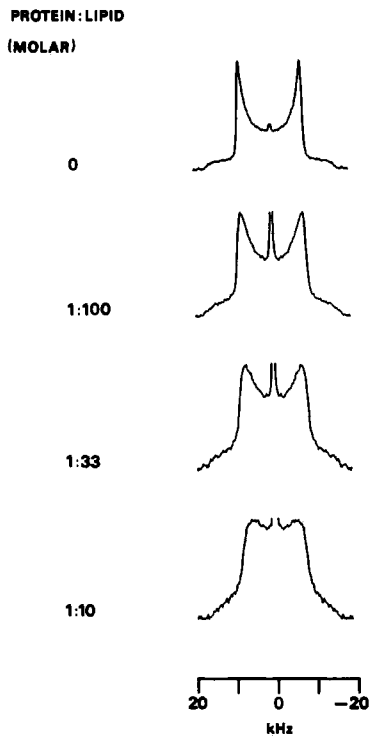

FIGURE 4: 46.1-MHz ^2H NMR spectra of dioleoylphosphatidyl-L-[2-$^2H_1$]serine dispersions with different amounts of added apocytochrome *c* at 30 °C.

phosphatidyl-L-[2-$^2H_1$]serine molecules. The peak separation of the ^2H NMR spectrum, quadrupolar splitting or $\Delta\nu_q$, is a measure of the segmental order of the C–D bond and can be described in terms of an order parameter (S_{CD}):

$$\Delta\nu_q = \frac{3}{4}\left(\frac{e^2qQ}{h}\right)S_{CD} \qquad (1)$$

where e^2qQ/h is the static quadrupolar coupling constant (170 kHz for a C–^2H bond). In this paper we will use "order" and "disorder" to describe the lipid organization judged from the quadrupolar splitting of the ^2H NMR spectra. It is important to notice that the order parameter calculated from ^2H NMR spectra contains contributions from both the average orientation of the C–^2H bond with respect to the axis of motional averaging and the amplitude of this motion.

The ^2H NMR spectrum of headgroup-deuterated DOPS dispersion has a clearly defined quadrupolar splitting of 13 kHz at 30 °C (Figure 4) in agreement with previous observations (Browning & Seelig, 1980). Addition of increasing amounts of apocytochrome *c* to headgroup-deuterated DOPS dispersions results in ^2H NMR spectra with an isotropic component of low intensity (<4%) most likely due to deuterons present at natural abundance in the aqueous buffer used to renature the protein. To facilitate a comparison of the underlying broader spectral components, the isotropic component is not entirely shown in the presented spectra. Interestingly, addition of apocytochrome *c* furthermore results in ^2H NMR spectra with a reduced quadrupolar splitting and increased line width (Figure 4), consistent with the ^{31}P NMR data.

Fatty Acyl Chain Region. To obtain information on the influence of apocytochrome *c* on the order of the acyl chain region, we decided to specifically label an unsaturated phosphatidylserine molecule (DOPS) at the 11-position with two deuterons per acyl chain: 1,2-[11,11-$^2H_2$]dioleoylphosphatidylserine. ^2H NMR spectra of the acyl chain deuterated DOPS dispersions have one clearly defined $\Delta\nu_q$, with a value of 6.3 kHz at 30 °C (Figure 5), indicating that all four

