Interaction of Spin-Labeled Apocytochrome *c* and Spin-Labeled Cytochrome *c* with Negatively Charged Lipids Studied by Electron Spin Resonance

Margot M. E. Snel,[‡] Ben de Kruijff,[§] and Derek Marsh*,[‡]

Max-Planck-Institut für biophysikalische Chemie, Abteilung Spektroskopie, Postfach 2841, 37018 Göttingen, Germany, and Centre for Biomembranes and Lipid Enzymology, Department of Biochemistry of Membranes, Utrecht University, Padualaan 8, 3584 CH Utrecht, The Netherlands

Received January 3, 1994; Revised Manuscript Received April 7, 1994[⊗]

ABSTRACT: Apocytochrome *c* has been spin-labeled with a nitroxide derivative of maleimide on a cysteine residue at either position 14 or position 17 in the N-terminus. Yeast cytochrome *c* was spin-labeled with the same maleimide derivative on its single free cysteine residue at position 102 in the C-terminus. The ESR spectra of spin-labeled apocytochrome *c* have been characterized in different environments with respect both to the conformation of the protein and to its association with lipid. In buffer, the spectrum of spin-labeled apocytochrome *c* indicates high mobility, characteristic of the unfolded structure of the apoprotein, and that of spin-labeled cytochrome *c* is only slightly less mobile, suggesting that the site labeled is situated at the surface of the folded holoprotein. Upon binding the spin-labeled protein to negatively charged lipid membranes composed of dioleoylphosphatidylglycerol (DOPG), the ESR spectra of apocytochrome *c* evidence a large reduction in the mobility of the spin-label group, as also do those of yeast cytochrome *c*. In the case of apocytochrome *c*, this immobilization most likely arises from both an increase in secondary structure and a partial penetration of the protein into the lipid bilayer, in addition to the electrostatic interaction with the lipid headgroups, whereas for cytochrome *c* the immobilization observed arises primarily from an intimate association with the membrane surface. When the spin-labeled holocytochrome *c* is denatured by heating and is bound to DOPG bilayer membranes, a rather mobile ESR spectrum is observed, which demonstrates that the spin-label is located at the surface of the membrane in this case. The ESR spectra of spin-labeled apocytochrome *c* bound to mixed bilayers of dimyristoylphosphatidylglycerol and dimyristoylphosphatidylcholine (DMPC) consist of both an immobile and a mobile component. The proportion of the mobile component is increased by increasing the mole fraction of the zwitterionic DMPC in the mixed bilayers. The mobile component represents a localization of apocytochrome *c* at the membrane surface, whereas the immobile component most probably represents the penetration of the precursor protein into the membrane interior. The immobile component assigned to membrane penetration of the precursor protein is still present at negatively charged lipid contents comparable to those in the native mitochondrial system. The results are discussed in relation to the conformation of apocytochrome *c*, its interaction with lipid, and the import of the apoprotein into mitochondria.

The precursor of mitochondrial cytochrome *c*, apocytochrome *c*, is synthesized in the cytosol and imported into the mitochondrial intermembrane space, where it functions in the respiratory chain (Hartl et al., 1989). Studies on protein import into mitochondria have shown that apocytochrome *c* follows a unique pathway. In contrast to other mitochondrial precursor proteins, apocytochrome *c* is synthesized without a cleavable N-terminal presequence. Furthermore, neither proteinaceous surface receptors, ATP, nor a membrane potential is required for the import of apocytochrome *c* [for a review, see Stuart and Neupert (1990)]. Studies with model membranes have provided strong indications that apocytochrome *c* is imported via the lipid phase of the mitochondrial outer membrane. These studies with lipid membranes (e.g., Rietveld et al., 1986a; Demel et al., 1989; Jordi et al., 1989a), combined with the mitochondrial import data (Hennig & Neupert, 1981; Nicholson et al., 1987, 1988), have led to a model in which the basic precursor binds preferentially to the anionic lipids of the outer mitochondrial membrane. Subsequently, a translocation-competent apocytochrome *c* is formed which inserts into the membrane and exposes its N-terminus to the cytochrome *c* heme lyase, which is present in the intermembrane space. This latter enzyme attaches a heme group covalently to the cysteines at positions 14 and 17 in the N-terminus, upon which the protein folds into the nontranslocatable holocytochrome *c* that is then released into the intermembrane space.

Information is now available on the protein structure, dynamics, and topology of apocytochrome *c* and of cytochrome *c* in a lipid environment, yet the molecular import mechanism is still by no means elucidated. Circular dichroism (CD)[1] studies have revealed that apocytochrome *c* undergoes a conformational change from random coil in aqueous solution

* To whom correspondence should be addressed.
‡ Max-Planck-Institut für biophysikalische Chemie.
§ Utrecht University.
⊗ Abstract published in *Advance ACS Abstracts,* May 15, 1994.

[1] Abbreviations: BHT, butylated hydroxytoluene; CD, circular dichroism; CW, continuous wave; DMPC, 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine; DMPG, 1,2-dimyristoyl-*sn*-glycero-3-phosphoglycerol; DOPC, 1,2-dioleoyl-*sn*-glycero-3-phosphocholine; DOPG, 1,2-dioleoyl-*sn*-glycero-3-phosphoglycerol; DOPS, 1,2-dioleoyl-*sn*-glycero-3-phosphoserine; DTT, dithiothreitol; ESR, electron spin resonance; FTIR, Fourier transform infrared spectroscopy; HEPES, *N*-(2-hydroxyethyl)-piperazine-*N*′-2-ethanesulfonic acid; 5-MSL, 3-maleimido-2,2,5,5-tetramethylpyrrolidine-*N*-oxyl; 12-Pglycol, dodecylphosphoglycol; photo-CIDNP, photochemically induced dynamic nuclear polarization; NMR, nuclear magnetic resonance; PIPES, piperazine-1,4-bis(2-ethanesulfonic acid); 12-PN, dodecylphosphocholine; SBTI, soybean trypsin inhibitor; SDS, sodium dodecyl sulfate.

0006-2960/94/0433-7146$04.50/0

to a partially α-helical conformation upon binding to negatively charged phospholipids or association with detergents mimicking anionic or zwitterionic phospholipids (Walter et al., 1986; Jordi et al., 1989a; de Jongh & de Kruijff, 1990). The folding state of apocytochrome *c* at these water–lipid interfaces is highly dynamic as shown by a rapid hydrogen–deuterium exchange of protein backbone amide groups monitored by NMR and FTIR (de Jongh et al., 1992; Muga et al., 1991a). The topology of apocytochrome *c* associated with SDS micelles has been investigated by photo-CIDNP ^{1}H NMR (Snel et al., 1991), and it was demonstrated that the aromatic residues, which are distributed over the entire protein, and especially the histidine residues, that are located in the N-terminus of apocytochrome *c*, are located at the interface of the SDS micelle. A surface location for the tryptophan residue (position 59) in apocytochrome *c* bound to detergent micelles and to phosphatidylserine bilayers was also found in time-resolved fluorescence measurements (Vincent & Gallay, 1991). With respect to the holoprotein, NMR and FTIR studies have suggested a loosening or unfolding of the cytochrome *c* tertiary structure upon its interaction with negatively charged lipid surfaces consisting either of cardiolipin (Spooner & Watts, 1991a,b), phosphatidylglycerol (Muga et al., 1991b), or of mixed dodecylphosphoglycol/dodecylphosphocholine micelles (de Jongh et al., 1992), which resulted in a highly dynamic folding state as shown by the rapid hydrogen–deuterium exchange of the protein backbone amide groups.

Electron spin resonance (ESR) spectroscopy has been used to investigate the interaction of apocytochrome *c*, cytochrome *c*, and of fragments derived from these proteins with phosphatidylserine bilayers containing spin-labeled phospholipids (Görrissen et al., 1986; Jordi et al., 1989b). These studies suggested that both terminal regions of apocytochrome *c* penetrate into the lipid bilayer, whereas cytochrome *c* was found not to penetrate at all. The latter work was confined, however, to measurements only of the lipid mobility. Vital information on the effects on the mobility of the protein is currently lacking. This can be provided by ESR measurements on the spin-labeled protein. Horse heart apocytochrome *c* has unique cysteine residues in its N-terminus (positions 14 and 17) to which the heme group is attached in the holoprotein and which provide a convenient site for specific labeling with a maleimide nitroxide derivative. This positions the spin-label exactly in that part of the protein that is thought to be important for membrane translocation. In this work, we present the first results of this type in which it is possible to visualize directly the interaction of a specific part of the apoprotein with the lipid membrane. The behavior of the spin-labeled N-terminus of intact apocytochrome *c* in interaction with negatively charged bilayer membranes consisting of dimyristoylphosphatidylglycerol and mixtures of dimyristoylphosphatidylglycerol (DMPG) and dimyristoylphosphatidylcholine (DMPC), or of dioleoylphosphatidylglycerol (DOPG), has been investigated by ESR spectroscopy. Also the ESR spectral changes associated with the conformational rearrangement which the spin-labeled apoprotein undergoes upon association with detergents or upon dissolving in 2-chloroethanol (Toniolo et al., 1975) were studied. Large and highly significant effects on the spin-labeled protein segments are observed, which are interpreted consistently in terms of the conformational changes taking place in the protein and its penetration into the lipid bilayer. In addition, two modes of binding are found in the lipid mixtures that are characterized by very different mobilities of the spin-labeled protein segments.

