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Specificity of the Interaction of Amino- and Carboxy-Terminal Fragments of the Mitochondrial Precursor Protein Apocytochrome *c* with Negatively Charged Phospholipids. A Spin-Label Electron Spin Resonance Study†

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ABSTRACT: The contribution of the various regions of the mitochondrial precursor protein apocytochrome *c* to the interaction of the protein with phosphatidylserine dispersions has been studied with chemically and enzymatically prepared fragments of horse heart apocytochrome *c* and phospholipids spin-labeled at different positions of the *sn*-2 chain. Three amino-terminal heme-less peptides, two heme-containing amino-terminal fragments, one central fragment, and three carboxy-terminal fragments were studied. The electron spin resonance spectra of phospholipids spin-labeled at the C5 position of the fatty acid chain indicate that both amino-terminal and carboxy-terminal fragments of the apocytochrome *c* molecule cause a restriction of motion of the lipids, whereas the heme-containing peptides and protein have less effect. In addition, a second motionally more restricted lipid component, which is observed for apocytochrome *c* interacting with phosphatidylserine dispersions containing lipids spin-labeled at the C12 or C14 position [Görrissen, H., Marsh, D., Rietveld, A., & de Kruijff, B. (1986) *Biochemistry 25*, 2904–2910], was observed both on binding the carboxy-terminal fragments and on binding of the amino-terminal fragments of the precursor protein. Interestingly, even a small water-soluble peptide consisting of the 24 carboxy-terminal residues gave rise to a two-component spectrum, with an outer hyperfine splitting of the restricted lipid component of 59 G, indicating a considerable restriction of the chain motion. This suggests that both the carboxy- and amino-terminal parts of the protein penetrate into the center of the bilayer and cause a strong perturbation of the fatty acyl chain motion. The implications of these findings for the mechanism of apocytochrome *c* translocation across membranes are discussed.

Most mitochondrial proteins are synthesized on free ribosomes in the cytoplasm as precursors with an amino-terminal extension (Hay et al., 1984; van Loon et al., 1988). These precursor proteins first must bind to mitochondria and subsequently insert into or translocate across one or two mitochondrial membranes, depending on the specific intramitochondrial location of the mature protein. The information for targeting the precursor proteins to mitochondria and also for intramitochondrial sorting is contained within the amino-terminal presequences (van Loon et al., 1988). The known mitochondrial presequences do not exhibit significant sequence homology (von Heyne, 1986), which would be expected if receptor proteins are involved. Chemically synthesized mitochondrial presequences are able to penetrate spontaneously into phospholipid monolayers and bilayers (Roise et al., 1986; Epand et al., 1986; Tamm, 1986), and a strong correlation exists between the model membrane penetrating capacity of the presequences and their ability to direct passenger proteins into the mitochondria (Roise et al., 1988). This is consistent with an involvement of lipid in mitochondrial precursor protein import. In order to understand the actual molecular mechanism of translocation of a precursor protein across a membrane, we have extensively studied apocytochrome *c*, the heme-free precursor of cytochrome *c* [for review, see Rietveld and de Kruijff (1986)].

Cytochrome *c* functions as a part of the mitochondrial respiratory chain and is located at the outside of the mitochondrial inner membrane. The protein is synthesized in the cytoplasm on free ribosomes, in a precursor form, apocytochrome *c*, that unlike most mitochondrial precursor proteins contains no amino-terminal presequence (Matsuura et al., 1981). This property allows the chemical preparation of large amounts of an import-competent precursor protein (Fisher et al., 1973; Zimmerman et al., 1979). After synthesis, apocytochrome *c* has to be imported into the mitochondrion. The import pathway probably does not involve translocation through the aqueous pores in the outer mitochondrial membrane (Manella et al., 1987). From biochemical analysis of the import of apocytochrome *c* into mitochondria (Zimmerman

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et al., 1979; Hennig et al., 1984; Nicholson et al., 1987) and biochemical and biophysical studies on apocytochrome c–lipid interactions in model systems (Rietveld et al., 1983, 1986a,b), complementary models for apocytochrome c import have been proposed. In these models the precursor protein first binds with high affinity, preferentially to the negatively charged lipid component of the outer mitochondrial membrane, followed by penetration and translocation of at least a part of the protein to the opposite interface of the membrane. Subsequently, the heme group is coupled to the two cysteine residues in the amino-terminal part of the precursor by an enzymatic reaction. This coupling converts the precursor protein into its folded mature form, after which the protein is transported to the inner mitochondrial membrane.

Model membrane studies with peptides derived from apocytochrome c have demonstrated that both amino- and carboxy-terminal peptides of the precursor are able to penetrate into model membranes, whereas only amino-terminal peptides are able to translocate, at least partially, across the bilayer (Zhou Li-Xin et al., 1988; Jordi et al., 1988). Covalent attachment of the heme to either the protein or the derived amino-terminal peptides results in a decreased penetration into and the inability to translocate across the bilayer (Jordi et al., 1988). Electron spin resonance (ESR)[1] experiments with spin-labeled phospholipids showed that apocytochrome c causes a perturbation of the bulk lipid mobility or ordering considerably greater than that observed on binding of the mature protein, cytochrome c (Görrissen et al., 1986). In addition, using lipids spin-labeled close to the terminal methyl ends of the fatty acid chains, a second more motionally restricted lipid component reminiscent of that observed for integral membrane proteins was observed on binding apocytochrome c but not cytochrome c, demonstrating that the precursor protein penetrates deeply into the bilayer. In this study we report on the interaction of fragments of apo- and holocytochrome c with negatively charged phospholipids by performing ESR measurements. The results of these experiments show that both amino-terminal and carboxy-terminal fragments of apocytochrome c cause a comparable decrease in the lipid mobility and the carboxy- as well as the amino-terminal fragments of apocytochrome c also induce a direct motional restriction of the terminal methyl ends of the fatty acid chains. Covalent attachment of the heme group results in a marked decrease of the spectral perturbations induced by both the entire protein and the derived peptides.