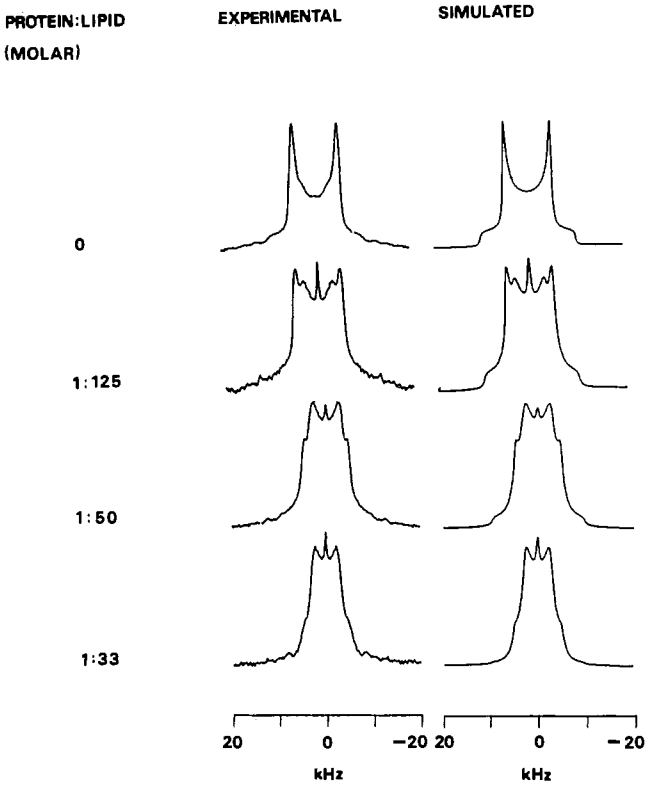

FIGURE 5: Experimental 46.1-MHz ^2H NMR spectra of 1,2-[11,11-$^2H_2$]dioleoylphosphatidylserine dispersions with different amounts of added apocytochrome *c* at 30 °C and computer-simulated ^2H NMR spectra of the same samples. The computer simulations were carried out with the following parameters: P:L = 0, $\Delta\nu_q$ = 6.3 kHz, δ = 200 Hz, P:L = 1:125, 0.74 ($\Delta\nu_q$ = 6.3 kHz, δ = 300 Hz) + 0.26 ($\Delta\nu_q$ = 4.1 kHz, δ = 400 Hz) + 0.003 ($\Delta\nu_q$ = 0 kHz, δ = 200 Hz), P:L = 1:50, 0.4 ($\Delta\nu_q$ = 6.1 kHz, δ = 250 Hz) + 0.6 ($\Delta\nu_q$ = 3.7 kHz, δ = 500 Hz) + 0.001 ($\Delta\nu_q$ = 0 kHz, δ = 200 Hz), P:L = 1:33, 0.92 ($\Delta\nu_q$ = 6.3 kHz, δ = 200 Hz) + 0.08 ($\Delta\nu_q$ = 3.5 kHz, δ = 450 Hz) + 0.003 ($\Delta\nu_q$ = 0 kHz, δ = 200 Hz), where P:L is the added protein:lipid ratio, $\Delta\nu_q$ is the quadrupolar splitting, and δ is the half-width at half-height of the Lorentzian broadening.

deuterons in the DOPS molecule experience similar motional freedom. Addition of apocytochrome *c* to the liposomes in a protein:lipid molar ratio of 1:125 results in, besides the appearance of an isotropic component of unknown origin (<1% of the total signal), a second spectral component with a smaller value of $\Delta\nu_q$ (Figure 5). The intensity of this component increases with increasing amounts of protein added. The spectra were simulated to calculate the relative amounts of the two components in the spectra. The amount of the original spectral component decreases linearly with the amount of protein bound until at a ratio of 1 apocytochrome *c* bound per 37 DOPS molecules only one spectral component is observed (data not shown). Apparently, at intermediate concentrations of protein, where both spectral components can be observed, lipids interacting with the protein are in slow exchange (exchange frequency < approximately 0.4 ms) with the bulk of the lipids. One possibility is that two macroscopic distinct phases are formed upon addition of apocytochrome *c* to DOPS liposomes. To test this, we have performed sucrose density centrifugation experiments with apocytochrome *c*/DOPS mixtures of different protein:lipid molar ratios. Loading protein-free DOPS liposomes on top of a sucrose gradient results in a single band after centrifugation at a density of 1.0170 g/mL. At protein:lipid molar ratios of 1:200–1:30 two bands with a different density were observed. Figure 6 shows a typical sucrose gradient tube of a sample in which apocytochrome *c* was added to acyl chain deuterated DOPS in

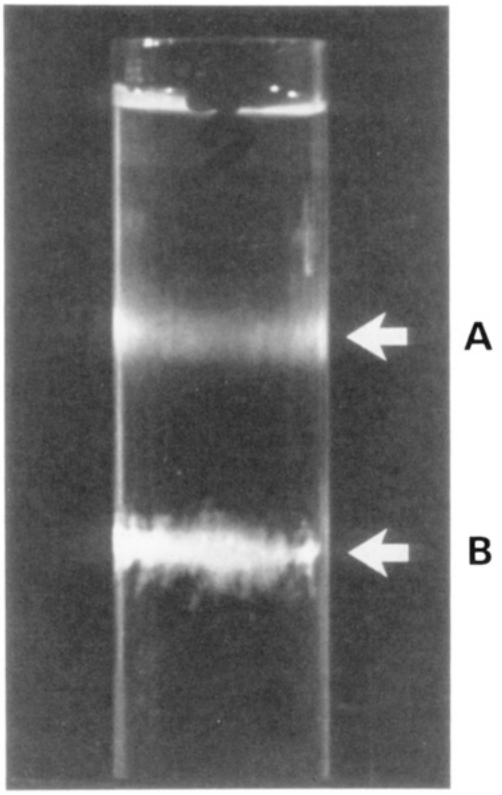

FIGURE 6: A typical sucrose density centrifugation tube of a 1,2-[11,11-$^2H_2$]DOPS dispersion with 1:100 (molar) added apocytochrome *c* showing the low- (A) and high-density band (B).

Table I: Analysis of the Two Fractions Obtained after Sucrose Density Centrifugation of a 1,2-[11,11-$^2H_2$]DOPS Dispersion with 1:100 (Molar) Added Apocytochrome *c*[a]

	low-density band (density 1.0263 g/mL)	high-density band (density 1.0526 g/mL)
P_i recovered (%)	47	39
P:L (molar)	0.0012	0.018
^{31}P NMR		
$\Delta\sigma$ (ppm)	48	41
line width (ppm)	5	13
^2H NMR		
$\Delta\nu_q$ (kHz)	5.2	3.8

[a] P_i is the amount of phosphorus determined in the fraction as a percentage of the amount loaded on the gradient, P:L is the determined protein:lipid molar ratio, $\Delta\sigma$ and line width are the residual chemical shift anisotropy and line width at half peak height of the ^{31}P NMR spectra, and $\Delta\nu_q$ is the quadrupolar splitting of the ^2H NMR spectra at 30 °C.

a 1:100 molar ratio. The sample was separated into two distinct bands. Hydration of a dry lipid film with a protein solution gave essentially identical results (data not shown). Occasionally, the protein-rich band was split into two high-density bands in addition to the protein-poor band (data not shown). The results of the ^{31}P and ^2H NMR measurements of the collected fractions are summarized in Table I. The band with the highest density has a high protein:lipid ratio, and the ^{31}P NMR spectrum has an increased line width and decreased CSA compared to the band with the low density. The quadrupolar splitting of the ^2H NMR spectrum of the high-density band band is 3.8 kHz, a value typical for the second component in two-component spectra and considerably smaller than the value of the low-density band.