Further studies were performed in order to compare the membrane interactions of spin-labeled apocytochrome *c* with those of the holoprotein, cytochrome *c*. The latter, in contrast to apocytochrome *c*, interacts only electrostatically with negatively charged phospholipids (Rietveld et al., 1983). For this comparison, yeast cytochrome *c* was spin-labeled on the single free cysteine residue at position 102 at the C-terminal of the protein, a residue that is not contained in horse heart cytochrome *c*. X-ray diffraction studies have shown that the overall folding of the polypeptide chains of horse heart and yeast cytochrome *c* are very similar and that the cysteine at position 102 in the C-terminal helix (88–103) is directed toward a hydrophobic region in cytochrome *c* (Dickerson et al., 1971; Louie et al., 1988). Chemical modifications of cysteine-102, including spin-labeling, have been shown not to change the electron-transfer activity of the native yeast cytochrome *c* (Drott et al., 1970; Zuniga & Nall, 1983).

The spin-label on the cysteine residue in the C-terminus of yeast holocytochrome *c* also offers, in principle, the possibility to study whether this part of the intact apoprotein penetrates the membrane. The precursor, yeast apocytochrome *c*, could not be used for this purpose, because it contains three free cysteine sulfhydryl groups at positions 14, 17 (to which the heme group is attached in the holoprotein), and 102 that cannot readily be spin-labeled specifically at the C-terminal residue alone. However, it has been reported that upon denaturing horse heart cytochrome *c* by heat treatment, the effects of its interaction with lipid bilayers and monolayers closely resemble those of apocytochrome *c* (Jordi et al., 1990; Demel et al., 1989). Taking advantage of this fact, spin-labeled yeast cytochrome *c* was denatured and used as a model for apocytochrome *c* spin-labeled specifically at the C-terminus. The specific spin-labeling of the cysteines in the N-terminus of apocytochrome *c* and in the C-terminus of yeast cytochrome *c*, in combination with the denaturing of this spin-labeled holocytochrome *c*, therefore allows investigation of the interaction of these two specific parts of the proteins with negatively charged lipid membranes.

EXPERIMENTAL PROCEDURES

Materials. Cytochrome *c* from horse heart (type VI) and from *Saccharomyces cerevisiae* (bakers' yeast) was obtained from Sigma (St. Louis, MO). 3-Maleimido-2,2,5,5-tetramethylpyrrolidine-*N*-oxyl (5-MSL) was obtained from Aldrich (Milwaukee, WI); sodium dodecyl sulfate (SDS) and 2-chloroethanol were from Merck (Darmstadt, Germany). The 2-chloroethanol was freshly distilled before use. DMPG was synthesized from DMPC (Fluka, Buchs, Switzerland) by a headgroup exchange reaction catalyzed by phospholipase D (Comfurius & Zwaal, 1977), and DOPG was obtained from Avanti (Birmingham, AL). The detergents dodecylphosphoglycol (12-Pglycol) and dodecylphosphocholine (12-PN) were a kind gift of Harmen de Jongh and were synthesized as micelle-forming phospholipid analogs as described in de Jongh and de Kruijff (1990). Apocytochrome *c* was prepared by removal of the heme group from horse heart cytochrome *c* according to the method of Fisher et al. (1973) and was stored at −30 °C after extensive dialysis at 4 °C against 10 mM ammonium acetate, pH 5.0, containing 0.01% v/v 2-mercaptoethanol and subsequent lyophilization. All other chemicals were analytical grade or better.

Spin-Labeling of Apocytochrome c. To dissociate any disulfide-bridged dimers present in the preparation, lyophilized apocytochrome *c* was dissolved in 8 M urea containing 25 mM dithiothreitol (DTT) at 2 mg/mL [determined spectro-

photometrically: ϵ_{277} = 0.92 mL·mg^{-1}·cm^{-1} (Stellwagen et al., 1972)] and incubated at room temperature for 1 h. Urea and DTT were removed by dialysis at 4 °C against 10 mM ammonium acetate, pH 5.0, over an 18-h period with three buffer changes. After determination of the protein concentration, typically 1.5 mg/mL, 18 μL of a 5 mM ethanolic 5-MSL maleimide spin-label solution was added per milliliter of apocytochrome c solution, corresponding to 0.7 mol of 5-MSL/mol of apocytochrome c. The mixture was incubated at room temperature for 30 min and subsequently passed over a Sephadex G-25 column equilibrated with 10 mM ammonium acetate, pH 5.0, to remove unreacted spin-label. After lyophilization, the spin-labeled apocytochrome c was stored at −30 °C. Renatured spin-labeled apocytochrome c was obtained by subjecting the protein to the renaturation procedure described by Hennig and Neupert (1983) but using 0.25 M 2-mercaptoethanol instead of DTT. Aliquots of the renatured protein (0.5 and 1.0 mg at a concentration of approximately 1.7 mg/mL) in 10 mM HEPES, 50 mM NaCl, pH 7.0, and 0.01% v/v 2-mercaptoethanol were stored in liquid nitrogen and used only once, immediately after being thawed on ice.

To estimate the amount of 5-MSL incorporated into apocytochrome *c*, the weakly immobilized ESR spectrum of spin-labeled apocytochrome *c* was compared with a free 5-MSL ESR spectrum of known concentration (see forthcoming Figure 2a,b). The number of spins is directly proportional to the product of the amplitude of the first-derivative peak and its peak-to-peak line width squared. Estimation and comparison of these products for the central peak, which is least affected by broadening factors, in both ESR spectra, yielded a labeling level of approximately 0.2 mol of 5-MSL/mol of apocytochrome *c*.

Statistically, this spin-labeling procedure could yield three types of spin-labeled horse heart apocytochrome *c*, namely, singly labeled apocytochrome *c* with the spin-label either at position 14 or at position 17 and doubly labeled apocytochrome *c* with the spin-label at both positions. Formation of the doubly spin-labeled derivative is unlikely because in the preparation less than an equimolar amount of 5-MSL spin-label is added and the degree of labeling that is found finally is not indicative of this. Also, spin–spin interactions between the neighboring spin-labels must be expected for the doubly spin-labeled derivative, and indeed were observed for apocytochrome *c* that contained ~2 mol of spin-label per mole of apoprotein, but not for apocytochrome *c* spin-labeled according to the procedure described above. Therefore, it is concluded that this spin-labeling procedure does not yield appreciable amounts of doubly spin-labeled apocytochromes *c*, but it does yield a mixture of singly spin-labeled apoproteins with the spin-label at either position 14 or position 17.

Spin-Labeling of Yeast Cytochrome c. Thirty milligrams of yeast cytochrome *c* in 5 mL of 10 mM sodium phosphate buffer, pH 7.0, was treated with a 2-fold molar excess of DTT to dissociate any disulfide-bridged dimers present in the cytochrome *c* preparation. The solution was kept under argon and stirred magnetically for 1 h at room temperature. After dialysis at 4 °C against 10 mM sodium phosphate, pH 7.0, to remove DTT, a 1.5-fold molar excess of an ethanolic solution of 5-MSL spin-labeled maleimide was added (final ethanol concentration <1.5%), and the mixture was incubated in the dark for 2 h at room temperature. Subsequently, the solution was adsorbed onto a (carboxymethyl)cellulose column equilibrated with 10 mM sodium phosphate buffer, pH 7.0. The unreacted 5-MSL was washed from the column with 2 column volumes of the equilibrating buffer. The spin-labeled yeast cytochrome *c* was eluted with 0.5 M sodium phosphate buffer, pH 7.0, and dialyzed at 4 °C against 10 mM HEPES/150 mM NaCl, pH 7.0. The spin-labeled protein was kept at 4 °C at a concentration of 0.45 mM, as determined using an extinction coefficient of ϵ_{410} = 106.8 × 10^3 M^{-1}·cm^{-1} (Drott et al., 1970). The optical absorption spectrum of spin-labeled cytochrome *c* parallelled the spectrum of native cytochrome *c* and was in agreement with the spectra published by Drott et al. (1970), suggesting that the protein structure is relatively undisturbed by the presence of the spin-label group. The number of spin-labels attached to yeast cytochrome *c* was estimated as described above for spin-labeled apocytochrome *c*, and yielded a value of ~0.15 mol of 5-MSL/mol of cytochrome *c*.

