Modulation of Cytochrome *c*-Membrane Interaction by the Physical State of the Membrane and the Redox State of Cytochrome *c*

Ukchun Kim, Yu Shin Kim, and Sanghwa Han*

Department of Biochemistry, Kangwon National University, Chunchon 200-701, Korea
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Association of cytochrome *c* with anionic membranes involved both electrostatic and hydrophobic interactions and their relative contributions depended on the physical state of the membrane and the redox state of cytochrome *c*. Hydrophobic interaction was favored by the membranes in gel phase, by the membranes with a large curvature, and by the membranes with a high surface charge density. Ferrocytochrome *c* was less dissociable by NaCl than ferricytochrome *c* suggesting that a lower protein stability is beneficial for hydrophobic interaction. Hydrophobic interaction induced larger structural perturbations on cytochrome *c* as monitored by the loss of the Fe-Met bond and by the increase in the distance between heme and Trp-59. When bound to anionic membranes, spin-labeled cytochrome *c* showed an electron paramagnetic resonance spectrum with two or more components, providing a direct evidence for multiple conformations of bound cytochrome *c*.

Introduction

Cytochrome *c* is an electron transport protein in the mitochondrial intermembrane space. Being positively charged at neutral pH, cytochrome *c* can form a complex with its redox partners, *i.e.*, cytochrome *c* reductase, cytochrome *c* oxidase, and cytochrome *c* peroxidase. Although the inner mitochondrial membrane is abundant in negatively charged cardiolipin, it is not evident that cytochrome *c* binds to the membrane because ionic strength in the intermembrane space may be high enough to prevent formation of an electrostatic complex.[1] Recent studies have shown, however, that a fraction of cytochrome *c* remains bound to the membrane even at high ionic strength.[2,3] Therefore the cytochrome *c*-membrane interaction is biologically relevant and may have influence on the electron transfer between cytochrome *c* and its redox partners.

Interaction of cytochrome *c* and anionic model membranes has been studied extensively by a variety of physical methods.[4] Structural changes were detected in both cytochrome *c* and the membranes. Cytochrome *c* is destabilized upon association with anionic membranes as evidenced by a lowered thermal denaturation temperature.[5] The tertiary structure is significantly perturbed but little disruption is imposed on the secondary structure.[5-7] The Fe-Met bond is weakened or broken.[7-9] Changes in the membrane include phase separation[10,11] and formation of nonbilayer structures.[9,12-14]

Inspection of numerous reports on the cytochrome *c*-membrane interaction reveals that different laboratories use different recipes for the preparation of membranes. For example, membranes can be multilamellar, large unilamellar, or small unilamellar if one uses, respectively, a dispersion, extrusion, or sonication method. Surface charge density can be varied by incorporating different amount of anionic phospholipids such as posphatidylglycerol (PG), phosphatidylserine (PS), and cardiolipin. Membranes can also be prepared in different phases depending on the saturation level of the fatty acid chains. In order to account for the quantitative discrepancies among different groups, we carefully examined the membrane phase, surface charge density, and curvature as important factors that influence the cytochrome *c*-membrane interaction. We also found that the stability of cytochrome *c*, depending on its redox state and source (horse or yeast), determined the nature of the interaction.

Experimental Section

Materials. Phospholipids were purchased from Avanti Polar Lipids (Alabaster, AL). 1-Palmitoyl-2-[6-(pyren-1-yl)]hexanoyl-*sn*-glycero-3-phosphocholine (PPHPC), a pyrene-labeled PC, was from Molecular Probes (Eugene, OR). 1-Oxyl-2,2,5,5-tetramethylpyrroline-3-methyl)methanethiosulfonate (MTSSL), a thiol-specific spin label, was obtained from Reanal (Budapest, Hungary). All other chemicals were from Sigma (St. Louis, MO).

Isolation of cytochrome *c* and spin labeling. Yeast B-7528 transformant containing the cloned *CYC1* gene was aerobically grown in 5 L of YPL medium (1% yeast extract, 2% peptone, 1% lactate) to the stationary phase at 30 °C. Cytochrome *c* was purified from the harvested cells according to published methods.[15,16] Extinction coefficient ϵ_{550} = 27.7 $mM^{-1}cm^{-1}$ was used to determine the concentration of reduced cytochrome *c*.