The quantification of $\Delta\nu_q$ of the two spectral components on addition of increasing amounts of apocytochrome *c* is shown in Figure 7. Apocytochrome *c* added in protein:lipid molar ratios between 1:125 and 1:30 results in ^2H NMR spectra in

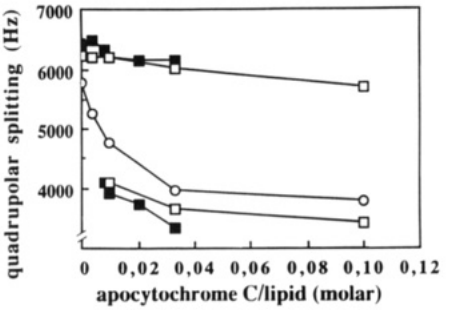

FIGURE 7: Effect of addition of different amounts of apocytochrome *c* on the quadrupolar splitting of 46.1-MHz ^2H NMR spectra of dispersions of acyl chain deuterated DOPS (■) and equimolar mixtures of dioleoylphosphatidylserine and dioleoylphosphatidylcholine with the deuterons attached either to the fatty acyl chains of the DOPS (□) or to the DOPC molecules (○) at 30 °C.

which in addition to the original spectral component of the protein-free lipid, with a $\Delta\nu_q$ of approximately 6.3 kHz, a second spectral component is observed. The $\Delta\nu_q$ of this component decreases slightly with increasing amounts of apocytochrome *c* added whereas at an added protein:lipid molar ratio of 1:10 no defined $\Delta\nu_q$ is observed. The results of the sucrose gradient experiments and the two-component ^2H NMR spectra together unequivocally prove that on addition of apocytochrome *c* to DOPS liposomes in relatively low protein:lipid molar ratios (between 1:125 and 1:30) two macroscopic distinct phases with different lipid organizations are formed.

Insight into the dynamics of the deuterons in the acyl chain region in the absence and presence of protein can be obtained by ^2H NMR spin–lattice relaxation time measurements (T_1). T_1 experiments at different temperatures demonstrated that the motions determining T_1 fall in the fast correlation regime (data not shown). The T_1 of DOPS dispersions at 30 °C was determined to be 21.6 ± 1 ms whereas binding of apocytochrome *c* to the liposomes in an added molar ratio of 1:10 reduces T_1 to 19.4 ± 1 ms, indicating a slight decrease in the rate of motion of the deuterons in the fatty acyl chains. T_1 measurements with peptides 1–65 and 81–104 yielded similar small reductions of T_1. The activation energy for these motions were determined to be 15.7 ± 0.5 and 16.4 ± 0.5 kJ/mol in the temperature range of 5–30 °C for DOPS and apocytochrome *c*/DOPS (1:10), respectively.

Mixed Lipid Systems. The outer mitochondrial membrane, which is the target membrane for apocytochrome *c*, contains besides negatively charged also zwitterionic phospholipids. To investigate the effect of the protein on the order of the fatty acyl chains of the negatively charged and zwitterionic lipid component in mixed modelmembranes, we have performed ^2H NMR experiments in which apocytochrome *c* was added to equimolar mixtures of dioleoylphosphatidylserine and dioleoylphosphatidylcholine in which either the DOPS or the DOPC were ^2H-labeled in the acyl chains. Addition of apocytochrome *c* to mixed model membranes affects both the quadrupolar splitting of the ^2H NMR spectrum of the deuterons of the negatively charged and the zwitterionic phospholipids (Figure 7). Addition of the precursor protein to equimolar DOPS/DOPC mixtures in which the deuterons are attached to the acyl chains of the DOPS molecules results in similar two-component spectra as for DOPS liposomes. An equimolar DOPS/DOPC mixture in which the acyl chains of DOPC molecules are deuterated gives rise to ^2H NMR spectra in which the original spectral component is only poorly resolved on addition of apocytochrome *c*, and this spectral component is therefore not indicated in Figure 7. At a protein:lipid ratio

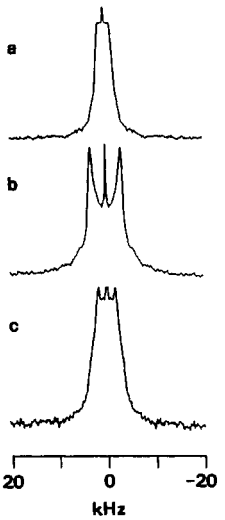

FIGURE 8: 46.1-MHz ^2H NMR spectra of 1,2-[11,11-$^2H_2$]DOPS dispersions in the presence of 1:10 protein:lipid (molar) ratio added of apocytochrome *c* (a), cytochrome *c* (b), and heat-denatured cytochrome *c* (c) at 30 °C.