Materials and Methods

Materials. Horse heart cytochrome c (type VI) was obtained from Sigma (St. Louis, MO). Apocytochrome c was prepared by removal of the heme group from cytochrome c (Fisher et al., 1973). Apocytochrome c derived peptides of sequence positions 1–38, 1–59, 1–65, 66–80, 39–104, 60–104, and 81–104, and cytochrome c derived heme-containing peptides of sequence positions 1–38 and 1–65, were prepared and their purity and identity checked via the methods cited and described previously (Jordi et al., 1988).

Proteins and peptides were lyophilized and weighed and solutions with a concentration of 2 mg/mL prepared in buffer containing 10 mM Hepes, 0.1 mM EDTA, 0.01% mercaptoethanol, and 10 or 50 mM NaCl, pH 7.0. The results for apocytochrome c reported in this study were identical with the data obtained previously using renatured apocytochrome c (Görrissen et al., 1988). Bovine spinal cord phosphatidylserine (PS) was from Lipid Products (South Nutfield, U.K.). Phosphatidylglycerols, spin-labeled at the C5 and C12 atom of the *sn*-2 chain, were synthesized essentially as described in Marsh and Watts (1982).

Sample Preparation. A dry film of 0.5 mg of PS (containing 1% w/w of spin-labeled lipid) was taken up in 1.25 mL of buffer. Phosphatidylserine forms large unilamellar vesicles under these conditions (Hauser, 1984). In experiments with polypeptides, the PS film was taken up in 1.25 mL of buffer containing 2 mg/mL of the various proteins or peptides. The peptides or proteins were allowed to bind at 30 °C for 30 min, and the protein–lipid complex was subsequently centrifuged at room temperature (20 min, 3000*g*) and transferred to a sealed-off 100-μL capillary. The complex was further concentrated in the capillary by centrifugation at room temperature (20 min, 3000*g*) prior to ESR experiments. The exact lipid/protein or lipid/peptide ratio in the sample was afterward determined by dissolving the complex in 1 N NaOH followed by phosphate analysis (Rouser et al., 1970) and protein determination (Lowry et al., 1951), using the various solutions of the proteins as standards. Control experiments showed that in the absence of phospholipids none of the proteins or peptides could be pelleted during centrifugation.

ESR Experiments. ESR spectra were recorded on a Varian E-12 9-GHz spectrometer equipped with nitrogen gas-flow temperature regulation. Samples were contained in 1-mm o.d. glass capillaries accommodated within a standard 4-mm quartz ESR tube, which contained silicon oil for thermal stability. Temperature was measured with a fine thermocouple situated in the silicon oil just above the top of the ESR cavity. All experiments were performed at 30, 35, and 40 °C. Spectra were digitized by using a Digital Equipment Corp. LPS system and dedicated PDP 11/10 computer with VT-11 display. Spectral subtraction and quantitation were performed with interactive graphics essentially as described (Marsh, 1982) with software written by Dr. W. Möller. The maximum outer hyperfine splittings ($2A_{max}$) and apparent order parameters (S_{eff}) were obtained from the spin-label ESR spectra as described by Görrissen et al. (1986). These parameters contain contributions from both the amplitude and the rate of motion but are nonetheless useful for comparing the relative effects of the different proteins and peptides on the lipid chain dynamics (Lange et al., 1985).

Results

To study the polypeptide–lipid interactions of the various regions of the apocytochrome c molecule and to investigate the effect of covalent coupling of the heme group to the two cysteine residues 14 and 17 on these interactions, we have prepared and purified fragments of apo- and holocytochrome c (three amino-terminal apofragments, two heme-containing fragments, one central fragment, and three carboxy-terminal fragments) and studied their interaction with negatively charged phospholipids by performing electron spin resonance (ESR) measurements. Figure 1 shows schematically the size and position of the various peptides with respect to the parent proteins. Apo- and holocytochrome c are highly basic proteins of which the amino-terminal fragments have a higher net positive charge than the carboxy-terminal peptides.

To investigate the effects of the various proteins and peptides on the mobility of the lipid acyl chains, phosphatidylglycerols spin-labeled at either the C5 (close to the glycerol backbone) or the C12 position (close to the terminal methyl group) were used as reporter molecules in a PS matrix. Apocytochrome

[1] Abbreviations: *n*-PGSL, 1-acyl-2-[*n*-(4,4-dimethyloxazolidine-*N*-oxyl)stearoyl]-*sn*-glycero-3-phosphoglycerol; PS, bovine spinal cord phosphatidylserine; Hepes, 2-[4-(2-hydroxyethyl)-1-piperazinyl]ethanesulfonic acid; EDTA, ethylenediaminetetraacetic acid; ESR, electron spin resonance.