Spin labeling of cytochrome *c* at Cys-102 was described elsewhere.[17] Briefly, 1.5-fold excess of MTSSL was added to cytochrome *c* and unreacted MTSSL was removed by gel filtration after incubation for 1 h at room temperature. Labeling was complete with a 1 : 1 stoichiometry as quantitated by electron paramagnetic resonance (EPR).

Isolation of cytochrome *c* oxidase and polarographic

*To whom correspondence should be addressed. E-mail: hansh@cc.kangwon.ac.kr; Tel: +82-361-250-8516; Fax: +82-361-242-0459

assay. Cytochrome *c* oxidase was isolated from bovine hearts according to Yonetani.[18] The rate of electron transfer between cytochrome *c* and cytochrome *c* oxidase was measured by using an oxygen electrode (Rank Brothers Digital Oxygen System Model 10). Cytochrome *c* (40 μM) and cytochrome *c* oxidase (20 nM) were mixed in 10 mM *N*-(2-hydroxyethyl)piperazine-*N'*-2-ethanesulfonic acid (HEPES) buffer (pH 7.4) containing 0.5% laurylmaltoside. The reaction was initiated by adding 200 μM *N',N',N',N'*-tetramethyl-*p*-phenylenediamine and 5 mM ascorbate and the oxygen consumption was measured polarographically.

Preparation of vesicles. Large unilamellar vesicles (LUVs) were prepared by an extrusion method.[15] To make vesicles containing 30 mol% 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoglycerol (POPG) and 70 mol% 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC), 35 mg POPC and 15 mg POPG were dissolved in chloroform, dried under nitrogen, and evacuated for 2 h. PPHPC (0.5 mol%) was included in the mixture for the samples used in fluorescence resonance energy transfer (FRET) measurements. The resulting lipid film was hydrated in 1 mL of 10 mM HEPES at pH 7 at room temperature and extruded through a polycarbonate filter (200 nm pore size) with a LiposoFast homogenizer (Avestin, Ottawa, Canada). Small unilamellar vesicles (SUVs) were prepared by sonicating the corresponding large unilamellar vesicles (LUVs).

Spectroscopic measurements. Binding of cytochrome *c* to membranes was measured at 25 °C according to a method developed by Mustonen and coworkers.[20] An aliquot of cytochrome *c* was added to 25 μM (total phospholipid concentration) of PPHPC-containing vesicles in 10 mM HEPES and the pyrene fluorescence was recorded on an Aminco-Bowman Series 2 lumunescence spectrometer (SLM-Aminco, Urbana, IL) with a 16 nm band-pass. Excitation wavelength was 344 nm with a 4 nm band-pass.

Visible absorption spectra were obtained by using an SLM-Aminco DW2000 spectrophotometer. The absorption spectrum of vesicles alone was subtracted from that of the vesicles with bound cytochrome *c* to correct for light scattering by the vesicles. Then a two-point baseline correction was applied to obtain a flat background. The absorption maximum, however, shifted to a slightly longer wavelength as a consequence.

Tryptophan fluorescence of a sample was obtained in a 3 mm × 3 mm cell. The sample was excited at 280 nm with a 4 nm band-pass and emission was recorded with an 8 nm band-pass. The emission spectrum was corrected for light scattering by the vesicles alone as in the absorption spectrum. Attenuation of the excitation was estimated by measuring the absorbance of the same sample at 280 nm and using the Beer-Lambert law. Similar corrections were made to the emission. Experimental spectra before corrections are presented in Figure 3. The values in Table 1 were obtained by integrating the corrected spectra from 310 nm to 370 nm.

EPR spectra of spin-labeled cytochrome *c* were obtained in a quartz flat cell at 25 °C using a Bruker ER-200 X-band spectrometer operating at 9.76 GHz. Modulation frequency was 100 kHz.

Results

Dependence on the membranes physical states. At neutral pH, cytochrome *c* and the inner mitochondrial membrane are oppositely charged and expected to interact electrostatically with each other. We model the inner mitochondrial membrane by LUVs with a composition of 30 mol% POPG and 70 mol% POPC. The vesicles are in liquid crystalline phase at room temperature and have approximately the same amount of negative charges as that of the inner mitochondrial membrane. Binding of cytochrome *c* was measured by the fluorescence quenching due to resonance energy transfer from pyrene embedded in the membrane to the heme of cytochrome *c*, as described by Mustonen and coworkers.[20]