ESR Sample Preparation. Unless otherwise stated, all experiments and preparations were performed in a buffer containing 10 mM HEPES/50 mM NaCl, pH 7.0. For preparation of samples of spin-labeled apocytochrome *c* (apo *c*-MSL) bound to dispersions of DMPG/DMPC, a dry (mixed) lipid film, consisting of 1 mg of total lipid, was hydrated in buffer and put through 5 cycles of freezing and thawing. One milligram of renatured apo *c*-MSL solution was added, and the samples (1-mL total volume) were incubated at 37 °C for 30 min. The resulting protein–lipid complexes were collected by centrifugation in a Labofuge II (Heraeus, 1000*g*, 10 min) and resuspended in 200 μL of buffer by freezing and thawing (5 cycles). After centrifugation, the pellets were transferred to the 100-μL capillaries used for ESR measurements. The ESR samples were further concentrated by centrifugation (1000*g*, 5 min), and the excess supernatant was removed.

Complexes of apo *c*-MSL with DOPG were prepared as described above, starting with 0.5 mg of DOPG containing 0.1 mol % butylated hydroxytoluene (BHT) to prevent lipid peroxidation. After incubation of the hydrated lipid with an aqueous solution containing 0.5 mg of renatured apo *c*-MSL (total volume 0.5 mL), the lipid–protein complexes were isolated by centrifugation in a Biofuge A (Heraeus, 1400*g*, 10 min), transferred to a 100-μL ESR capillary, and concentrated further by centrifugation (1000*g*, 10 min), and excess supernatant was removed.

Complexes of spin-labeled yeast cytochrome *c* (cyt *c*-MSL) with DOPG were prepared in 10 mM HEPES/150 mM NaCl, pH 7.0, buffer as described above for DOPG/apo *c*-MSL but were incubated for 40 min at 37 °C. The higher concentration of NaCl was used to obtain lipid/protein ratios in the final samples that are comparable to those in the DOPG/apo *c*-MSL samples.

For preparation of complexes of denatured yeast cyt *c*-MSL with DOPG, heat-denatured yeast cyt *c*-MSL was produced by incubating 1.9 mg/mL yeast cyt *c*-MSL in buffer for 1 h at 95 °C. After being cooled, the solution was centrifuged (Biofuge A, 15000*g*, 3 min) to remove any aggregated protein, and 0.5 mg of the denatured protein was then added to 0.4 mg of DOPG hydrated in buffer. ESR samples were pelletted in capillaries as described for the nondenatured yeast cyt *c*-MSL/DOPG.

These methods of sample preparation, involving centrifugation and removal of excess supernatant, are designed to minimize the content of any unbound protein. In most cases, any protein free in solution was distinguishable in the ESR spectrum. For samples with apocytochrome *c*, all the protein was bound; for samples with cytochrome *c*, the major part of the protein was bound, but a free component was also detected (see Results).

After the ESR measurements, the membrane pellets were dissolved in 25 μL of 1 M NaOH for determining the lipid and protein contents. The phospholipid concentration was determined by the method of Eibl and Lands (1969). Protein assays were performed according to the modified method of Lowry (Peterson, 1977) with bovine serum albumin as standard.

ESR Spectroscopy. ESR measurements were performed on a Varian E-12 Century Line 9-GHz ESR spectrometer. Sample capillaries were centered in a standard 4-mm quartz tube containing light silicone oil for thermal stability; temperature was regulated with a pure nitrogen gas flow system. Conventional, in-phase, absorption spectra were recorded with a modulation frequency of 100 kHz and a modulation amplitude of 1.25-G peak-to-peak. The total scan width was 100 G. Continuous-wave power saturation studies were performed as described by Snel and Marsh (1993), with instrumental calibrations and data analysis as given in the same reference.

Circular Dichroism. Spectra were acquired at room temperature on a Jasco-600 spectropolarimeter, using a 0.2-mm path-length quartz cell while flushing the cuvette chamber with nitrogen gas. For each sample, one to four CD spectra were accumulated over the range from 260 to 190 nm. After subtraction of the spectra from the protein-free control sample, the data were stored with a resolution of 0.2 nm. Spectral analysis was performed essentially as described by de Jongh and de Kruijff (1990).

Translocation Assay. The translocation of unlabeled and spin-labeled apocytochrome *c* across lipid bilayer membranes consisting of dioleoylphosphatidylserine (DOPS) and dioleoylphosphatidylcholine (DOPC) was assayed essentially as described by Jordi et al. (1989a). DOPS/DOPC (1/1 mol/mol) large unilamellar vesicles with trypsin enclosed were prepared by hydrating the dry lipid film (15 μmol of total lipid) with 1.5 mL of buffer that contained 10 mM PIPES, 50 mM NaCl, pH 7.0, and 2 mg/mL trypsin at 0 °C. Subsequently, the lipid suspension was extruded (10 times) through a polycarbonate Millipore filter (0.4-μm pore size), centrifuged for 25 min at 35000*g* (4 °C), and washed twice with buffer without trypsin to remove the trypsin outside the vesicles. The phospholipid content was determined (Rouser et al., 1970), and 1 μg of soybean trypsin inhibitor (SBTI)/nmol of lipid phosphate (P_i) was added from a 25 mg/mL SBTI stock solution to inhibit any residual trypsin present.

The translocation assays were started by adding 66 μg of apocytochrome *c* or spin-labeled apocytochrome *c* (renatured in 10 mM PIPES/50 mM NaCl, pH 7.0) to the trypsin-containing vesicles (625 nmol of P_i) at 0 °C in a total volume of 300 μL. Samples were drawn immediately after mixing the lipid vesicles with the apoproteins (t = 0 min) and after incubation at 30 °C for 120 min. Prior to electrophoresis, the protein samples were denatured by mixing them with an equal volume of a 4% (w/v) SDS sample buffer, followed by heating at 95 °C for 10 min. Samples were run on SDS–polyacrylamide slab gels (15% w/v) according to the method of Laemmli (1970). Gels were stained for protein with Coomassie blue R-250, and the amount of unproteolyzed apocytochrome *c* normalized to the amount of SBTI added was determined by scanning the gel with a Vitatron densitometer.

RESULTS

Characterization of Spin-Labeled Apocytochrome c. Apocytochrome *c* in aqueous solution has a circular dichroism spectrum typical for a protein in an unordered conformation, whereas the protein dissolved in 2-chloroethanol displays a CD spectrum characteristic of high α-helical content (Stellwagen et al., 1972; Fisher et al., 1973; Rietveld et al., 1985; Toniolo et al., 1975). To check whether attachment of the spin-label affects the folding properties of apocytochrome *c* in these solvents, CD measurements were performed which are shown in Figure 1. Clearly, no significant differences exist between the labeled and unlabeled proteins in either solvent, which was confirmed by a quantitative spectral analysis (data not shown). The CD spectra are in agreement with those reported in the studies cited above, and indicate that the spin-labeling does not affect the conformational properties of the protein.

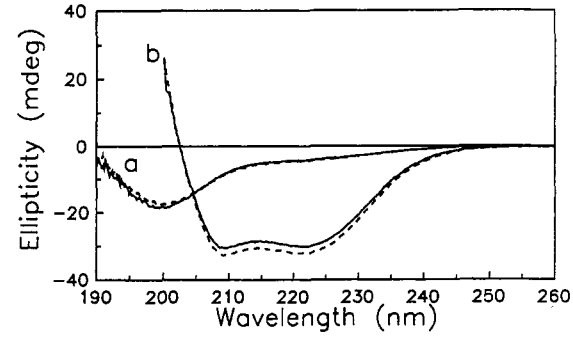

FIGURE 1: CD spectra of 50 μM apocytochrome *c* (solid line) and spin-labeled apocytochrome *c* (dashed line) in (a) 10 mM PIPES/50 mM NaCl, pH 7.0, and (b) 2-chloroethanol, recorded at room temperature.

Apocytochrome *c* has been shown (partially) to cross negatively charged model membranes spontaneously (Rietveld et al., 1986a), a property that is specific for apocytochrome *c* and that is not shared by the mitochondrial precursor protein consisting of the presequence of yeast cytochrome oxidase subunit IV fused to mouse dihydrofolate reductase (Jordi et al., 1992). The alternative, mitochondrial translocation assay for apocytochrome *c* (Nicholson et al., 1987) involves attachment of the heme group and therefore cannot be applied in the present case. Therefore, to verify the capability of spin-labeled apocytochrome *c* to cross the membrane, translocation experiments were performed with lipid bilayers. Both labeled and unlabeled apocytochromes *c* were found to translocate across DOPS/DOPC (1/1 mol/mol) lipid bilayers. After 2 h, 47% of the unlabeled and 43% of the spin-labeled apocytochromes *c*, relative to the amount added, were digested by the vesicle-enclosed trypsin, in full agreement with the results of Jordi et al. (1989a). Even spin-labeled apocytochrome *c* that was doubly labeled (~2 mol of spin-label/mol of apocytochrome *c*), and for which spin–spin interactions were observed in the ESR spectra, was able to translocate across the lipid bilayers to the same extent (43% digested by trypsin).