added of 0.1 the amount of the original spectral component is <5% of the total signal (estimated from comparison with the simulated spectra). The larger reduction of $\Delta\nu_q$ in ^2H NMR spectra of the deuterated DOPS compared to DOPC at a protein:lipid ratio added of 0.1 indicates a larger perturbing effect on the order of the acyl chains of the negatively charged lipid component in mixed model membranes. In ^2H NMR spectra of mixtures of negatively charged and zwitterionic phospholipids which more closely mimic the composition of the outer mitochondrial membrane with respect to the percentage of negatively charged phospholipids (DOPC:DOPS 4:1), apocytochrome *c* also has a large perturbing effect on the order of the acyl chains of the negatively charged lipid component, resulting in $\Delta\nu_q$ of 4.6 kHz at an added protein:lipid molar ratio of 0.1 (data not shown). A control experiment confirmed that addition of apocytochrome *c* to 1,2-[11,11-$^2H_2$]DOPC liposomes has no effect at all on the ^2H NMR spectrum (data not shown).

Protein Specificity. To determine if the large perturbing effects induced by apocytochrome *c* are precursor specific and to get insight into which part of the protein influences the order of the acyl chains, we have studied the interaction of the mature protein cytochrome *c* and various peptide fragments of apo- and holocytochrome *c* with acyl chain deuterated DOPS dispersions. In strong contrast to apocytochrome *c* (Figure 8a), addition of cytochrome *c* in a protein:lipid molar ratio of 0.1 does not affect the ^2H NMR spectra, besides the induction of a small amount of isotropic signal of unknown origin (<1% of total signal) (Figure 8b). Also, no significant changes in the ^{31}P NMR spectra are observed as previously reported (de Kruijff & Cullis, 1980). Five cycles of freezing and thawing of the sample, to allow optimal access of cytochrome *c* to all lipids, does not alter the ^{31}P NMR line shapes (data not shown). These differences between the precursor and the mature protein cannot be explained by a different amount of bound protein since, under these conditions, 1 cytochrome *c* is bound per 30 DOPS molecules, at which protein:lipid ratio apocytochrome *c* has large effects on the order of the headgroup and acyl chains, judged from both the ^{31}P and ^{2}NMR spectra.

Cytochrome *c* is a tightly folded, protease-insensitive globular protein compared to apocytochrome *c* (Dickerson et al., 1971). To investigate if the conformation of the protein results in the inability to perturb the order of the phosphatidylserine molecules, we have changed the folding of cytochrome *c* by heat treatment or incubation in 8 M urea (see Materials and Methods). Heat treatment results in a protease-sensitive denatured protein (data not shown). Circular dichroism measurements carried out according to Jordi et al. (1989a) indicated a decrease in α-helical content in agreement with Meyer (1968) (data not shown). The unfolded cytochrome *c* causes a large reduction in the quadrupolar splitting (Figure 8c) similar to that of the precursor protein (Figure 8a). The ^{31}P NMR spectrum shows an increase in line width and decrease of the CSA similar to the results with apocytochrome *c* (Table II). Urea-treated cytochrome *c* has less perturbing effect on the ^{31}P and ^2H NMR spectra (data not shown) probably because, in contrast to heat denaturation, unfolding by urea is reversible after dilution and the protein can refold when added from 8 M urea prior to binding to the liposomes (without urea).

Table II: Residual Chemical Shift Anisotropy ($\Delta\sigma$) and Line Width at Half Peak Height of the ^{31}P NMR Spectra of DOPS Bilayers in the Absence and Presence of Various Polypeptides in 1:10 Molar Ratio Added at 30 °C

polypeptide	$\Delta\sigma$ (ppm)	line width (ppm)
none	52	4
apocytochrome *c*	41	16
cytochrome *c*	51	5
heat-denatured cytochrome *c*	44	13
1–38	45	7
1–59	44	12
1–65	45	8
1–65H	44	10
presequence COX IV	45	9

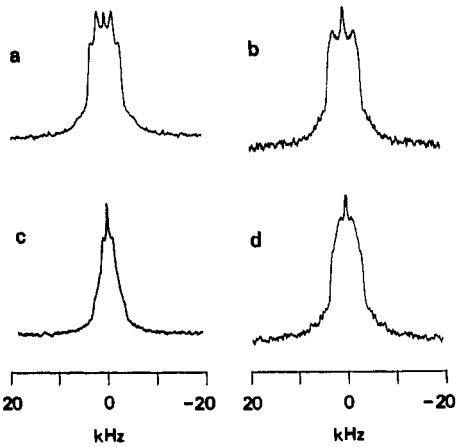

FIGURE 9: 46.1-MHz ^2H NMR spectra of 1,2-[11,11-$^2H_2$]DOPS dispersions in the presence of a 1:10 protein:lipid (molar) ratio added of (a) the amino-terminal peptide (residues 1–38), (b) the carboxy-terminal peptide (residues 81–104, (c) the presequence of COX IV, and (d) the carboxy-terminal fragment (residues 39–104) at 30 °C.