Addition of 500 nM ferricytochrome *c* to 25 μM LUVs containing 30 mol% POPG at low ionic strength reduced the pyrene fluorescence by ~65% (Figure 1, *circles*). Under these conditions, the membrane surface is completely covered by cytochrome *c* as previously reported.[17,21] Increasing ionic strength caused dissociation of bound cytochrome *c*. Fluorescence was not fully recovered, however, even at extreme concentrations of NaCl. At [NaCl] of 150 mM, an estimated ionic strength of the intermembrane space,[1] fluorescence intensity was recovered to 83%. In other words 26% of cytochrome *c* remained bound to the membrane at the physiological ionic strength (see Table 1). As the POPG content of the vesicles increased, the bound cytochrome *c* became less dissociable by addition of NaCl (data not shown). When cyto-

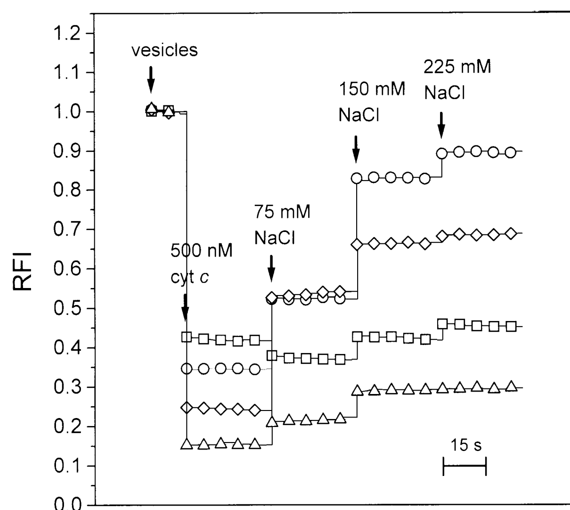

Figure 1. Dependence of cytochrome *c*-membrane interaction on the physical state of the membranes. 500 nM cytochrome *c* was added to 25 μM (total phospholipid concentration) of vesicles containing 0.1 mol% pyrene-labeled PC in 10 mM HEPES at pH 7. Emission of pyrene at 390 nm was measured with excitation at 345 nm to estimate binding of cytochrome *c*. Quenching of the pyrene fluorescence by bound cytochrome *c* was expressed as a relative fluorescence intensity (RFI) referenced to the fluorescence of vesicles alone. NaCl was added and dissociation of cytochrome *c* was monitored. *Circles*, LUVs containing 30 mol% POPG and 70 mol% POPC; *diamonds*, SUVs containing 30 mol% POPG and 70 mol% POPC; *triangles*, SUVs containing 30 mol% DPPG and 70 mol% DPPC; *squares*, LUVs containing 100 mol% POPG.

Table 1. Characteristics of cytochrome *c* binding to anionic membranes

vesicles	pyrene fluorescence[a]	absorption at ~700 nm[b]	tryptophan fluorescence[c]	fraction of cytochrome *c* bound at [NaCl]=150 mM
30:70 POPG:POPC: LUV	0.35	0.94	0.05	0.26
30:70 POPG:POPC: SUV	0.25	0.79	0.20	0.45
30:70 DPPG:DPPC: SUV	0.15	0.79	0.30	0.82
100:0 POPG:POPC: LUV	0.42	0.44 (0.19)[d]	0.18 (0.39)	~1.0

[a]Fluorescence intensity relative to that of vesicles alone. [b]Absorption at ~700 nm relative to the native state. [c]Integrated fluorescence intensity after correction, relative to the unfolded state. [d]Number in parenthesis is the value after 30 min incubation.

chrome *c* was bound to the LUVs containing 100% POPG, the fluorescence was not recovered even at very high ionic strength (Figure 1, *squares*). In contrast, Rytömaa and Kinnunen[21] found that bound cytochrome *c* was largely dissociated from the LUVs prepared from 100% egg PG upon addition of 150 mM NaCl. We attribute the discrepancy to the different sources of cytochrome *c*, namely, horse in their work and yeast in our work (see Discussion). Other phospholipids such as egg PG and 1,2-dioleoyl-*sn*-glycero-3-phosphoglycerol (DOPG) showed similar effects of increasing the surface charge density (data not shown).