It therefore can be concluded that the attachment of the spin-label does not affect the conformational and translocation characteristics of apocytochrome *c*. Thus it can be assumed that this derivative reliably reflects the properties of the native protein upon interaction with lipids.

Spin-Labeled Apocytochrome c in Different Environments. To obtain insight into the behavior of spin-labeled apocytochrome *c* in different environments, ESR spectra were recorded of the spin-labeled apoprotein in buffer alone and in the presence of detergents that form a water–lipid interface, and of spin-labeled apocytochrome *c* dissolved in 2-chloroethanol. These spectra are given in Figure 2. Comparison of the ESR

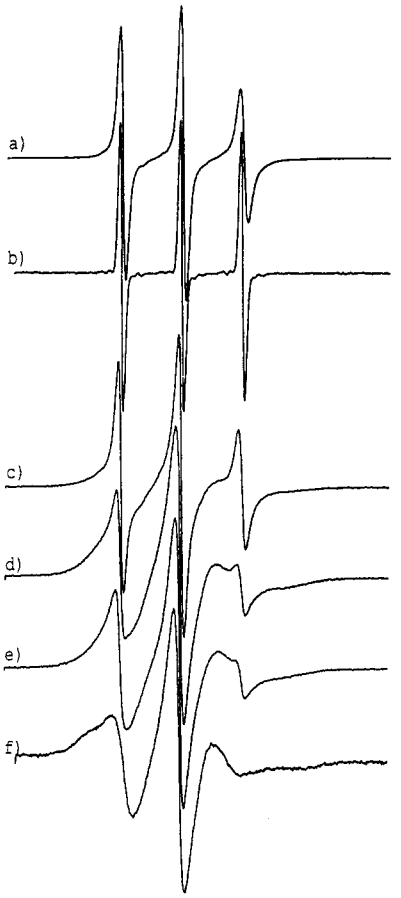

FIGURE 2: ESR spectra recorded at 30 °C of (a) 0.16 mM spin-labeled horse heart apocytochrome *c* (apo *c*-MSL) in buffer alone, (b) 31 μM maleimide spin-label (5-MSL) in 10 mM HEPES, pH 7.0, (c) apo *c*-MSL at a concentration of 0.12 mM in the presence of 12 mM dodecylphosphocholine/dodecylphosphoglycol (9/1 mol/mol), (d) apo *c*-MSL at a concentration of 0.12 mM in the presence of 12 mM dodecylphosphoglycol, (e) apo *c*-MSL at a concentration of 0.12 mM in the presence of 12 mM SDS, and (f) apo *c*-MSL dissolved at a concentration of 0.5 mM in 2-chloroethanol. Total scan width is 100 G.

spectra of spin-labeled apocytochrome *c* (Figure 2a) and the free 5-MSL spin-label (Figure 2b) in buffer shows a differential increase in the line widths of the spectrum for the spin-labeled protein, which reflects the increase in rotational correlation time from ~0.04 ns for 5-MSL to ~0.6 ns on covalent binding of the spin-label to apocytochrome *c*. The spectra of spin-labeled apocytochrome *c* in the presence of detergents (Figure 2c–e) consist essentially of a superposition of a mobile component similar to that of the free protein and a component of intermediate mobility (visible in the spectra as an increase in line broadening and hyperfine anisotropy), the relative proportion of which depends on the particular detergent. Addition of a mixture of the zwitterionic dodecylphosphocholine (12-PN) and the anionic dodecylphosphoglycol (12-Pglycol), 9/1 mol/mol, induces a limited amount of the second component (Figure 2c), whereas the anionic detergents 12-Pglycol alone (Figure 2d) and SDS (Figure 2e) result in a much greater proportion of this less mobile spin-labeled apocytochrome *c* spectrum. At the same temperature (30 °C), the protein in 2-chloroethanol evidences an even greater degree of immobilization of the spin-label (Figure 2f) than that induced by binding to the detergent micelles. This might be explained, at least partially, by an extensive aggregation of apocytochrome *c* in 2-chloroethanol, as observed by H. de Jongh and B. de Kruijff (personal communication) in centrifugation experiments in which the apoprotein could be

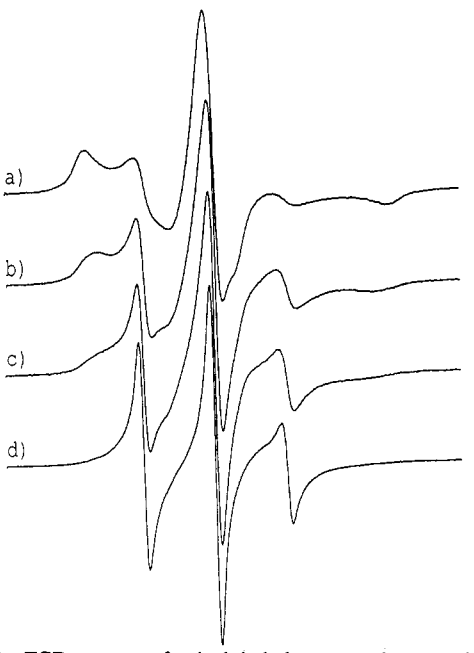

FIGURE 3: ESR spectra of spin-labeled apocytochrome *c* bound to DMPG bilayers at a lipid/protein ratio of 13 mol/mol in 10 mM HEPES/50 mM NaCl, pH 7.0, recorded at different temperatures: (a) 5 °C, (b) 25 °C, (c) 45 °C, (d) 70 °C. Total scan width is 100 G.

pelleted under these conditions, in addition to the conformational effects evident in the CD spectra (cf. Figure 1).

ESR Spectra of Spin-Labeled Apocytochrome c in Lipid Complexes. The ESR spectra of spin-labeled apocytochrome *c* bound to negatively charged DMPG bilayers at different temperatures are shown in Figure 3. Clearly, a large reduction in mobility of the spin-label takes place on binding of the protein to negatively charged lipid membranes (cf. Figure 2a). Two components can be distinguished in the spectra of the lipid-bound protein: an "immobile" component, which lies in the slow-motion regime of spin-label ESR spectroscopy and is predominant at low temperatures, and a three-line, "mobile" component, which lies in the faster motional regime of ESR spectroscopy and is clearest in appearance at the higher temperatures. At low temperature, the major part of the spectrum exhibits a large outer hyperfine splitting and relatively small line widths of both the low- and high-field resonance lines, which is characteristic for spin-labels in the slow-motion regime that are strongly immobilized [cf. Freed (1976)]. Increasing the temperature enhances the appearance of the second component in the ESR spectra (i.e., the more weakly immobilized spin-labels which have little or no hyperfine anisotropy) and, as evidenced by the reduction in outer hyperfine splitting and increase in line widths, gives rise to a progressive increase in mobility of the less mobile component, which is characteristic for spin-labels that nonetheless remain in the slow-motion regime of conventional nitroxide ESR spectroscopy [cf. Freed (1976)].

Muga et al. (1991a) have demonstrated by Fourier transform infrared spectroscopy that the interaction of apocytochrome *c* with DMPG is modulated strongly by the zwitterionic lipid DMPC, an effect that was found previously for the interaction of apocytochrome *c* with other negatively charged phospholipids (Rietveld et al., 1986a). ESR spectra of spin-labeled apocytochrome *c* bound to mixed DMPG/DMPC bilayers of different compositions are presented in Figure 4. All of these spectra also consist of two components. At lower mole fractions of DMPC (Figure 4a–c), the ESR

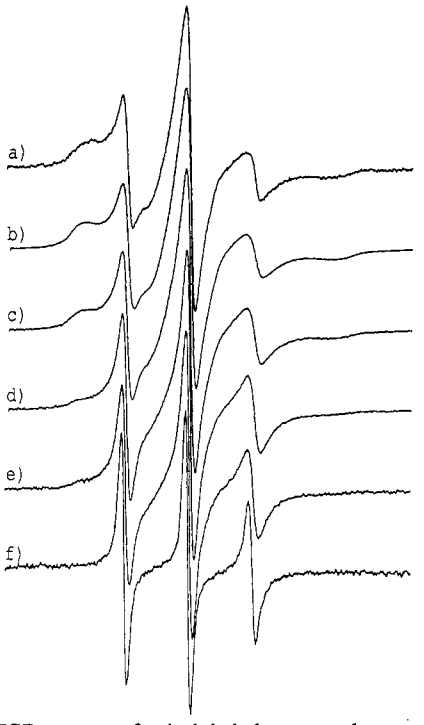

FIGURE 4: ESR spectra of spin-labeled apocytochrome *c* bound to bilayers composed of mixtures of DMPG with DMPC in 10 mM HEPES/50 mM NaCl, pH 7.0 (a–e), and of spin-labeled apocytochrome *c* alone in the same buffer solution (f), recorded at 30 °C. The mole fraction of DMPC in the mixed bilayers is (a) 0, (b) 0.25, (c) 0.5, (d) 0.75, and (e) 0.85. Corresponding lipid/protein ratios of these samples are given in Figure 5A. Total scan width is 100 G.