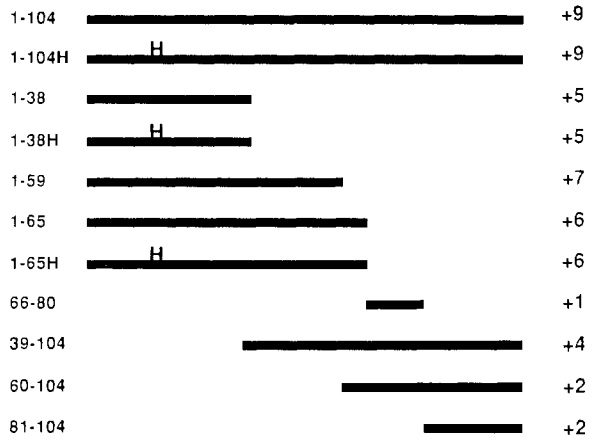

FIGURE 1: Location of the various peptides with respect to apocytochrome *c* (1–104) and cytochrome *c* (1–104H). The net charges of the various proteins and peptides at pH 7.0 as calculated from the side chains of the amino acids (Arg and Lys positive, Asp and Glu negative) are given at the right of the figure, and the location of the heme group is indicated by the letter H.

c interacts strongly with both these negatively charged lipids (Rietveld et al., 1986a). Additionally, it was shown, by using spin-labeled PG and PS molecules, that there is no headgroup effect (Görrissen et al., 1986). In order to obtain the maximal spectral perturbations, PS lipid films were hydrated with a 5-fold weight excess of protein or peptide, since the binding curves of the various peptides or proteins to PS vesicles indicate that at this protein/lipid ratio saturation binding is obtained for 50 mM NaCl, except for the fragments 60–104 and 66–80 (Jordi et al., 1989). Analysis of the protein/lipid ratio of the ESR samples essentially indicated that saturation of lipid binding sites has occurred in the ESR experiments. The values of the binding stoichiometries are given later in Table I. Fragment 66–80 does not bind to PS vesicles at all (Jordi et al., 1988), probably due to its low net positive charge, and therefore did not induce any spectral perturbation for either spin-label position. The analysis of the small spectral changes induced by fragment 60–104 is complicated by the lack of saturation of the lipid binding sites. Data on fragments 66–80 and 60–104 will not be further described or discussed in detail.

Figure 2 shows the effect of adding saturating amounts of a small amino-terminal fragment of apocytochrome *c* (1–38), a small heme-containing fragment of cytochrome *c* (1–38H), and a small carboxy-terminal fragment (81–104) on the ESR spectra of the 5-PGSL spin-label in PS dispersions. Both amino-terminal and carboxy-terminal fragments of apocytochrome *c* induce both a broadening of the spectra and a large increase in outer hyperfine splitting whereas the heme-containing amino-terminal fragment of the mature protein causes a smaller spectral perturbation. From the spectra shown in Figure 2, the outer hyperfine splitting and effective order parameter, which contain contributions from both the amplitude and rate of motion of the spin-labeled lipids (Lange et al., 1985), can be obtained as described previously for the entire protein (Görrissen et al., 1986). Figure 3A shows that the apparent order parameter decreases with increasing temperature, corresponding to an increase in motional freedom of the lipid chains, both for the pure hydrated lipid and for the peptide–lipid complexes. This figure also clearly shows the different extents to which the various peptides decrease the lipid mobility. Figure 3B shows the results of the same experiments using a buffer containing 50 mM instead of 10 mM NaCl. The increase in NaCl concentration results in a decrease in effective order parameter at all temperatures for these peptides. In contrast, little effect of salt concentration

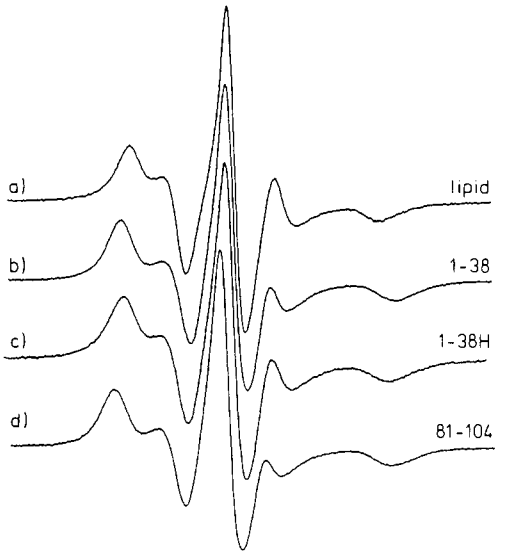

FIGURE 2: ESR spectra of the 5-PGSL spin-label in PS dispersions/10 mM Hepes, 10 mM NaCl, and 0.1 mM EDTA, pH 7.0 (containing 0.01% mercaptoethanol). (a) In the absence of protein; and in the presence of saturating amounts (5 mg of peptide/mg of lipid) of (b) the apopeptide (1–38), (c) the heme-containing peptide (1–38H), and (d) the carboxy-terminal fragment 81–104. Total scan width = 100 G; T = 30 °C.

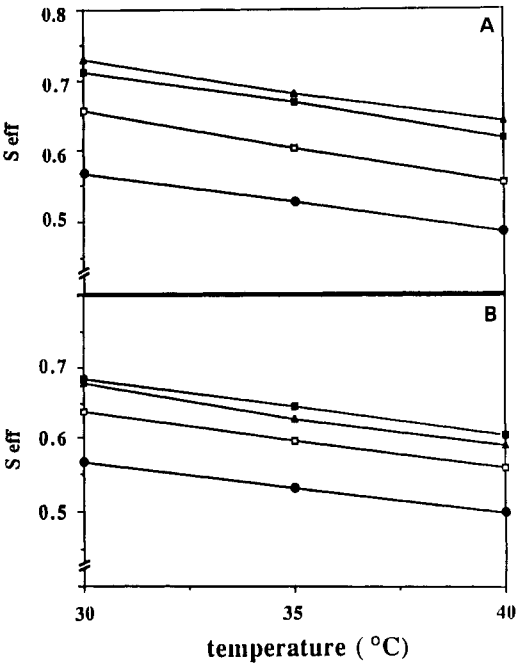

FIGURE 3: Effect of temperature and NaCl concentration on the apparent order parameter, S_{eff}, of the 5-PGSL spin-label in PS dispersions/10 mM Hepes, 10 mM (A) or 50 mM (B) NaCl, and 0.1 mM EDTA, pH 7.0 (containing 0.01% mercaptoethanol), in the absence (●) and in the presence of saturating amounts (5 mg of peptide/mg of lipid) of the apopeptide 1–38 (■), the heme-containing peptide 1–38H (□), and the carboxy-terminal peptide 81–104 (▲).