Aqueous dispersion of phospholipids of a desired composition produces multilamellar vesicles. One can extrude the vesicles through a filter with a known pore size to obtain LUVs or sonicate them to prepare SUVs. LUVs and SUVs have the same lamellarity but different curvature. Different methods of vesicle preparation were used in different studies without carefully considering the effects of membrane curvature on the cytochrome *c*-membrane interaction. Compared with LUVs containing 30 mol% POPG (Figure 1, *circles*), association of cytochrome *c* with the corresponding SUVs quenched the pyrene fluorescence more efficiently (Figure 1, *diamonds*). About 45% of cytochrome *c* was bound to the SUVs at 150 mM of NaCl. We repeated the measurements on other phospholipids including egg PG, 1,2-dipalmitoyl-*sn*-glycero-3-phosphoglycerol (DPPG), DOPG, and tetrastearoylcardiolipin to find that cytochrome *c* bound to SUVs was always more resistant, regardless of the membrane phase, to dissociation by NaCl than that bound to the corresponding LUVs (data not shown).

As mentioned above, the vesicles made of POPC and POPG (phase transition temperature T_m ~ −2 °C) are in liquid crystalline phase at room temperature. In order to see if the membrane phase affects the cytochrome *c*-membrane interaction, we prepared SUVs in gel phase (T_m ~ 41 °C) by sonicating the LUVs composed of 30 mol% DPPG and 70 mol% 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC). As shown in Figure 1 (*triangles*), fluorescence was quenched even more efficiently and 82% of cytochrome *c* remained bound to the membrane at high ionic strength. The value is nearly twice as large as that for the SUVs in liquid crystalline phase (see above). LUVs in gel phase also had a higher affinity for cytochrome *c* than those in liquid crystalline phase (data not shown).

Spectroscopic properties of bound cytochrome *c*. In Figure 2, the absorption spectra of cytochrome *c* bound to various vesicles were obtained in the region around 700 nm. A band at ~700 nm is indicative of the intact Fe-Met bond. The baseline of each spectrum was corrected and, as a consequence, the absorption maximum shifted to a slightly longer wavelength without significantly altering the relative intensity. Compared with cytochrome *c* in the native state (not shown), only 6% decrease in the absorption band was observed when cytochrome *c* was bound to the LUVs containing 30 mol% POPG (*circles*). About 20% of the Fe-Met bond was broken in cytochrome *c* bound to the SUVs containing 30 mol% POPG (*diamonds*) or 30 mol% DPPG (*triangles*). The LUVs (*squares*) and SUVs (not shown) of 100% POPG disrupted 56% and 85%, respectively, of the Fe-Met bond. Cytochrome *c* on the membranes of pure POPG showed a gradual decrease in the absorption upon longer incubation, suggesting that cytochrome *c* experienced further unfolding after the initial binding. Cytochrome *c* bound to the SUVs containing 100% POPG completely lost its Fe-Met bond within 30 min. In all the cases, the amount of high spin species was very small, if any, as judged by the absorpion at ~650 nm. This means that a strong ligand, possibly a histidine residue, replaces methionine so that Fe stays in a six-coordinate low-spin state.

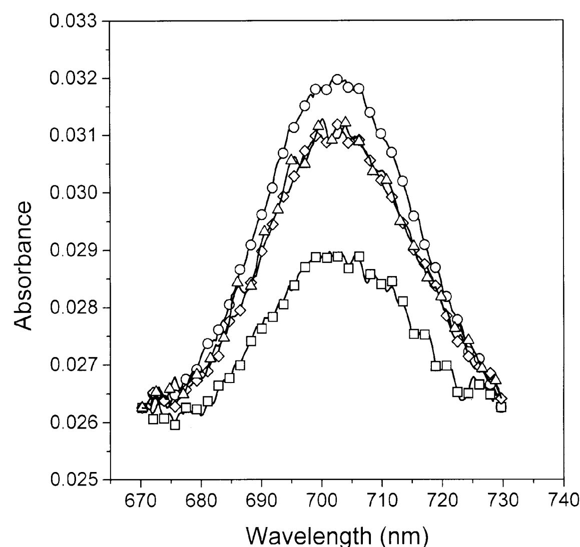

Figure 2. Disruption of the Fe-Met bond caused by cytochrome *c*-membrane interaction. 30 μM of cytochrome *c* was added to 2 mM vesicles and the absorption spectrum was measured immediately. For LUVs, the spectrum of vesicles alone was subtracted from the spectrum of a sample. The baseline was corrected in each spectrum. Symbols are the same as in Figure 1.