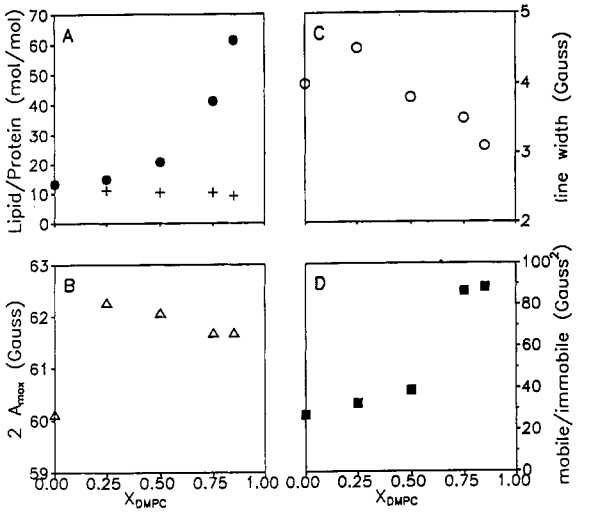

FIGURE 5: Dependence of protein binding and parameters of the spin-label ESR spectra given in Figure 4 on the mole fraction of DMPC (X_{DMPC}) in mixed bilayers of DMPG and DMPC to which spin-labeled apocytochrome *c* is bound. (A) Amount of spin-labeled apocytochrome *c* bound to the lipid mixtures expressed as (●) total molar ratio of lipid (DMPG + DMPC) to protein bound and as (+) number of DMPG molecules per bound apocytochrome *c* (±10%). Lipid–protein complexes were formed by adding 1 mg of protein to 1 of mg total lipid (DMPG + DMPC) and then isolating by centrifugation and washing (see Experimental Procedures). (B) Outer hyperfine splitting of the immobile spin-label component ($2A_{max}$ ± 0.2 G). (C) High-field peak-to-peak line width of the mobile spin-label component (±0.15 G). (D) Ratio of the amount of mobile to immobile spin-label component determined from the high-field and low-field line heights, respectively, where the former is corrected for differences in line width by multiplying by the square of its peak-to-peak line width.

spectra are composed mainly of the broader, immobile component. However, at higher mole fractions of DMPC, the second, more mobile component appears more dominantly in the spectra (Figure 4d,e). For comparison, the ESR spectrum of spin-labeled apocytochrome *c* alone in buffer is shown in Figure 4f. Attempts to subtract this solution spectrum from the spectra of the lipid-bound samples (not shown) indicate that the mobile component in the lipid-bound spectra is not unbound spin-labeled apocytochrome *c*, but is spin-labeled apocytochrome *c* bound in a different conformation. (Even the spectrum from nonrenatured apocytochrome *c*, that is not pretreated with urea/2-mercaptoethanol and is known to be aggregated in solution, was sharper than this membrane-bound component.)

Figure 5 summarizes the dependence on DMPC content in the membrane of several quantitative features of the ESR spectra of the bound protein that are shown in Figure 4 and relates them to the extent of protein binding to the lipid in the complexes. All samples were prepared by adding a constant amount of protein to a fixed amount of total lipid (1 mg/mg), and the lipid–protein complexes were then isolated by centrifugation and washing (see Experimental Procedures). Figure 5A shows that the total amount of lipid (DMPG + DMPC) per protein bound increases with the mole fraction of DMPC (X_{DMPC}) in the complexes with the different lipid mixtures. However, the number of DMPG molecules per spin-labeled apocytochrome *c* bound merely decreases a little from 13 to 9 when going from complexes with pure DMPG to those with the mixed-lipid system with $X_{DMPC} = 0.85$. The outer hyperfine splitting of the immobile component (Figure 5B) and the line width of the mobile component (Figure 5C) in the spectra of the bound spin-labeled protein are greater at $X_{DMPC} = 0.25$ than they are for complexes with DMPG alone, corresponding to a decrease in mobility of both components. Most probably, this is due to a modulatory effect of DMPC by which the separation of the negatively charged DMPG headgroups is better able to adapt to the optimum requirement for the protein, resulting in this case in a more closely packed membrane. Increasing the mole fraction of DMPC further from 0.25 to 0.85 causes a progressive decrease both in the outer hyperfine splitting of the immobile spectral component (Figure 5B) and in the line width of the mobile spectral component (Figure 5C). This indicates a higher rotational mobility of both spin-label components with increasing X_{DMPC}, correlating with the decreased net binding of apocytochrome *c* evidenced by the higher lipid/protein ratios of these samples (Figure 5A). In the same region, the ratio of the amount of the mobile to the immobile component increases, as is shown in Figure 5D. The change in the ratio between the two components points to a change in the binding conformation that spin-labeled apocytochrome *c* prefers at higher lipid to protein ratios. It will be noted that it is improbable that the two spectral components correspond also to an inequivalence between the two sites of labeling because they do not have a fixed relative stoichiometry.

The temperature dependences of the outer hyperfine splitting for spin-labeled apocytochrome *c* in different environments are summarized in Figure 6. Spin-labeled apocytochrome *c* bound to DMPG shows a large outer hyperfine splitting at low temperatures, which then decreases rather steeply at higher temperatures. This large outer hyperfine splitting at low temperatures is comparable to those observed for integral membrane proteins such as the calcium ATPase (68 G; Lewis & Thomas, 1986) and the sodium/potassium ATPase (66.5–67.5 G; Esmann et al., 1989) spin-labeled with maleimide derivatives. In order to compare the effects on spin-labeled apocytochrome *c* of saturated and unsaturated chains for

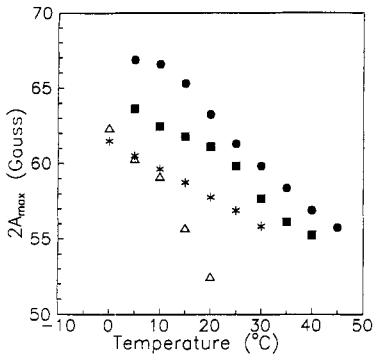

FIGURE 6: Temperature dependence of the outer hyperfine splitting ($2A_{max}$) for spin-labeled apocytochrome *c*: (●) bound to DMPG bilayers; (■) bound to DOPG bilayers; (△) in the presence of SDS (1/100 mol/mol); and (*) dissolved in 2-chloroethanol at a concentration of 0.5 mM. For experimental conditions, see Experimental Procedures.

negatively charged lipids with an identical headgroup, the temperature dependence of the outer hyperfine splitting for the spin-labeled apoprotein bound to DOPG is also included in Figure 6. In the region above the phase transition temperature of DMPG (T_t = 23 °C), the temperature dependence parallels that observed for the complex with DMPG. However, at lower temperatures, the outer hyperfine splittings obtained with the DOPG complex are smaller than those from the DMPG complex, since the former is still fully in the liquid-crystalline state (T_t = −20 °C). It can be concluded that the temperature behavior of spin-labeled apocytochrome *c* when it is bound either to the saturated DMPG or to the unsaturated DOPG is not essentially different. At low temperatures, the values of the outer hyperfine splitting for spin-labeled apocytochrome *c* in the presence of SDS and dissolved in 2-chloroethanol are rather similar. At higher temperatures, the outer hyperfine splitting decreases more steeply for spin-labeled apocytochrome *c* associated with SDS compared with that for the protein bound to negatively charged lipids. On the basis of the location in the headgroup region of the SDS micelle established for the N-terminal histidine residues (positions 18, 26, and 33) in apocytochrome *c* (Snel et al., 1991), a similar location for the spin-label attached to a cysteine residue at position 14 or 17 might be expected. This could therefore explain the higher rotational mobility at the higher temperatures in the complex with SDS. On the other hand, for spin-labeled apocytochrome *c* dissolved in 2-chloroethanol, the hyperfine splitting decreases less steeply with increasing temperature, which might be explained by an extensive aggregation of apocytochrome *c* in this solvent as is stated above.