was observed for peptide-free, hydrated phosphatidylserine, demonstrating that the decrease in apparent order parameter at higher NaCl concentrations is not caused by a direct effect of salt on the phospholipid. The observation that the peptides induce less spectral perturbation at a higher NaCl concentration can be explained by a decrease in peptide–phospholipid binding with increasing ionic strength (data not shown) as described previously for the apocytochrome *c*–phosphatidylserine interaction (Görrissen et al., 1986). Interestingly, the effect of increasing ionic strength is greater for the peptide 81–104 than for the peptide 1–38, even though the latter bears

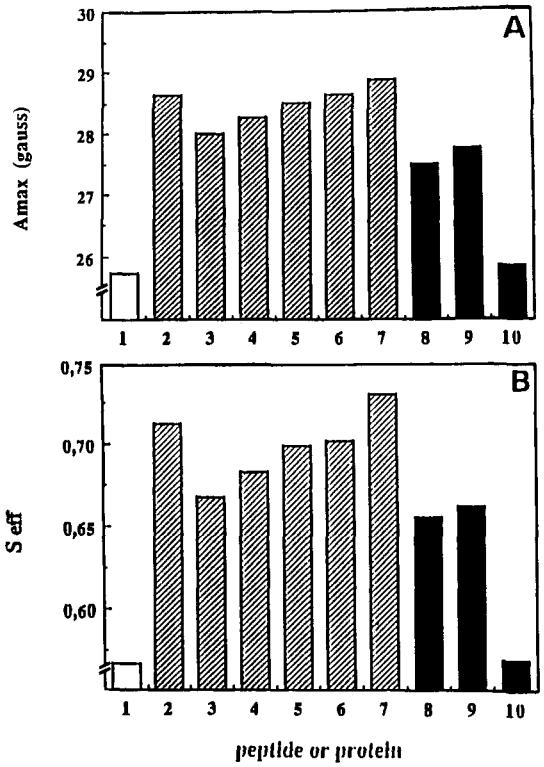

FIGURE 4: Outer hyperfine splitting, A_{max}, and the effective order parameter, S_{eff}, at 30 °C of the 5-PGSL spin-label in PS dispersions/10 mM Hepes, 10 mM NaCl, and 0.1 mM EDTA, pH 7.0 (containing 0.01% mercaptoethanol), in the absence (open bar) and presence of saturating amounts (5 mg of peptide or protein/mg of lipid) of apocytochrome c and derived peptides (hatched bars) and cytochrome c and derived peptides (black bars). Lipid alone (1), peptide 1–38 (2), peptide 1–59 (3), peptide 1–65 (4), apocytochrome c (5), peptide 39–104 (6), peptide 81–104 (7), peptide 1–38H (8), peptide 1–65H (9), and cytochrome c (10).

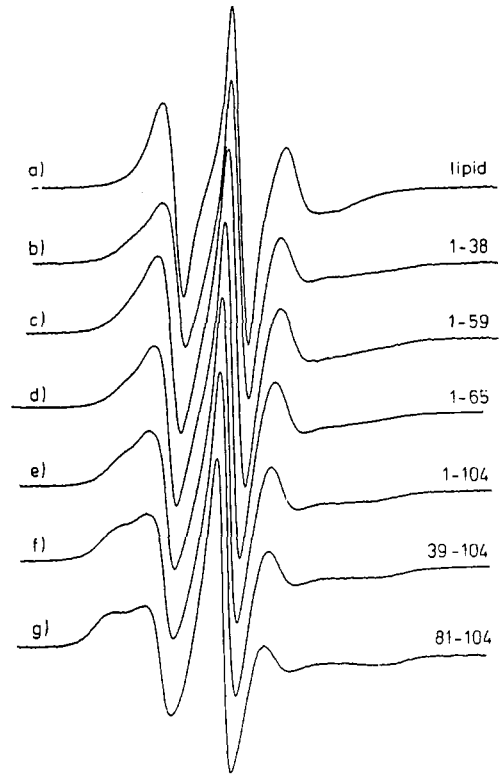

FIGURE 5: ESR spectra of the 12-PGSL spin-label in PS dispersions/10 mM Hepes, 10 mM NaCl, and 0.1 mM EDTA, pH 7.0 (containing 0.01% mercaptoethanol), in the absence (a) and presence of saturating amounts (5 mg of protein or peptide/mg of lipid) of apocytochrome c (e) and amino-terminal (b–d) and carboxy-terminal (f and g) fragments of the protein. Total scan width = 100 G; T = 30 °C.

a higher net positive charge. All other polypeptide–lipid complexes studied showed a characteristic decrease in outer hyperfine splitting and apparent order parameter with increasing temperature and a decrease in both parameters at higher NaCl concentrations (data not shown). For simplicity, only data will be shown which were obtained at 30 °C with a buffer containing 10 mM NaCl.