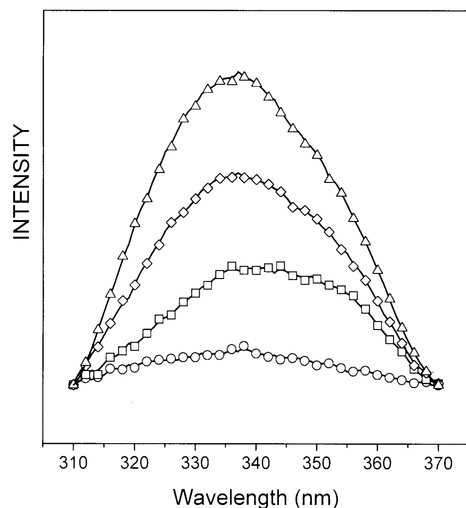

Figure 3. Tryptophan fluorescence of membrane-bound cytochrome *c*. 30 μM of cytochrome *c* was added to 2 mM vesicles and the fluorescence spectrum was measured immediately by exciting at 280 nm. The spectra presented here are uncorrected. The integrated intensity after correction for the attenuation of excitation and emission due to scattering is given in Table 1. Symbols are the same as in Figure 1.

Cytochrome *c* has a single tryptophan at position 59 whose fluorescence is efficiently quenched by nearby heme. When unfolded by guanidinium chloride, cytochrome *c* has an emission spectrum whose maximum is at ~350 nm (data not shown). Fluorescence intensity relative to that of the unfolded state was only 0.05 for cytochrome *c* bound to the LUVs containing 30 mol% POPG (Figure 3, *circles*), indicating that Trp-59 was kept near the heme as in the native state (see Table 1). The relative fluorescence was raised to 0.2 and 0.3 when cytochrome *c* was bound to the SUVs containing 30 mol% POPG (*diamonds*) and 30 mol% DPPG (*triangles*), respectively, suggesting that a fraction (or all) of cytochrome *c* suffered a significant structural change. No further spectral changes occurred upon longer incubation. The LUVs of 100% POPG (*squares*) yielded a relative fluorescence of 0.18, which is smaller than the SUVs containing 30 mol% POPG or DPPG. Note that the former was more efficient than the latter in disrupting the Fe-Met bond (see above). This means that the fluorescence and absorption report different structural changes of the bound cytochrome *c*. Again the vesicles of 100% POPG underwent a gradual increase in fluorescence, being consistent with the visible absorption data.

Examination of Figure 3 reveals that the fluorescence spectra are composed of two bands, one at ~338 nm and the other at ~350 nm, suggesting that the bound cytochrome *c* exists in multiple conformations. The two bands correspond to Trp-59 in hydrophobic and hydrophilic environments, respectively. The relative contribution of the hydrophilic component was greater for the vesicles containing 100% POPG than SUVs containing 30 mol% POPG or DPPG. The band at ~350 nm may arise from the bound cytochrome *c* in which the tertiary structure is disturbed enough to allow access of solvent molecules to Trp-59.

Yeast cytochrome *c* has a cysteine residue at position 102.

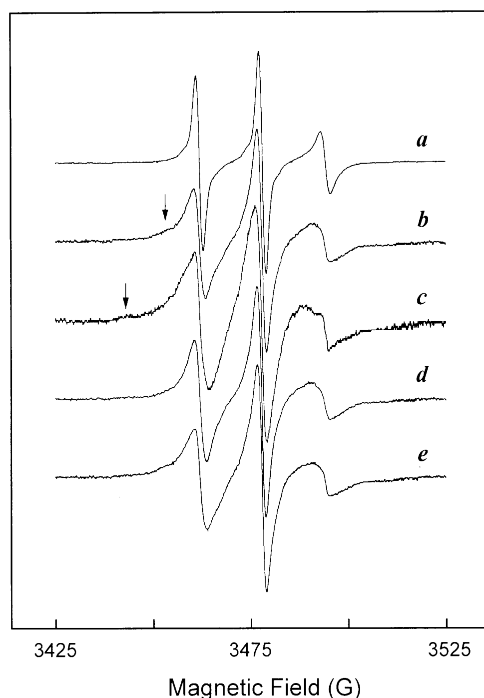

Figure 4. Changes in the EPR spectra of cytochrome *c* induced by the cytochrome *c*-membrane interaction. 30 μM of spin-labeled cytochrome *c* was added to 2 mM vesicles and the EPR spectra were recorded on an X-band spectrometer. The microwave frequency and modulation frequency were, respectively, 9.76 GHz and 100 kHz. All the spectra were normalized to the central line ($m_I = 0$) to emphasize the changes in the line shape. *a*, unbound; *b*, LUVs containing 30 mol% POPG and 70 mol% POPC; *c*, LUVs containing 100 mol% POPG; *d*, SUVs containing 30 mol% POPG and 70 mol% POPC; *e*, SUVs containing 30 mol% DPPG and 70 mol% DPPC. Arrows indicate immobile signals.