ESR Spectra of Spin-Labeled Yeast Cytochrome c in Lipid Complexes. The ESR spectrum of spin-labeled yeast cytochrome *c* in buffer is shown in Figure 7a. The spectrum indicates a reduction in mobility (with a rotational correlation time of $\tau_r \sim 0.9$ ns) relative to that of 5-MSL free in buffer (cf. Figure 2b), as a result of the covalent attachment of the spin-label. The spectrum and the rotational correlation time are also similar to those of yeast cytochrome *c* spin-labeled with a bromoacetamide derivative of the same nitroxide group as used in this study ($\tau_r \sim 1$ ns; Drott et al., 1970). Binding to negatively charged lipid bilayers produces a large change in the spectrum of the spin-labeled cytochrome *c*. Figure 7b,c shows the ESR spectra of spin-labeled yeast cytochrome *c* bound to negatively charged DOPG bilayers at two different lipid to protein ratios. As in the case of spin-labeled apocytochrome *c*, two components can be distinguished in these spectra: a broader, immobile component and a narrower,

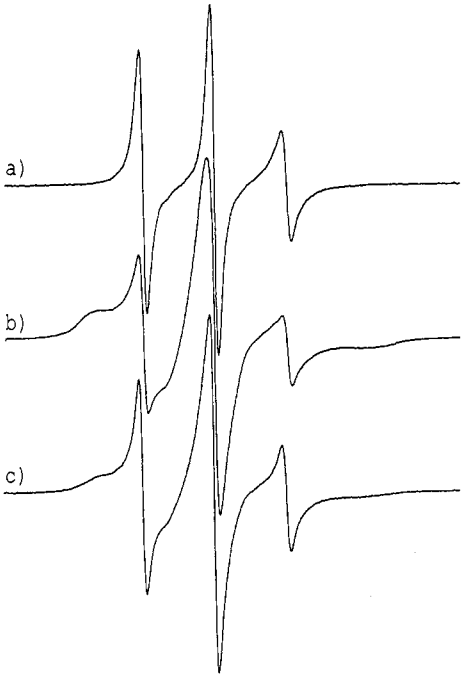

FIGURE 7: ESR spectra of spin-labeled yeast cytochrome *c* recorded at 30 °C. (a) At a concentration of 0.45 mM in 10 mM HEPES/150 mM NaCl, pH 7.0, buffer alone. (b) Bound to DOPG bilayers at a lipid/protein ratio of 13 mol/mol. (c) Bound to DOPG bilayers at a lipid/protein ratio of 21 mol/mol. Total scan width is 100 G.

mobile component. However, in this case, subtraction of the spectrum of the labeled protein in buffer from the spectra of the lipid-bound protein largely removes the mobile component (data not shown), demonstrating that this mobile component mostly represents unbound protein, which probably is released during the preparation of the ESR sample. Further, a reduction of approximately 2 G in the outer hyperfine splitting of the immobile component is observed in the spectra of spin-labeled yeast cytochrome *c* bound at higher lipid to protein ratios, indicating a higher rotational mobility of the labeled group at lower surface densities of protein.

To obtain further information on the origin of the immobilization of the spin-labeled cytochrome *c* segment on binding to lipid, the accessibility of the spin-labeled group to the paramagnetic relaxation agents molecular oxygen and chromium oxalate was probed with CW saturation ESR using the relaxation enhancement parameters defined by Snel and Marsh (1993). This accessibility parameter for spin-labeled cytochrome *c* bound to DOPG dispersions saturated with oxygen was 17×10^{12} s^{-2} G^{-1}, which is comparable to the value of 25×10^{12} s^{-2}G^{-1} found for a spin-label attached to the lipid headgroups in DOPG dispersions with bound cytochrome *c*, and much smaller than the corresponding values for spin-labels attached to the lipid hydrocarbon chains [cf. Snel and Marsh (1993)]. The accessibility parameter for bound cytochrome *c* in 1 mM chromium oxalate was considerably smaller and had a value of 4×10^{12} s^{-2} G^{-1} that is similar to that obtained for a spin-label attached to the phospholipid headgroup in bilayer dispersions of zwitterionic lipids (Snel & Marsh, 1993).

The ESR spectra of spin-labeled heat-denatured yeast cytochrome *c* in buffer and bound to DOPG are given in Figure 8a,b. The change in the spectrum on binding to lipid is considerably less than that for spin-labeled apocytochrome *c* (Figure 8c). Clearly the C-terminal of heat-denatured cytochrome *c* and the N-terminal of apocytochrome *c* behave differently on binding to DOPG bilayers.

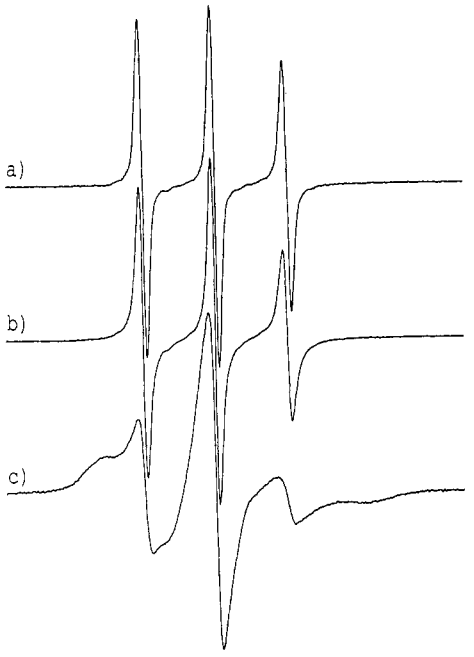

FIGURE 8: ESR spectra of heat-denatured, spin-labeled yeast cytochrome *c* recorded at 30 °C. (a) At a concentration of 0.15 mM in buffer alone. (b) Bound to DOPG bilayers at a lipid/protein ratio of 14.5 mol/mol. (c) ESR spectrum of spin-labeled apocytochrome *c* bound to DOPG at a lipid/protein ratio of 14.5 mol/mol. Total scan width is 100 G.

DISCUSSION

The results presented here clearly indicate that the ESR spectra of both apocytochrome *c* spin-labeled in the N-terminal region and holocytochrome *c* spin-labeled in the C-terminal region are extremely sensitive to the binding of these proteins to negatively charged lipid membranes. In addition, the ESR spectra of the apoprotein are generally sensitive to environment, possibly reflecting conformational changes in the protein. Further, the experiments with mixed lipid systems show that distinct modes of binding can be differentiated from the ESR spectra of the spin-labeled apoprotein. These different aspects are discussed separately below.

Apocytochrome c. The most striking observation with regard to the ESR spectra of N-terminal spin-labeled apocytochrome *c* is that they are characteristic of a high degree of immobilization when the protein is bound to negatively charged (mixed) model membranes. In principle, this immobilization could be due to a combination of factors: (1) electrostatic binding of the protein to the acidic lipids, (2) a conformational rearrangement of the protein upon binding, (3) aggregation of apocytochrome *c* on the membrane surface, and (4) penetration of the precursor into the hydrophobic bilayer. In the following, these four possibilities and their mutual interdependence will be discussed.

CD studies have demonstrated that apocytochrome *c* changes from its largely unordered conformation in aqueous solution to a partially α-helical conformation upon binding to negatively charged phospholipids, where the latter is mimicked by addition of (a mixture of) anionic (and zwitterionic) detergents (Walter et al., 1986; Jordi et al., 1989a; de Jongh & de Kruijff, 1990). The conformational rearrangements in apocytochrome *c* upon addition of various detergents have been quantitated, but they could be determined only with difficulty from CD studies for aggregated apocytochrome *c*–phospholipid complexes. Therefore, the ESR spectra of spin-labeled apocytochrome *c* in detergent systems were investigated to determine the possible effect of known conformational changes and thereby calibrate the changes observed in the ESR spectra of spin-labeled apocytochrome *c* when bound to negatively charged phospholipid bilayer membranes.

The N-terminus is one of the regions that is predicted to adopt an α-helical structure (Jordi et al., 1989a), presumably from residues 3 to 18. The extent of this predicted N-terminal helix seems quite likely because identification of secondary structural elements in horse heart cytochrome *c* by NMR (Wand et al., 1989; Feng et al., 1989) and by X-ray diffraction (Dickerson et al., 1971) has revealed an α-helical conformation for residues 3–14 and a distorted helical turn for residues 14–18. The latter most probably is caused by the heme group that is attached to cysteine residues 14 and 17 and therefore may be free to adopt a more regular helical structure in the apoprotein. Although the spin-labeling procedure used does not yield one specifically labeled derivative, but a mixture of singly labeled apocytochromes *c* with the spin-label at position 14 or 17, both spin-labeled residues are located in the N-terminal helix predicted for apocytochrome *c*, and therefore both of them are assumed to report on the behavior of the putative N-terminal helix.

In the spin-labeled apocytochrome *c*–detergent mixtures, at least 38% of the α-helix is induced (de Jongh & de Kruijff, 1990; de Jongh et al., 1992), suggesting that all of them contain the N-terminal helix. The changes in mobility evidenced by the ESR spectra of the apoprotein spin-labeled in the N-terminal region add further support to this view. It has been found, for instance, that for a small (17-residue) spin-labeled peptide the segmental mobility of the spin-label is suppressed in the α-helical conformation, as compared with the random-coil structure, the residual rotational motion corresponding in the former case solely to that of the entire helical rod (Todd & Millhauser, 1991). The (electrostatic) association with anionic detergents leading to formation of the N-terminal helix in apocytochrome *c* also is accompanied by a decrease in the mobility of the spin-label, which is observed in the ESR spectra (Figure 2) as a component with a mobility intermediate between that of the protein free in solution (Figure 2a) and that of the protein bound to negatively charged lipid bilayers (Figure 3b). This intermediate degree of mobility is due most probably to the looser structure of the apocytochrome *c*–detergent complex, as compared to the apocytochrome *c*–phospholipid complexes. The proportion of this component of intermediate mobility appearing in the spectra is modulated by the presence of the zwitterionic detergent. Similar ESR spectra are observed for spin-labeled apocytochrome *c* associated with the anionic detergents SDS and 12-Pglycol; although a different amount of α-helix is induced in the protein by these two detergents, it is likely that the N-terminal region containing the spin-label is α-helical in both cases. The above implies that the secondary structural rearrangement in the protein and the electrostatic association with the membrane which induces it can contribute to the high degree of immobilization observed in the ESR spectra of spin-labeled apocytochrome *c* bound to negatively charged membranes.