The outer hyperfine splittings and the apparent order parameters of the 5-PGSL spin-label in PS dispersions with saturating amounts of the various peptides are given in Figure 4. All amino-terminal fragments of apocytochrome c cause an increase in the outer hyperfine splitting and apparent order parameter. The maximum effect is caused by the small fragment 1–38 and is comparable in magnitude to, or even slightly larger than, the change in spectral parameters induced by the entire precursor protein. Both carboxy-terminal peptides 39–104 and 81–104 cause large increases in outer hyperfine splitting and apparent order parameter which are similar to or even slightly larger than those for the entire apoprotein. The presence of the heme group has a pronounced effect on the peptide–lipid interactions. Both heme-containing fragments 1–38H and 1–65H cause a definite increase in A_{max} and S_{eff} for 5-PGSL but to a smaller extent than the heme-free analogues, and the mature protein cytochrome c has an even smaller effect on the order parameter and A_{max} of the spin-labeled phosphatidylglycerol molecules. The relative differences between the apo- and holoprotein or peptides, defined as

$$d = \frac{S_{eff}(\text{apopeptide}) - S_{eff}(\text{pure lipid})}{S_{eff}(\text{heme peptide}) - S_{eff}(\text{pure lipid})}$$

follow the order 1–104 > 1–38 > 1–65. In conclusion, both amino-terminal and carboxy-terminal fragments of apocytochrome c cause a strong restriction of motion at the C5 position of the *sn*-2 acyl chain, whereas the heme-containing peptides and protein have less effect.

In order to investigate which region of the apocytochrome c molecule causes a specific motional restriction of the lipid chains toward the center of the bilayer, ESR experiments were performed with phosphatidylglycerol molecules spin-labeled at the C12 position of the *sn*-2 acyl chain (see Figure 5). For these spin-labels, which give rise to a narrow spectrum in peptide-free PS dispersions (Figure 5a), a second broader spectral component in the outer wings of the narrow component is observed on binding of apocytochrome c (Figure 5e), in agreement with previous observations (Görrissen, et al. 1986). Carboxy-terminal peptides (Figure 5f,g), as well as the amino-terminal peptides (Figure 5b–d), of apocytochrome c induce a clearly resolved second motionally restricted lipid component in the ESR spectra of the 12-PGSL spin-label. Differences between the carboxy-terminal and amino-terminal peptides at saturating peptide concentrations are very clear when comparing the ESR spectra of the peptides 1–65 and 39–104. These peptides of comparable size (fragment 1–65 contains the first 65 amino acid residues whereas fragment 39–104 contains the last 66 residues of the apocytochrome c molecule) induce different ESR spectra on binding to the lipids. The carboxy-terminal peptide gives rise to a much greater proportion of the motionally restricted lipid spectrum. These differences between the amino- and carboxy-terminal peptides can be explained, at least in part, by the different molar binding stoichiometries, although on a w/w basis the binding of the 1–38 and 81–104 residue fragments are rather similar.

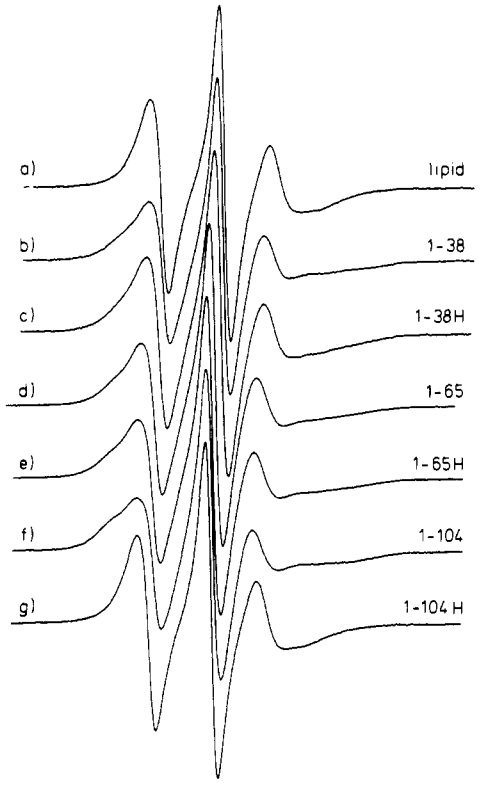

FIGURE 6: ESR spectra of the 12-PGSL spin-label in PS dispersions/10 mM Hepes, 10 mM NaCl, and 0.1 mM EDTA, pH 7.0 (containing 0.01% mercaptoethanol), in the absence (a) and presence of saturating amounts (5 mg of peptide or protein/mg of lipid) of apocytochrome *c* (f), cytochrome *c* (g), and amino-terminal apopeptides (b and d) and heme-containing (c and e) peptides. Total scan width = 100 G; T = 30 °C.

To visualize directly the effect of covalent attachment of the heme group to the various proteins and peptides, ESR spectra of the 12-PGSL phosphatidylglycerol spin-label in the absence or presence of both the heme-containing fragments and the apopeptides or -proteins are shown in Figure 6. The second, motionally restricted lipid component is less clearly seen for the heme-containing fragment 1–38H (Figure 6c) than for the corresponding apopeptide (Figure 6b), and for cytochrome *c* (Figure 6g) it is completely absent. However, in this case also, the binding stoichiometries of the two fragments are rather different. In contrast, the large amino-terminal apofragments and heme-containing fragments 1–65 and 1–65H have rather similar ESR spectra (Figure 6d,e), indicating that for this fragment the covalent attachment of the heme group has less effect. Significantly, in this case, the extents of binding of the two fragments are more similar. Therefore, differences in spectral perturbation observed between the apofragments and heme-containing proteins or peptides cannot be explained only by the effect of the heme group itself but must be attributed also to the degree of binding.