We can attach a thiol specific spin label to Cys-102 and measure its EPR spectrum to monitor the local mobility of the spin label, which is affected by the surroundings such as protein side chains and solvent viscosity. Therefore the spin-label EPR technique can be used to detect conformational changes due to membrane association of cytochrome *c*.[17,22]

As shown in Figure 4a, an EPR spectrum of fast rotational motion was observed for unbound cytochrome *c*. When bound to the LUVs containing 30 mol% POPG, a significant line broadening occurred due to motional restriction (Figure 4b). Interestingly the spectrum consists of two major components, an immobile (*arrow*) and a mobile signal, suggesting that the bound cytochrome *c* exists in multiple conformations. When cytochrome *c* was bound to the LUVs of 100% POPG, a further broadening in lineshape was observed with another immobilized signal (Figure 4c, *arrow*). Cytochrome *c* on the SUVs of 30 mol% POPG (Figure 4d) or DPPG (Figure 4e) showed a similar spectrum to that on the LUVs containing 30 mol% POPG. Addition of NaCl produced the spectrum of unbound cytochrome *c* with a simultaneous decrease in the mobile and immobile signals (not shown).

Dependence on the redox state of cytochrome *c*. There have been controversial reports on the effects of the redox state of cytochrome *c* on its binding affinity for anionic mem-

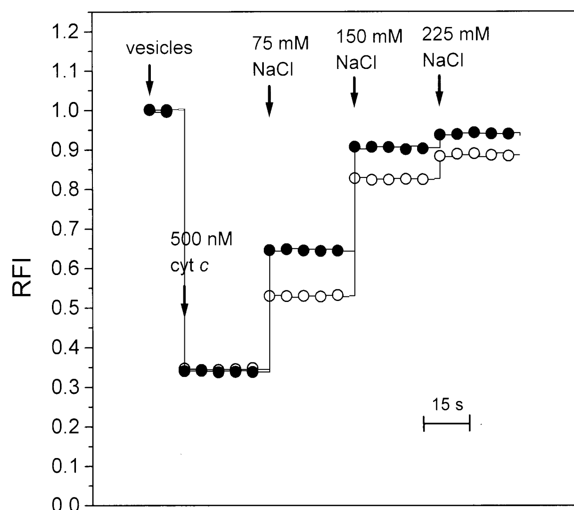

Figure 5. Dependence of cytochrome *c*-membrane interaction on the redox state of cytochrome *c*. 500 nM cytochrome *c* was added to 25 μM (total phospholipid concentration) of LUVs (30 mol% POPG and 70 mol% POPC) containing 0.1 mol% pyrene-labeled PC in 10 mM HEPES at pH 7. Measurements in Figure 1 were repeated. *Open circles*, ferricytochrome *c*; *closed circles*, ferrocytochrome *c*.

branes. Rytömaa *et al.*[23] briefly mentioned no dependence on the redox state whereas Ksenzhek *et al.*[24] reported a different affinity for a different redox state. We observed a definitive difference in the binding affinity between ferrocytochrome *c* and ferricytochrome *c*. In Figure 5, both ferrocytochrome *c* (*squares*) and ferricytochrome *c* (*circles*) quenched the pyrene fluorescence to an almost identical extent when bound to the LUVs containing 30 mol% POPG. Upon addition of 75 mM NaCl, ferrocytochrome *c* emitted ~10% more fluorescence than ferricytochrome *c* indicating that ferrocytochrome *c* has a lower binding affinity. The redox-state dependence was observed for both SUVs and LUVs regardless of surface charge density. Same conclusion was drawn from the measurements on other phospholipids such as DPPG, egg PG, DOPG, and tetrastearoylcardiolipin (data not shown).