Previously, Rietveld et al. (1986a) have shown that some oligomerization of apocytochrome *c* takes place on binding to bilayer membranes consisting of negatively charged phosphatidylserine and zwitterionic phosphatidylcholine lipids. This oligomerization, i.e., the formation of intermolecular disulfide-bridges between the cysteines of apocytochrome *c* monomers, depends on the phosphatidylserine content of the vesicles, being higher for larger proportions of phosphatidylserine, but it does not affect the kinetics of apocytochrome *c* translocation across

these membranes. Recently, aggregation of apocytochrome *c* on the surface of DMPG bilayer membranes was suggested by Muga et al. (1991a), and also conditions were described under which no aggregation was observed. In an ESR experiment performed under these latter conditions, i.e., with small unilamellar vesicles of DMPG at a lipid/protein ratio of 100/1 mol/mol, an immobilized component in the ESR spectrum of spin-labeled apocytochrome *c* was observed that was comparable to that found in the spectra of Figure 4 (data not shown). This implies that the strong immobilization found on binding apocytochrome *c* to negatively charged phospholipids is caused mainly by factors other than aggregation. (Additionally, it will be noted that one of the two –SH groups of horse heart apocytochrome *c* is blocked in the ESR experiments.)

Penetration of the precursor protein into negatively charged lipid bilayers has been indicated previously by the rather specific effect that it has on the mobility of spin-labeled lipid chains (Görrissen et al., 1986; Jordi et al., 1989b). Also, apocytochrome *c* has a marked ability, relative to cytochrome *c*, to penetrate lipid monolayers (Jordi et al., 1989a). More recently, penetration of the spin-labeled segment of apocytochrome *c* into phosphatidylglycerol bilayers has been revealed by spin–spin interactions with spin-labeled lipid chains (Snel et al., 1993), and by the interaction with lipid-permeant relaxation agents relative to that with lipid-impermeant relaxation agents (Snel, de Kruijff, and Marsh, unpublished results). This penetration into the highly viscous lipid chain environment will cause a restriction in mobility of the spin-label group that is responsible, at least in part, for the broadening and increase in hyperfine splitting of the ESR spectrum from spin-labeled apocytochrome *c* bound to negatively charged lipid bilayers. It is unlikely that this effect by itself is sufficient to account wholely for the spectral effects observed on binding to lipid. From the discussion given above, it is probable that a combination of factors contributes to the large immobilization observed on binding spin-labeled apocytochrome *c* to negatively charged lipid membranes, namely, electrostatic binding, structural rearrangement, and penetration of the precursor protein. In particular, the association with the membrane is necessary to induce (rigid) secondary structure in the protein which in turn is then likely to enhance the reduction in mobility arising from interaction with the membrane.

Another extremely significant observation is the presence of two components of different mobility in the ESR spectra of spin-labeled apocytochrome *c* bound to negatively charged mixed lipid bilayers (Figure 4). As stated above, the mobile component is not unbound protein, but most probably apocytochrome *c* bound differently to the membrane. Quenching of the tryptophan fluorescence in apocytochrome *c* with brominated lipids in vesicular systems indicated that apocytochrome *c* can adopt different orientations in a vesicle-associated form (Berkhout et al., 1987). On the basis of hydrogen–deuterium exchange of backbone amide groups in lipid-associated apocytochrome *c*, the membrane location of apocytochrome *c* was suggested recently to be the average of a rather dynamic situation in which there is a rapid exchange of two populations of the protein, one in the bilayer interior and one in a water-accessible environment on the membrane surface (Muga et al., 1991a). Also, the two components of different mobility observed in the ESR spectra can be explained by two such populations, where the membrane-penetrant population is represented by the immobile component and the membrane surface population by the mobile component.

The dependence of the extent of binding and the spectral parameters of the spin-labeled apocytochrome *c* on the composition of the mixed lipid bilayers reveals several interesting features of the mode of protein–lipid association. The stoichiometry of bound protein with respect to the negatively charged lipid component (i.e., DMPG) remains fairly constant over a wide range of total lipid/protein ratio (Figure 5A). This suggests that negatively charged lipids are recruited to the protein in the mixed bilayers. A selective interaction of apocytochrome *c* with negatively charged lipids has been observed previously in mixed lipid systems (Rietveld et al., 1986b). In the absence of DMPC, the number of DMPG lipids per bound apocytochrome *c* is somewhat higher than at high DMPC contents. This might indicate that, without DMPC "spacer" lipids, not all DMPG headgroups are able to interact optimally with the basic residues on the protein. Addition of DMPC then relieves this constraint and results initially in a more effective interaction between the lipid and the protein, as evidenced by the further decrease in mobility of the spin-labeled protein segments at $X_{DMPC} = 0.25$ (see Figure 5B,C). At high DMPC contents, the stoichiometry of negatively charged lipids with respect to protein is approximately 9, which corresponds to the net positive charge on the protein, suggesting that this might be a minimal requirement for protein binding. The slight decrease in the negatively charged lipid stoichiometry with increasing DMPC content may result from a finite selectivity for this component [cf. Sankaram et al. (1989)] and is reflected in the increase in mobility of the spin-labeled protein at $X_{DMPC} > 0.25$ (cf. Figure 5B,C). Lastly, the ratio of the mobile to immobile spin-labeled protein components increases only slightly up to $X_{DMPC} = 0.5$, but beyond this increases rapidly with additional DMPC content. It appears that above equimolar contents of DMPC the more peripheral mode of association of apocytochrome *c* with the lipid bilayers becomes more favored, relative to that in which the precursor protein penetrates the membrane.

The outer mitochondrial membrane of rat liver contains approximately 15% lipids that are negatively charged (Hovius et al., 1990). Their distribution across the outer membrane is asymmetric: cardiolipin is found exclusively in the outer leaflet, whereas the inner leaflet is enriched in phosphatidylserine and phosphatidylinositol (Hovius et al., 1993). Apocytochrome *c* is thought to bind to these acidic lipids in the outer mitochondrial membrane. At the higher mole fractions of DMPC (i.e., 15 and 25 mol % of DMPG), the ratio of the mobile to the immobile component in the ESR spectra is seen to increase steeply (Figure 5D). This suggests that, at anionic lipid contents comparable to those in the *in vivo* situation, apocytochrome *c* binds partly at the membrane surface, although part of the protein also penetrates the membrane as is indicated by the immobile component that is still present in the ESR spectra. Nicholson et al. (1988) have shown that apocytochrome *c* bound to mitochondria is accessible to externally added proteases but at the same time penetrates far enough through the outer membrane to interact with cytochrome *c* heme lyase, the enzyme that attaches a heme group to apocytochrome *c* and which is located within the intermembrane space. The present spin-label results obtained on lipid model membranes correlate very well with these observations on the native mitochondrial system.

Yeast Cytochrome c. In previous studies, including those with ESR spectroscopy, it has been shown that cytochrome *c* interacts mainly electrostatically with acidic lipids without penetrating the membrane (Rietveld et al., 1983; Görrissen

et al., 1986). More recently, it has been suggested that the tertiary structure of cytochrome *c* loosens or unfolds upon interaction with negatively charged membranes (Spooner & Watts, 1991a,b; Muga et al., 1991b; de Jongh et al., 1992). The ESR spectra of spin-labeled yeast cytochrome *c* bound to negatively charged DOPG model membranes consist largely of an immobilized component. The spin-label is attached to the cysteine residue in the C-terminal helix, and the ESR spectrum observed suggests that yeast cytochrome *c* binds with this particular region directed toward the membrane, which could replace the hydrophobic region of the protein to which cysteine-102 is directed in the holoprotein (Louie et al., 1988). Support for this suggestion comes from measurements of accessibility to oxygen and chromium oxalate which probe distinctly separate regions of the lipid dispersion [cf. Snel and Marsh, (1993)]. The accessibilities found are similar to those obtained for a spin-label attached to the phospholipid headgroups, suggesting that the spin-labeled cytochrome *c* is located at the headgroup region of the membrane. It was found further that binding of cytochrome *c* reduced the accessibility of oxygen to the phospholipid headgroups [cf. Snel and Marsh (1993)]. This is consistent with an intimate contact between cytochrome *c* and the membrane surface such as could induce immobilization of the spin-label attached to the protein. Significant in this respect also is the observation that holocytochrome *c* is associated more intimately with bilayers of the unsaturated DOPG than with those of the saturated DMPG, as evidenced by the ESR spectra of spin-labeled phospholipids (Snel, de Kruijff, and Marsh, unpublished observations).