To obtain quantitative data on the proportion of motionally restricted lipids and the degree of motional restriction, spectral subtractions were performed for the ESR spectra of the various peptide–lipid or protein–lipid complexes containing the 12-PGSL phosphatidylglycerol spin-label. The results of spectral subtraction of the two-component ESR spectrum for the lipid complex with the large carboxy-terminal peptide 39–104 are shown in Figure 7. The fluid component in the spectrum of the peptide–lipid complex at 30 °C (Figure 7a) closely resembles the ESR spectrum obtained from peptide-free hydrated lipid at 24 °C (Figure 7c) and therefore demonstrates a higher degree of motional restriction. Subtraction of 40%

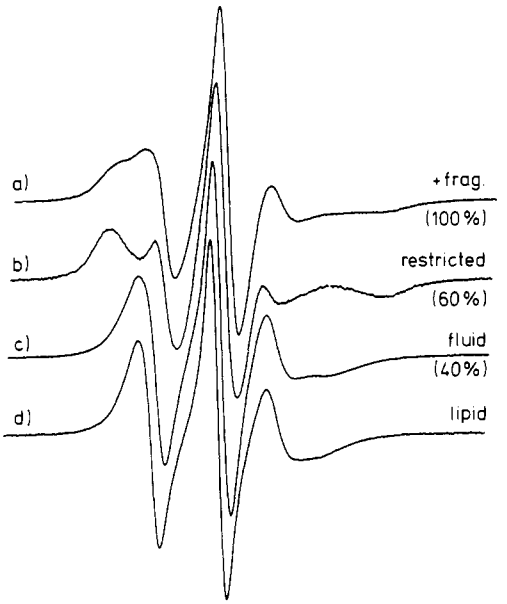

FIGURE 7: Spectral subtraction of the composite ESR spectrum of the 12-PGSL spin-label in PS dispersions/10 mM Hepes, 10 mM NaCl, and 0.1 mM EDTA, pH 7.0 (containing 0.01% mercaptoethanol), in the presence of a saturating amount (5 mg of peptide/mg of lipid) of the carboxy-terminal fragment 39–104. (a) 12-PGSL in PS + peptide 39–104, T = 30 °C; (b) difference spectrum obtained by subtracting 40% of spectrum c from the spectral intensity of the composite spectrum a; (c) fluid spectral component used for the subtraction (spectrum from 12-PGSL in PS dispersions/10 mM Hepes, 10 mM NaCl, and 0.1 mM EDTA, pH 7.0 at 24 °C); (d) 12-PGSL in PS, T = 30 °C. Total scan width = 100 G.

Table I: Results of the Spectral Subtractions of the Composite ESR Spectra of the 12-PGSL Spin-Label[a]

peptide or protein	n_t	F (%)	n_b	$2A_{max}$ (G)	T (°C)
apocytochrome *c*	10	43	4.3	56	22
1–38	3.8	37	1.4	55	26
1–59	12	37	4.4	55	26
1–65	15	33	4.9	54	26
cytochrome *c*	19	0	0		
1–38H	11	40	4.4	53	28
1–65H	9	39	3.5	55	26
39–104	4.8	61	2.9	57	24
81–104	2.4	69	1.7	59	22

[a] Experiments were carried out with 12-PGSL spin-label in PS Dispersions/10 mM Hepes, 10 mM NaCl, and 0.1 mM EDTA, pH 7.0 containing 0.01% mercaptoethanol), in the presence of the various proteins or peptide (5 mg/mg lipid) at 30 °C. n_t is the total number of PS lipid molecules bound per protein or peptide; F is the fraction of restricted lipid component, expressed as a percentage of the total spectral intensity; n_b is the effective number of motionally restricted lipids per peptide; $2A_{max}$ is the outer hyperfine splitting of the restricted lipid component (in gauss); and T is the temperature at which the spectrum of the peptide-free lipid dispersion used for the subtraction of the fluid spectral component was recorded.

of the double-integrated intensity from the ESR spectrum of the peptide–ligand complex, using this fluid component spectrum, yields a typical single-component spectrum representing the motionally restricted lipid component of the peptide–lipid complex (Figure 7b). This motionally restricted component therefore comprises 60% of the total spin-labeled lipid and has an outer hyperfine splitting of 57 G at 30 °C.

The data from digital subtractions with the ESR spectra of the 12-PGSL spin-label, together with the binding stoichiometries of the various protein or peptide complexes, are summarized in Table I. From the fraction of the restricted lipid component and the amount of polypeptide bound to the lipid, the effective number of motionally restricted lipids per protein or peptide, n_b, can be calculated. These values are also

given in Table I. However, interpretation of the stoichiometry in such systems is not straightforward. First, it was reported that for apocytochrome c the stoichiometry decreases with increasing probe concentration (Görrissen et al., 1986). Comparison with latter work suggests that the present results refer to a region in which the stoichiometry of the motionally restricted lipid component is not unduly affected by the extent of binding. Second, it was demonstrated by using a radiolabeled apocytochrome c that approximately 50% of the protein could be washed off a phosphatidylserine monolayer without changing the surface pressure (Pilon et al., 1987), indicating the presence of an adsorbed protein layer that is not interacting with the lipids but that is included in the calculation of the stoichiometry. Furthermore, we do not know what percentage of the various peptides interacts with the lipids. This point will be returned to under Discussion.