Addition of a small water-soluble amino-terminal peptide fragment of apocytochrome *c* (amino acid residues 1–38) to acyl chain deuterated DOPS liposomes also induces a second spectral component with a strongly reduced $\Delta\nu_q$, indicating large disordering effects on the acyl chain region (Figure 9a). The quantification of $\Delta\nu_q$ of the two spectral components on addition of increasing amounts of this N-terminal peptide is shown in Figure 10. Two other amino-terminal peptide fragments (amino acid residues 1–59 and 1–65) have very similar effects on the ^2H NMR spectra of acyl chain deuterated DOPS dispersions (Figure 10). Interestingly, an amino-terminal fragment of cytochrome *c* (amino acid residues 1–65 with the heme attached) is also able to induce a second component, although with a larger $\Delta\nu_q$, demonstrating that the presence of the heme itself does not cause the inability to

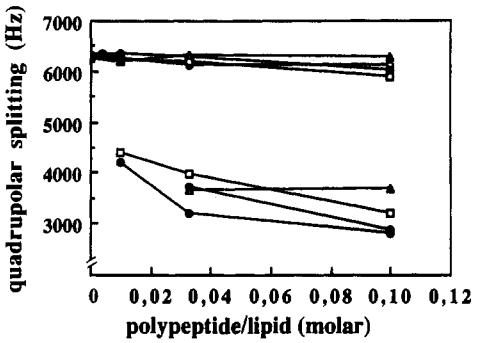

FIGURE 10: Effect of addition of different amounts of the amino-terminal peptides (residue nos.) 1–38 (□), 1–59 (○), 1–65 (●), and 1–65 with heme attached (△) on the quadrupolar splitting of 46.1-MHz ^{2}H NMR spectra of 1,2-[11,11-$^2H_2$]DOPS bilayers at 30 °C.

perturb acyl chain order (Figure 10). Carboxy-terminal peptides also influence the chain order of DOPS bilayers but do not result in clearly defined quadrupolar splittings in the ^{2}H NMR spectra (Figure 9b,d). To test if the perturbing effect of (amino-terminal fragments of) apocytochrome *c* on the headgroup and acyl chain order is a unique property of this mitochondrial precursor protein, we have investigated the interaction of a chemically synthesized peptide corresponding to amino acid residues 2–17 of the mitochondrial presequence of COX IV with acyl chain deuterated DOPS liposomes. This small peptide with a net positive charge of 4 has very large effects on ^{2}H NMR spectra of acyl chain deuterated DOPS dispersions (Figure 9c) and does not have any effect on the ^{31}P and ^{2}H NMR spectra of a DOPC dispersion (data not shown). ^{31}P NMR spectra of DOPS dispersion in the presence of the various peptides all have qualitatively similar line shapes, characterized by a decreased CSA and increased line width at half peak height (Table II).

Macroscopic Structure. To further characterize the macroscopic structure of the hydrated apocytochrome *c*/DOPS mixtures, we have performed freeze–fracture electron microscopy and small-angle X-ray diffraction measurements. A hydrated DOPS lipid film forms large vesicles in which no multilamellar structures could be detected by freeze–fracture electron microscopy. Upon addition of apocytochrome *c*, the lipids convert into multilamellar structures with particles on the fracture face (data not shown) essentially similar as reported for the apocytochrome *c*/bovine brain phosphatidylserine mixtures with a protein concentration of 50 mg/mL (Rietveld et al., 1983). The hydrated DOPS dispersions do not give rise to the sharp reflections that are characteristic of multilamellar vesicles (Figure 11a), in agreement with the freeze–fracture electron microscopy data. In the presence of apo- or holocytochrome *c* reflections with *d* values relating as 1:$^1/_2$ are visible with a repeat distance of 83 Å for apocytochrome *c* and 84 Å for cytochrome *c* (panels B and C, respectively, of Figure 11). The reflections of apocytochrome *c* are less sharp as those observed for the cytochrome *c*/DOPS mixtures consistent with more induced disorder of the phosphate region compared to the holoprotein.

Discussion

Our freeze–fracture electron microscopy data and X-ray measurements indicate that DOPS spontaneously forms large unilamellar vesicles on hydration at neutral pH, in agreement with previous data (Hauser, 1984). Addition of apocytochrome *c* to these vesicles at relatively low protein:lipid molar ratios (1:200–1:30) results in the formation of two distinct phases with different protein:lipid ratios, which can be separated by sucrose density centrifugation experiments.

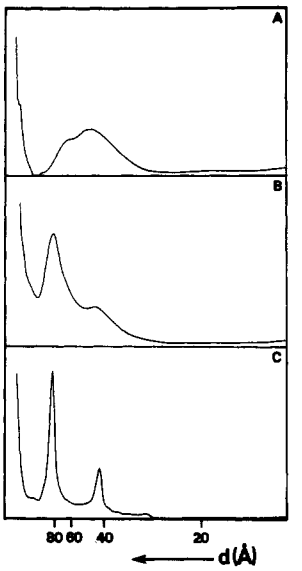

FIGURE 11: Small-angle X-ray diffraction patterns of aqueous dispersions of DOPS in the absence (A) and presence of a 1:10 protein:lipid (molar) ratio added of apocytochrome *c* (B) and cytochrome *c* (C).