Discussion

Multiple conformations of bound cytochrome *c*. Upon binding to anionic membranes, cytochrome *c* undergoes structural changes that can be monitored by various spectroscopic methods including visible absorption,[7,8] fluorescence,[7] circular dichroism,[3,7] infrared,[5,6] resonance Raman,[12] NMR, 8,9,11-14,25 and EPR[17,22,26] spectroscopies. It is generally agreed upon that the bound cytochrome *c* has a loosened tertiary structure with little change in the secondary structure. The Fe-Met bond is weakened or broken and cytochrome *c* suffers partial unfolding. The altered spectra such as increase in tryptophan fluorescence or decrease in the absorption at ~700 nm can be interpreted in two ways. It may arise from a homogeneous population having an altered spectral property or an averaged spectral property of a heterogeneous population. The EPR spectra of spin-labeled cytochrome *c* bound to the membrane provided a clue to resolve the problem. As shown in Figure 4, the EPR spectrum of spin labeled cytochrome *c* bound to the LUVs containing 30 mol% POPG has at two major components, a mobile and an immobile signal. The immobile signal does not necessarily mean that the spin label is squeezed into the interface of the membrane and binding domain of cytochrome *c*. It could arise from a conformational change that brings the spin label into a more restricted region within the protein. No matter what structure each spectral component corresponds to, it is of no doubt that each signal represents a different conformation so that multiple EPR signals provide evidence for multiple conformations. The tryptophan fluorescence spectrum (Figure 3) is also a composite of at least two components supporting the above interpretation.

Cortese *et al.*[2] reported that, in mitochondrial preparations, some cytochrome *c* could not be removed by washing with a buffer of high ionic strength, suggesting that a fraction of cytochrome *c* was nonelectrostatically associated with the membrane. Recently, they identified a few different conformational states of the bound cytochrome *c* depending on how cytochrome *c* was allowed to interact with the membrane: an electrostatically bound (EB) state and two membrane bound (MB and MBL) states.[3] The MB and MBL states were produced by incubating cytochrome *c* with the membrane at high and low ionic strength, respectively, but only the MB state had a low a-helix content. At low ionic strength, the EPR spectrum of spin-labeled cytochrome *c* bound to the membrane containing 30 mol% POPG consist of two major components. It is tempting to assign the immobile and mobile signals to the MBL and EB states, respectively, although a structurally more perturbed state does not necessarily have a more immobilized EPR signal. Our EPR data are consistent with the proposal by Cortese *et al.*[3] since the EB and MBL states are predominant at low ionic strength. The MBL state is likely to suffer larger structural perturbation than the EB state since the former should allow its exposed interior to interact hydrophobically with the membrane. We found that the EPR intensity of the immobile signal decreased together with that of the mobile signal (data not shown) when ionic strength was increased. This strongly supports the model proposed by Cortese *et al.*[3] that an equilibrium was established between the two states with only the EB state being dissociable by NaCl. Since the MBL state has to be converted to the EB state before it can dissociate from the membrane,[3] the relative contribution of hydrophobically bound state (MBL) is likely to determine dissociability of the bound cytochrome *c* by NaCl.

Although roughly correlated, disruption of the Fe-Met bond is not quantitatively related to the increase in tryptophan fluorescence (see Figures 2 and 3, and Table 1). For example, the SUVs containing 30 mol% POPG and SUVs containing 30 mol% DPPG show a similar degree of the Fe-Met bond disruption but significantly different tryptophan fluorescence. The LUVs containing 100% POPG caused the bound cytochrome *c* to have much lower absorption at ~700 nm than the SUVs containing 30 mol% DPPG but cytochrome *c* bound

to the SUVs fluoresces more intensely. It is likely that certain species with disrupted Fe-Met bond may still be compact enough to keep Trp-59 close to the heme. At the same time certain unfolded species may have stronger fluorescence than others.

Effects of the physical state of the membranes. Membranes can be in gel or liquid crystalline phase depending on the fatty acid chains and temperature. They can be multilamellar, large unilamellar, or small unilamellar if dispersion, extrusion, or sonication method, respectively, is used for the preparation. Both the membrane phase and curvature are expected to affect the cytochrome *c*-membrane interaction but not much attention has been paid to by researchers in the field.