The results of Table I emphasize some of the differences in peptide–lipid interaction for the amino-terminal and carboxy-terminal fragments of apocytochrome c. First, it is immediately clear that, although the fraction of the restricted lipid component as a percentage of the total integrated spectral intensity is higher at saturation binding for the carboxy-terminal fragments than for the amino-terminal fragments of apocytochrome c, the molar stoichiometries are generally in the reverse order. This result directly reflects the different binding stoichiometries given in Table I. Second, the fluid lipid component observed in ESR spectra using carboxy-terminal peptides has a higher degree of motional restriction than observed for the amino-terminal peptides, since it resembles ESR spectra of peptide-free hydrated lipid obtained at lower temperatures. This parallels to a large extent the results with the 5-PGSL spin-label in Figure 4. In addition, there are important differences between the outer hyperfine fine splittings of the restricted lipid components: binding of amino-terminal fragments gives rise to ESR spectra with restricted lipid components with a smaller outer hyperfine fine splitting than obtained for the entire apocytochrome c molecule. In strong contrast, the carboxy-terminal fragments induce ESR spectra with restricted lipid components having splittings that are even larger than obtained for the entire apocytochrome c molecule. Especially, the ESR spectrum induced by the small fragment 81–104 more strongly resembles ESR spectra typical of integral membrane proteins (Marsh, 1985). This is even more striking in view of the fact that this fragment contains no large hydrophobic sequence.

The covalent attachment of the heme group to apocytochrome c results in the complete disappearance of the restricted lipid component in agreement with previous observations (Görrissen et al., 1986). Data for the small amino-terminal fragment 1–38H and for the large fragment 1–65H demonstrate the induction of a motionally restricted component on binding either fragment, hence indicating that it is the globular structure of holocytochrome c, rather than the attachment of the heme group, which inhibits the induction of this component.

Discussion

The mitochondrial precursor protein apocytochrome c binds with high affinity to negatively charged phospholipids and mixtures of zwitterionic and negatively charged phospholipids (Rietveld et al., 1986a). Apocytochrome c is a largely unstructured protein in aqueous solution (Fisher et al., 1973). Since peptides derived from apocytochrome c are also largely unstructured (Jordi et al., 1989), it can be expected that reliable information on the importance of the various regions of the apocytochrome c molecule for interaction with lipids can be obtained by studying the individual polypeptide fragments. In this study we report on the influence of the different polypeptides derived from apo- and holocytochrome c on the dynamics of the phospholipids as studied by ESR measurements with spin-labeled phospholipids.

We first discuss primarily the relative importance of the various regions of the apocytochrome c molecule for interaction with lipids and then focus on the role of the covalently attached heme in these interactions. Phospholipids spin-labeled at the 5-position of the *sn*-2 acyl chain give rise to ESR spectra in the presence of the various polypeptides that both are broader and have larger splittings than obtained for the peptide-free model membranes, corresponding to a restriction of motion in the presence of the proteins or peptides. This restriction of motion at the C5 position appears to be a property present over the entire length of the apocytochrome c molecule as no large differences between the various fragments are observed. This effect most probably arises from an increase in the lipid packing density caused primarily by the surface component of the lipid–protein association. In contrast, covalent attachment of the heme to both the N-terminal peptides and the protein results in a decreased effect on the ESR spectra of lipids labeled at the C5 position.

Experiments with phospholipids spin-labeled more toward the terminal methyl end of the chain (at the 12-position of the *sn*-2 acyl chain) demonstrate that both the carboxy- and amino-terminal fragments cause a strong restriction of motion of the spin-label. Interaction with these fragments results in clearly resolved two-component ESR spectra which to some extent resemble those obtained with integral membrane proteins (Marsh, 1985) and indicate deep penetration of the polypeptides into the bilayer.

Discussion of the stoichiometries of the motionally restricted lipids induced by the various fragments cannot be made without reference to the different extents of binding. From the values of the binding stoichiometries (Table I), the different peptide–lipid complexes can be divided into three classes. The first class (fragments 1–38, 39–104, and 81–104) have lipid/protein molar ratios at saturation binding which are considerably less than that for apocytochrome c. The second class (fragments 1–59, 1–65, 1–38H, and 1–65H) exhibit a lower extent of binding and have lipid/protein molar ratios of the same order of or somewhat larger than that for apocytochrome c. The third class corresponds to very low binding and comprises the fragment 66–80, which exhibits no binding, and the fragment 60–104, which has a lipid/peptide molar ratio similar to that for cytochrome c, i.e., considerably greater than that for apocytochrome c.

Of the first class, the complementary fragments 1–38 and 39–104 are particularly noteworthy, since their binding stoichiometries add up to a value close to that for the whole apoprotein. This suggests that the mode of binding of the fragments is very similar to that of the corresponding segments in the intact protein. It is therefore particularly significant that the stoichiometries of the motionally restricted spin-label component induced by these two fragments also sum up to the value for the whole apoprotein. This strongly indicates that some of the residues contributing to the motionally restricted lipid component induced by apocytochrome c are located in the segment 1–38, the remainder being located in the complementary segment 39–104, in a proportion indicated by the relative values of n_b for the corresponding peptide fragments. Unfortunately, data for the fragment complementary to fragment 81–104 are not available. However, the binding stoichiometry of the latter is consistent with it contributing

in a complementary manner to that of the longer fragment 39–104. Assuming this to be the case, the value of n_b for fragment 81–104 suggests that residues in this fragment induce the motional restriction of approximately 1.7 of the 2.9 lipids directly perturbed by the 39–104 fragment. In this interpretation, fragment 81–104 is expected to have a particularly strong capacity for perturbing the lipid chains, as is evidenced also by the large value of A_{max} for the motionally restricted component. This arises because a significant number of membrane-penetrating residues are confined within the relatively short 24-residue segment.