According to the studies mentioned above, cytochrome *c* does not penetrate the bilayer deeply, and no appreciable changes in the secondary structure have been demonstrated on binding of the protein to negatively charged lipid bilayers (Muga et al., 1991b). This means that the membrane association of a spin-labeled helix can produce an immobilized component in the ESR spectrum of a protein that already has appreciable secondary structure but without necessarily involving deep penetration of the protein into the hydrophobic region of the membrane. This implies that part of the immobilization of the N-terminal section of apocytochrome *c* observed on association with negatively charged lipid bilayers could arise in addition to the induction of secondary structure also from the electrostatic association with the membrane *per se*. It will be noted, however, that the position of labeling relative to the terminal residue is rather different in these two cases.

Following previous results (Jordi et al., 1990), heat-denatured cytochrome *c* might be supposed to model the interactions of apocytochrome *c*, including those of the C-terminus. The spin-label spectra in Figure 8 evidence a rather high mobility, indicating that the C-terminus of heat-denatured cytochrome *c* is not as closely associated with the lipid as is a C-terminal peptide fragment of apocytochrome *c* that has been suggested to penetrate bilayers of negatively charged lipids (Jordi et al., 1989b). Either the C-terminal region is modulated in its interaction by attachment to the rest of the apoprotein, or the apocytochrome *c*-like effects of heat-denatured cytochrome *c* are caused in part by other regions of the protein. In so far as the heat-denatured holoprotein is a good model, the C-terminal is unlikely to penetrate the membrane.

Conclusions. It has been demonstrated that the ESR spectra of spin-labeled apocytochrome *c* are extremely sensitive to environment, reflecting both conformational changes in the protein and changes in protein dynamics on association with negatively charged lipid bilayers and detergent micelles. In mixed lipid systems, evidence is obtained from the ESR spectra for two distinct modes of binding of the apoprotein at high mole fractions of zwitterionic lipid, which correlate with observations on the native mitochondrial system during import of the precursor protein. The ESR spectra of holocytochrome *c* spin-labeled at its C-terminus also exhibit large changes in mobility of the labeled segment on binding to anionic lipid bilayers, providing new evidence on the mode of association with the membrane. The results obtained with the heat-denatured spin-labeled yeast cytochrome *c* indicate a surface localization of the spin-label at the C-terminus, whereas the N-terminus of spin-labeled apocytochrome *c* not only is located at the surface but also penetrates the membrane, to an extent that is dependent on the lipid composition.

REFERENCES

Berkhout, T. A., Rietveld, A., & de Kruijff, B. (1987) *Biochim. Biophys. Acta 897*, 1–4.

Comfurius, P., & Zwaal, R. F. A. (1977) *Biochim. Biophys. Acta 488*, 36–42.

De Jongh, H. H. J., & de Kruijff, B. (1990) *Biochim. Biophys. Acta 1029*, 105–112.

De Jongh, H. H. J., Killian, J. A., & de Kruijff (1992) *Biochemistry 31*, 1636–1643.

Demel, R. A., Jordi, W., Lambrechts, H., van Damme, H., Hovius, R., & de Kruijff, B. (1989) *J. Biol. Chem. 264*, 3988–3997.

Dickerson, R. E., Takano, T., Eisenberg, D., Kallai, O. B., Samson, L., Cooper, A., & Margoliash, E. (1971) *J. Biol. Chem. 246*, 1511–1535.

Drott, H. R., Lee, C. P., & Yonetani, T. (1970) *J. Biol. Chem. 245*, 5875–5879.

Eibl, H., & Lands, W. E. M. (1969) *Anal. Biochem. 30*, 51–57.

Esmann, M., Hankovszky, H. O., Hideg, K., & Marsh, D. (1989) *Biochim. Biophys. Acta 978*, 209–215.

Feng, Y., Roder, H., Englander, S. W., Wand, A. J., & Di Stefano, D. L. (1989) *Biochemistry 28*, 195–203.

Fisher, W. R., Taniuchi, H., & Anfinsen, C. B. (1973) *J. Biol. Chem. 248*, 3188–3195.

Freed, J. H. (1976) in *Spin Labeling. Theory and Applications* (Berliner, L. J., Ed.) Vol. 1, pp 53–132, Academic Press, New York.

Gao, Y., Boyd, J., Williams, R. J. P., & Pielak, G. J. (1990) *Biochemistry 29*, 6994-7003.

Görrissen, H., Marsh, D., Rietveld, A., & de Kruijff, B. (1986) *Biochemistry 25*, 2904–2910.

Hartl, F.-U., Pfanner, N., Nicholson, D. W., & Neupert, W. (1989) *Biochim. Biophys. Acta 988*, 1–45.

Hennig, B., & Neupert, W. (1981) *Eur. J. Biochem. 121*, 203–212.

Hennig, B., & Neupert, W. (1983) *Methods Enzymol. 97*, 261–274.

Hovius, R., Lambrechts, H., Nicolay, K., & de Kruijff, B. (1990) *Biochim. Biophys. Acta 1021*, 217–226.

Hovius, R., Thijssen, J., van der Linden, P., Nicolay, K., & de Kruijff, B. (1993) *FEBS Lett. 330*, 71–76.

Jordi, W., Li-Xin, Z., Pilon, M., Demel, R. A., & de Kruijff, B. (1989a) *J. Biol. Chem. 264*, 2292–2301.

Jordi, W., de Kruijff, B., & Marsh, D. (1989b) *Biochemistry 28*, 8998–9005.

Jordi, W., de Kroon, A. I. P. M., Killian, J. A., & de Kruijff, B. (1990) *Biochemistry 29*, 2313–2321.

Jordi, W., Hergersberg, C., & de Kruijff, B. (1992) *Eur. J. Biochem. 204*, 841–846.

Lewis, S. M., & Thomas, D. D. (1986) *Biochemistry 25*, 4615–4621.

Louie, G. V., Hutcheon, W. L. B., & Brayer, G. D. (1988) *J. Mol. Biol. 199*, 295–314.
Muga, A., Mantsch, H. H., & Surewicz, W. K. (1991a) *Biochemistry 30*, 2629–2635.
Muga, A., Mantsch, H. H., & Surewicz, W. K. (1991b) *Biochemistry 30*, 7219–7224.
Nicholson, D. W., Köhler, H., & Neupert, W. (1987) *Eur. J. Biochem. 164*, 147–157.
Nicholson, D. W., Hergersberg, C., & Neupert, W. (1988) *J. Biol. Chem. 263*, 19034–19042.
Peterson, G. L. (1977) *Anal. Biochem. 83*, 346–356.
Rietveld, A., Sijens, P., Verkleij, A. J., & de Kruijff, B. (1983) *EMBO J. 2*, 907–913.
Rietveld, A., Ponjee, G. A. E., Schiffers, P., Jordi, W., van de Coolwijk, P. J. F. M., Demel, R. A., Marsh, D., & de Kruijff, B. (1985) *Biochim. Biophys. Acta 818*, 398–409.
Rietveld, A., Jordi, W., & de Kruijff, B. (1986a) *J. Biol. Chem. 261*, 3846–3856.
Rietveld, A., Berkhout, T. A., Roenhorst, A., Marsh, D., & de Kruijff, B. (1986b) *Biochim. Biophys. Acta 858*, 38–46.
Rouser, G., Fleischer, S., & Yamamoto, A. (1970) *Lipids 5*, 494–496.
Sankaram, M. B., de Kruijff, B., & Marsh, D. (1989) *Biochim. Biophys. Acta 986*, 315–320.
Snel, M. M. E., & Marsh, D. (1993) *Biochim. Biophys. Acta 1150*, 155–161.
Snel, M. M. E., Kaptein, R., & de Kruijff, B. (1991) *Biochemistry 30*, 3387–3395.
Snel, M. M. E., de Kruijff, B., & Marsh, D. (1993) *Biophys. J. 64*, A16.
Spooner, P. J. R., & Watts, A. (1991a) *Biochemistry 30*, 3871–3879.
Spooner, P. J. R., & Watts, A. (1991b) *Biochemistry 30*, 3880–3885.
Stellwagen, E., Rysavy, R., & Babul, G. (1972) *J. Biol. Chem. 247*, 8074–8077.
Stuart, R. A., & Neupert, W. (1990) *Biochimie 72*, 115–121.
Todd, A. P., & Millhauser, G. L. (1991) *Biochemistry 30*, 5515–5523.
Toniolo, C., Fontana, A., & Scoffone, E. (1975) *Eur. J. Biochem. 50*, 367–374.
Vincent, M., & Gallay, J. (1991) *Eur. J. Biophys. 20*, 183–191.
Walter, A., Margolis, D., Mohan, R., & Blumenthal, R. (1986) *Membr. Biochem. 6*, 217–237.
Wand, A. J., Di Stefano, D. L., Feng, Y., Roder, H., & Englander, S. W. (1989) *Biochemistry 28*, 186–194.
Zuniga, E. H., & Nall, B. T. (1983) *Biochemistry 22*, 1430–1437.