We consistently found that cytochrome *c* was more resistant to NaCl when bound to the membranes in gel phase than in liquid crystalline phase, regardless of the membrane curvature. That is, the proportion of hydrophobically bound cytochrome *c* was larger for the membranes in gel phase than in liquid crystalline phase. As shown in Figures 2 and 3 and summarized in Table 1, cytochrome *c* bound to the membranes in gel phase and in the liquid crystalline phase has similar absorbance at ~700 nm but the former has stronger tryptophan fluorescence due to a larger degree of unfolding. Since the membrane in gel phase is less flexible than that in liquid crystalline phase, cytochrome *c* bound to the former has to adapt to the flat membrane surface which forces cytochrome *c* to unfold. Cytochrome *c* bound to the membrane in liquid crystalline phase, on the other hand, will probably induce structural changes in the membrane instead of being perturbed by the membrane. A similar argument was made by Heimburg *et al.*[12] when dioleoylglycerol was added to make the membrane flexible.

Recently Pinheiro *et al.*[7] measured various spectroscopic properties of cytochrome *c* bound to the SUVs of 100% dioleoylPS. A decrease in the absorption at ~700 nm and an increase in the tryptophan fluorescence were taken as an evidence for cytochrome *c* undergoing partial unfolding that led to hydrophobic interaction. As a mechanism for the membrane-induced unfolding, they invoked the lowered pH near the membrane surface. It is known that the pH near the headgroup of the membrane is lower than that of the bulk.[27] However, their argument does not support our findings that the LUVs of 100% POPG are less effective in unfolding the bound cytochrome *c* than the corresponding SUVs. If pH was the only factor, one would expect the LUVs to be more effective than the SUVs since the surface charge density can be slightly reduced by a larger curvature of the SUVs. We always observed that the vesicles with a larger curvature were more effective in inducing hydrophobic interaction, regardless of the constituting phospholipids. As shown in Figures 2 and 3, cytochrome *c* on the SUVs containing 30 mol% POPG has lower absorbance at ~700 nm and higher tryptophan fluorescence than the corresponding LUVs. Highly curved membranes will expose their hydrophobic interior to the bound cytochrome *c* promoting hydrophobic interaction. The local pH effect may play a role at high surface charge density. Preference for curved surfaces was also observed in other proteins.[28,29]

Stability as a determinant of hydrophobic interaction. In agreement with the results obtained by Rytömaa and Kinnunen,[19] quenching of the pyrene fluorescence by the bound cytochrome *c* was saturated at ~500 nM of cytochrome *c* for 25 mM (total phospholipid concentration) of LUVs containing 30 mol% POPG. This can be translated into ~50 phospholipid molecules (or ~15 POPG molecules) per bound cytochrome *c* molecule. Our previous EPR measurements on LUVs containing 30 mol% DPPG gave ~65 phospholipid molecules per cytochrome *c* molecule.[17]

Rytömaa and Kinnunen[19] found that the pyrene fluorescence was recovered up to 92% when 75 mM NaCl was added. No further increase in fluorescence was observed at higher ionic strength. In our experiment, the fluorescence went up only to 53% at [NaCl]=75 mM and reached 90% only when [NaCl] was higher than 200 mM. Moreover with the LUVs containing 100% POPG, cytochrome *c* was not dissociable in the present study whereas it was almost fully dissociated at [NaCl] =150 mM in the work of Rytömaa and Kinnunen.[19] As we always find yeast cytochrome *c* less dissociable than horse cytochrome *c* (data not shown), the above discrepancy can be attributed to the different sources from which cytochrome *c* is isolated.

If the interaction is totally electrostatic in nature, horse cytochrome *c* would have a higher affinity than yeast cytochrome *c* because the former has a larger net positive charge. It implies then that hydrophobic interaction contributes to the binding of cytochrome *c*. Indeed hydrophobic interaction has been postulated in the cytochrome *c*-membrane interaction by many investigators.[2,3,7,30] It is generally agreed upon that cytochrome *c* binds to anionic membranes via electrostatic interaction which subsequently becomes partially hydrophobic. Then why does yeast cytochrome *c* interact more hydrophobically with the membrane than horse cytochrome *c* Hydrophobic interaction involves partial unfolding of bound cytochrome *c* that leads to exposure of its interior. Therefore cytochrome *c* with a lower stability will unfold more easily so as to interact hydrophobically with the membrane. Horse cytochrome *c* is more stable than yeast cytochrome *c* so that hydrophobic interaction is favored by yeast cytochrome *c*. Ferrocytochrome *c* is less positively charged and more stable than ferricytochrome *c*. Then it is obvious that ferricytochrome *c* suffers a larger extent of unfolding than ferrocytochrome *c* so that it is less dissociable by NaCl.

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