For the second class of peptides, it is more difficult to make such direct comparisons, since the binding stoichiometries do not correlate in this complementary fashion with that of the whole protein. On average, at saturation binding, the lipid coverage is less dense with respect to protein mass for these peptides than for the whole protein. However, the values of n_b for these fragments are very similar to those for the whole protein, with relatively little effect of attachment of the heme group. This indicates a considerable contribution from residues in the range 1–65 to the total motionally restricted lipid component induced by apocytochrome *c*. At least part of this contribution comes from residues in the range 1–38, as discussed above, but the high values of n_b suggest that membrane-penetrating residues of the 39–104 section of the protein that are not contained in the short carboxy-terminal segment 81–104 may be restricted mainly to the 39–65 segment of the protein.

The spectral characteristics of the fluid and motionally restricted components for the carboxy-terminal fragments are particularly interesting. The extent of motional restriction of both lipid components is significantly greater than observed for the other peptides, as judged from the outer hyperfine splitting of the spectral components. In particular, for the small C-terminal peptide residues 81–104, a two-component spectrum is obtained with an outer hyperfine splitting of the restricted lipid component of 59 G, a value close to that typical for integral membrane proteins (Marsh, 1985). The fact that the C-terminal region of the protein has a hydrophobic interaction with the acyl chains is supported by the finding that this part is partially protected against proteolytic digestion after interaction with vesicles (Dumont & Richards, 1984). Furthermore all peptides induce an increase in surface pressure in a phosphatidylserine monolayer, whereas only the C-terminal fragments cause a pressure increase in a phosphatidylcholine monolayer (Jordi et al., 1989). Although hydrophobic cluster analysis of the amino acid sequence of apocytochrome *c* shows that no large potentially membrane spanning stretches are present, the carboxy-terminal region in particular contains small hydrophobic stretches (residues 74–78, 80–85, and 94–98). Circular dichroism experiments showed that apocytochrome *c* forms α-helical structures on binding to vesicles (Walter et al., 1986). Peptide 81–104 also contains a predicted α-helix, residues 88–101 (Rietveld et al., 1985), which partially overlaps with the hydrophobic segment, residues 94–98. Circular dichroism measurements showed that this fragment indeed adopts a partially α-helical conformation in a negatively charged detergent solution (Jordi et al., 1989). Since we demonstrate here that this peptide is able to penetrate deeply into a model membrane of negatively charged phospholipids, an α-helix with its long axis perpendicular to the plane of the bilayer is perhaps the most likely conformation for this part of the protein. Both the size of the helix and the presence of hydrophilic and or charged residues indicate that it is not a typical membrane-spanning α-helix and is therefore probably less firmly inserted in the bilayer. This might facilitate release of the protein from the outer mitochondrial membrane into the mitochondrial intermembrane space.

Circular dichroism measurements and structure prediction programs indicate that a second α-helix, residues 58–69, is formed in the carboxy-terminal part of the protein in the presence of a negatively charged detergent (Jordi et al., 1989). However, the fact that we did not have a peptide fragment containing this entire predicted α-helix without the other C-terminal α-helix complicates interpretation of the peptide–lipid interaction of this part of the molecule. Conformational analysis and circular dichroism data indicate that a third α-helix is formed in the amino-terminal region of the protein, in the presence of a negatively charged detergent (Jordi et al., 1989). The ESR measurements discussed above indicate that this part of the protein also causes a motional restriction at the terminal methyl ends of the fatty acids. We have shown that the amino-terminal and not the carboxy-terminal peptides are able at least partially to translocate across the bilayer in such a way that they can be digested by a protease present at the opposite interface (Jordi et al., 1989). During import of apocytochrome *c* into mitochondria the amino-terminal region of the precursor also has to translocate across the outer mitochondrial membrane because the heme group is covalently attached to cysteine residues 14 and 17 in the intermembrane space.

We now discuss further the effect of the covalent attachment of the heme group at cysteine residues 14 and 17 on the peptide–lipid interaction. In general, the heme attachment results in a decreased effect of the peptides on the ESR spectra for the C5 spin-label positions. Recently, we have reported that the covalent attachment of the heme group decreases the effect of the peptides or protein on the gel-to-fluid transition enthalpy of dielaidoylphosphatidylserine dispersions (Zhou Li-Xin et al., 1988) and causes a reduced penetration of the polypeptides into phosphatidylserine monolayers (Jordi et al., 1989). Neither observation can be explained by a decreased binding of the polypeptides, therefore indicating that the heme-containing peptides and proteins interact less efficiently with model membranes. For all systems, the decrease in membrane-perturbing ability on attachment of the heme is most pronounced for cytochrome *c* and much less for the large amino-terminal fragment 1–65H, demonstrating that it is not the relatively hydrophobic heme group itself, but rather a change in the three-dimensional structure of the protein induced by the heme, which is responsible for the observed differences. Most interestingly, both circular dichroism spectra (Jordi et al., 1989) and protease experiments (W. Jordi, unpublished results) indicate that whereas cytochrome *c* has more secondary structure and a more compact conformation than apocytochrome *c*, covalent attachment of the heme to peptides 1–38 and 1–65 has less effect on the peptide conformation. This probably reflects the fact that the carboxy-terminal region of the amino acid sequence is also involved in interaction with the heme (Dickerson et al., 1971) and is necessary for determining the final cytochrome *c* conformation. These data indicate a correlation between the capacity for perturbing the lipid chain dynamics in model membranes 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 recent review, see Eilers and Schatz (1988)]. Extrapolating from these model membrane studies would imply that the unfolding of a precursor is an essential step in translocation of any protein across a membrane since it allows the protein

to interact efficiently with the membrane lipids.

Registry No. Cytochrome *c*, 9007-43-6; heme, 14875-96-8.

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