This heterogeneity in the samples demonstrates a slow rate of redistribution of apocytochrome *c* between the DOPS vesicles. On addition of apocytochrome *c* at protein:lipid molar ratios of 1:30, only one macroscopic phase is observed. The molar ratio protein (bound):lipid in this phase gradually increases on addition of more protein and saturates at approximately 1 protein/10 DOPS molecules in agreement with previous studies on different model membrane systems (Rietveld et al., 1986a; Jordi et al., 1989a). The electron microscopy and X-ray data indicate that at high protein:lipid molar ratios (1:10) the large unilamellar vesicles convert into multilamellar structures with an interbilayer repeat distance of 83 Å. Recently, we have demonstrated that addition of polylysine to DOPS dispersions results in closely packed multilamellar structures with a repeat distance of 58 Å (Frits de Wolf, unpublished observations), a value close to the previously reported 52 Å for polylysine–cardiolipin complexes (de Kruijff et al., 1985). These values are substantially smaller than the value for the interbilayer repeat distance for the apocytochrome *c*–DOPS complexes, suggesting the presence of substantial amounts of apocytochrome *c* in between the bilayers.

To study the order of the DOPS headgroups, we have performed ^{31}P and ^{2}H NMR experiments. For the protein-free hydrated DOPS dispersions, both the quadrupolar splitting, $\Delta\nu_q$, measured for the headgroup deuteron and the CSA in the ^{31}P NMR are larger than those for correspondingly deuterated phosphatidylcholine molecules (Browning & Seelig, 1980). In addition, the DOPS headgroup deuteron exhibits a short T_1 (Browning, 1981) compared to the α- and β-deuterons in PC headgroups (Gally et al., 1974; Tamm & Seelig, 1983; Sixl & Watts, 1982), indicating a slow rate of reorientation of the PS headgroup. These data are consistent with a relatively rigidly packed PS headgroup. The interaction of apocytochrome *c* with DOPS dispersions results in ^{31}P NMR spectra typical of phospholipids undergoing fast axial rotation in extended liquid-crystalline bilayers with a decreased residual CSA and increased line width. Although at low protein:lipid molar ratios (1:100–1:30) the existence of different macroscopic phases with different ^{31}P NMR spectra was demonstrated by separating the two phases, the individual components could not be resolved in the ^{31}P NMR spectra of the unfrac-

tionated sample. The changes in ^{31}P NMR line shape could in principle be due to local changes in the structure of the phosphate region of the PS molecule (Seelig, 1978). However, we favor an alternative interpretation since the combination of a decreased $\Delta\sigma$ and increased line width is typical for an increased rate of isotropic motional averaging of the residual chemical shift anisotropy within a bilayer orientation (Burnell et al., 1985). From a comparison with simulated ^{31}P NMR spectra of such systems (Burnell et al., 1985) the τ_c of such motions would be approximately 2×10^{-4} s. Consistent with this interpretation is the observation that the interaction of apocytochrome c with headgroup-deuterated DOPS molecules results in ^2H NMR spectra with increased line widths. In conjunction with the ^{31}P NMR results, this indicates an increased motional freedom of the entire phospholipid headgroup.

At added protein:lipid molar ratios >1:16 the additional binding of the protein does not lead to further spectral changes in the ^{31}P NMR spectra. A possible explanation for this phenomenom is that at protein:lipid molar ratios >1:16 a layer of protein is formed which is bound to the vesicles via protein–protein interactions but is not interacting with the lipids (Pilon et al., 1988).

The order and mobility of the fatty acyl chain region of the DOPS liposomes were studied with acyl chain deuterated DOPlS molecules. At relatively low protein:lipid molar ratios (1:125–1:30) the presence of two macroscopic phases with different protein:lipid ratios gives rise to ^2H NMR spectra with two clearly resolved quadrupolar splittings. From simulated ^2H NMR spectra the relative amounts of the two components can be calculated. From the relationship between the relative amounts of the two spectral components and the protein:lipid molar ratio it can be determined that, at a protein:lipid molar ratio of 1:37, the order of all DOPS molecules is influenced by the protein. At this protein:lipid molar ratio, the lipid molecules give rise to ^2H NMR spectra with a 44% reduced quadrupolar splitting. From these data and eq 1, it can be calculated that the order parameter S_{CD} decreases from 0.050 in protein-free DOPS dispersions to 0.027 at a protein:lipid molar ratio of 1:37. It is important to emphasize that this C–^2H bond order parameter is determined both by the orientation of the C–^2H bond with respect to the axis of motional averaging and by the amplitude of fluctuations of the C–^2H bond with respect to this axis. Spin–lattice relaxation time measurements indicate that these changes in the order parameter are accompanied by a slight immobilization of the acyl chains. Together with the ^{31}P and ^2H NMR results on the headgroup-deuterated DOPS, these data are consistent with an increased motional freedom of the entire phospholipid molecule on interaction with the protein. The overall bilayer organization, however, is maintained on binding of large amounts of apocytochrome c as evidenced from the line shape of the ^{31}P NMR spectra, from freeze–fracture electron microscopy data and from the relationship between the spacings of the reflections in small-angle X-ray measurements.

Previously we have studied the interaction of apocytochrome c (Görrissen et al., 1985) and various derived peptide fragments (Jordi et al., 1989b) with bovine brain phosphatidylserine dispersions using spin-label electron spin resonance spectroscopy. In these studies we used phospholipids spin-labeled at different positions on the *sn*-2 chain. There are a number of striking differences between the ESR and ^2H NMR results. First, the stoichiometry is different: at protein:lipid molar ratios of 1:10 only 40% of the spin-labeled lipids are restricted in motion in ESR spectra, whereas in ^2H NMR spectra at protein:lipid molar ratios >1:37, the spectral component of the protein-free lipid is absent. This indicates that at protein:lipid molar ratios >1:37, when a single macroscopic phase is present, the lipids interacting with the protein are in fast exchange with respect to the ^2H NMR time scale (milliseconds) but slow with respect to the ESR time scale (nanoseconds). A second difference is that the outer hyperfine splitting in the ESR spectra increases on interaction with the protein whereas the quadrupolar splitting in the ^2H NMR spectra decreases. However, a direct comparison between the order parameter calculated from the outer hyperfine splitting and calculated from the quadrupolar splitting is complicated because (1) detailed line shape simulations have shown that the ESR spectra can contain important contributions from slow molecular motions (Lange et al., 1985) whereas the calculation of the order parameter (Görrissen et al., 1986) assumes fast molecular motion; (2) the outer hyperfine splitting of the ESR spectra also contains contributions from the rate of motion in contrast to the quadrupolar splitting in ^2H NMR spectra, and this rate of motion is slightly decreased judged from the spin–lattice relaxation time measurements; and (3) the ESR technique has a much shorter time scale than the ^2H NMR technique and will not be sensitive to an isotropic averaging with a τ_c of approximately 2×10^{-4} s.

Addition of apocytochrome c to equimolar mixtures of dioleoylphosphatidylserine and dioleoylphosphatidylcholine with the deuterons attached to the fatty acyl chains of either the DOPS or the DOPC molecule shows that the protein influences the order of both the negatively charged and the zwitterionic phospholipids, thereby demonstrating that no large phase separation of the lipids occurs within a mixed model membrane on binding of the protein. Apocytochrome c has a small preferential interaction with the negatively charged lipid, judged from the larger spectral perturbations of the negatively charged lipid component (Figure 7). This observation cannot be explained by the intrinsic properties of the phosphatidylserine molecules since acyl chain deuterated phosphatidylserine dispersions give rise to ^2H NMR spectra with larger $\Delta\nu_q$ (6.3 kHz at 30 °C), as the correspondingly deuterated phosphatidylcholine (5.4 kHz at 30 °C). The relatively larger decrease in the $\Delta\nu_q$ of the negatively charged lipid component on addition of apocytochrome c is consistent with an enrichment of negatively charged phospholipids in the microdomain interacting with the protein, in agreement with previous ESR and fluorescence data (Berkhout et al., 1987; Rietveld et al., 1986b).

Let us now consider the protein–lipid interactions of apo- and holocytochrome c and see what unique properties of apocytochrome c enable this protein to influence the order of the fatty acyl chains of dioleoylphosphatidylserine dispersions. By using a variety of physical techniques, we have established that cytochrome c is more electrostatically bound to model membranes of negatively charged phospholipids whereas the interaction of apocytochrome c in addition has a more hydrophobic component (Rietveld et al., 1985; Jordi et al., 1989a; Görrissen et al., 1986; Li-Xin Zhou et al., 1988). We have investigated the effect of various polylysines (molecular weight 4K and 20K), and none of these electrostatically bound polypeptides had any effect on the ^2H NMR spectra of acyl chain deuterated dioleoylphosphatidylserine dispersions (data not shown), indicating that proteins which are only electrostatically bound to DOPS dispersions do not affect the order of the acyl chains.

Apocytochrome c is an unstructured, random coil protein whereas cytochrome c is a globular protein in which many of

the hydrophobic amino acid residues are in the interior of the protein facing the relatively hydrophobic heme group (Dickerson et al., 1971). We reasoned that unfolding of the compact structure of cytochrome *c* could result in a heme-containing protein in which some of the small hydrophobic stretches are exposed at the surface of the protein and are able to interact with the lipids (Demel et al., 1989). Here we show that an irreversibly unfolded cytochrome *c* is indeed able to influence the order of the headgroup and acyl chains of the phospholipids. The amino acid sequence of (apo)cytochrome *c* contains no long hydrophobic stretches typical of integral membrane proteins, suggesting the protein will only partially penetrate into the hydrophobic core of the bilayer. The chemically synthesized mitochondrial presequence of COX IV forms an amphipathic α-helix in a lipidic environment (Roise et al., 1986). The molecular area of this presequence in monolayers of negatively charged phospholipids is consistent with a partial penetration of the peptide with its long axis parallel to the plane of the monolayer (Tamm, 1986). Therefore, we propose that the large effects of both apocytochrome *c* and the amino acid residues 2–17 of the mitochondrial presequence of COX IV on the ^{2}H NMR spectra of acyl chain deuterated DOPS dispersions are caused by an increased motional freedom of the acyl chains due to spacing of the phospholipid headgroups by the penetrating polypeptide.

We would like to emphasize that the observed acyl chain disordering effects are not a general property of all polypeptides that penetrate into the bilayer, since a small hydrophobic peptide like gramicidin does not cause similar acyl chain perturbing effects (Chupin et al., 1987). Additionally, a large number of ^{2}H NMR studies have been published on the interaction of large integral membrane proteins such as cytochrome oxidase (Tamm & Seelig, 1983), rhodopsin (Bienvenue et al., 1982), and Ca^{2+}-ATPase (Seelig et al., 1981) with acyl chain deuterated phospholipids. In general, these proteins do not produce, besides a line broadening, a significant variation of the segmental order of the acyl chains. Possibly, the motional freedom of the acyl chains caused by spacing of the lipid headgroups is restricted due to the penetrating hydrophobic part of the polypeptide.

What might be the biological significance of these findings for the translocation of apocytochrome *c* across the outer mitochondrial membrane? Addition of apocytochrome *c* in a protein:lipid molar ratio of 1:10 to a DOPC/DOPS mixture which mimics that of the outer mitochondrial membrane with respect to the percentage of negatively charged phospholipids (Daum, 1985; Sperka-Gottlieb et al., 1988) results in a 26% reduction of the order parameter of the acyl chain deuteron of the negatively charged lipid component, indicating that also under these conditions lipid order is perturbed. The ratio of apocytochrome *c* to negatively charged phospholipids in the outer monolayer of the outer mitochondrial membrane is probably low due to the low cytosolic concentration of mitochondrial precursor proteins. The linear relationship between the protein:lipid ratio and the amount of the spectral component with a reduced quadrupolar splitting, for the dioleoylphosphatidylserine liposomes, suggests that, even at very low protein:lipid ratios, which are beyond the sensitivity of the technique, apocytochrome *c* perturbs the order of the acyl chains. The large perturbing effects of this precursor protein on the order of the phospholipids might facilitate the transient passage of a part of the precursor across the outer mitochondrial membrane. Interestingly, the amino terminus of the protein, which has to translocate across the outer mitochondrial membrane to enable the attachment of the heme to the protein by cytochrome *c* heme lyase in the intermembrane space, has similar acyl chain disordering effects. The fact that also a mitochondrial presequence has a large effect on the order of the phospholipids indicates that the phospholipid-disordering effects could be a property shared by other mitochondrial precursors. The mature protein cytochrome *c* causes no spectral changes at all due to its tight folding. These data demonstrate a clear correlation between the capacity for perturbing the phospholipid order and the unfolded conformation of the polypeptides. Rapidly accumulating evidence suggests that precursor proteins have to be unfolded prior to or during translocation across biological membranes [for a review, see Eilers and Schatz (1988)]. We propose, on the basis of these model membrane experiments, that the unfolded structure of apocytochrome *c* is essential for translocation across a membrane since it allows the protein to interact efficiently with the membrane lipids.

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Registry No. DOPS, 6811-55-8; DOPC, 4235-95-4.

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Spontaneous Formation of Small Unilamellar Vesicles by pH Jump: A pH Gradient across the Bilayer Membrane as the Driving Force†

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ABSTRACT: ^{31}P NMR and infrared spectroscopic methods have been used to study the formation of small unilamellar vesicles by the pH-jump method. It is shown that increasing the pH of different lamellar phospholipid dispersions (phosphatidic acids and phosphatidylserines) induces a pH gradient. This pH gradient is estimated to be 4 ± 1 pH units, and its direction is such that the inner monolayer of the vesicles is at lower pH. There is spectroscopic evidence for tighter packing of the lipid hydrocarbon chains in the inner monolayer, probably due to the constraints imposed by the high curvature of the small vesicles formed. These results are discussed in terms of the driving force of the spontaneous vesiculation.

Spontaneous vesiculation is defined as the formation of unilamellar vesicles upon dispersing a dry smectic (lamellar) lipid film in aqueous medium. The term spontaneous implies that vesicle formation occurs without energy being supplied to the dispersion. The phenomenon of spontaneous vesiculation is well documented in the literature (Hauser, 1984, 1985, 1987; Hauser & Gains, 1982; Hauser et al., 1983, 1985, 1986; Haines, 1983; Rydhag et al., 1982; Rydhag & Gabran, 1982; Gains & Hauser, 1983; Aurora et al., 1985; Li & Haines, 1986; Mantelli et al., 1985). A special case of spontaneous vesiculation is the pH-jump or pH-adjustment method originally described by Hauser and Gains (1982).

Raising the pH of a smectic (lamellar) phosphatidic acid dispersion transiently to values between 10 and 12 induces the formation of small unilamellar vesicles of diameter of about 20-60 nm (Hauser & Gains, 1982). Addition of an alkaline solution of pH 10-12 to a dry film of phosphatidic acid deposited on the glass wall of a round-bottom flask has the same effect (Hauser et al., 1983). The alkaline dispersion is then

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