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Lipid Specificity for Membrane Oxidation Catalyzed by Cytochrome c : An EPR Study

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Decay of the spin label attached to cytochrome *c* or to stearic acid has been measured by electron paramagnetic resonance (EPR) spectroscopy to monitor membrane oxidation induced by cytochrome *c*-membrane interaction. Binding of cytochrome *c* sequestered the acidic phospholipids and membrane oxidation was efficient in the order linoleic≫ oleic>stearic acid for a fatty acid chain in the acidic phospholipids. The spin label on cyt *c* was destroyed at pH 7 whereas that on stearic acid embedded in the membrane was destroyed at pH 4, presumably due to different modes of cyt *c*-membrane interaction depending on pH. Interestingly, cyt *c* also interacts with phosphatidylethanolamine, an electrically neutral phospholipid, to cause rapid membrane oxidation. Both EPR and fluorescence measurements indicated that electrostatic interaction is at least partially responsible for the process.

Introduction

Cytochrome *c* (Cyt *c*) is a peripheral membrane protein in the intermembrane space of mitochondria. Being abundant in lysine residues, the protein carries a large positive charge at physiological pH. The inner mitochondrial membrane, on the other hand, is rich in cardiolipin (CL) which makes the membrane surface electrically negative. Therefore cyt *c* is expected to interact electrostatically with the inner membrane.[1] The consequences of the cyt *c*-membrane interaction include alteration in the heme coordination state,[2] partial unfolding of the polypetide,[3] and disruption of the bilayer structure.[4,5] It is not clear if such interaction plays a role *in vivo*. Gupte and Hackenbrock[6] argued that ionic strength in the intermembrane space is too high for cyt *c* to interact electrostatically with the membrane. Recently, however, the same group[7] reported that a fraction of cyt *c* interacts with the membrane even under the condition of high ionic strength. Rytömaa and Kinnunen[8] suggested that the interaction of cyt *c* and acidic membranes is not electrostatic in nature at low pH. In their model, the protonated phosphate group of a phospholipid forms a hydrogen bonding network with an arginine residue of cyt *c*. Therefore cyt *c* may well interact with the inner mitochondrial membrane under certain conditions.

Using spin-label EPR techniques,[9] we were able to distinguish spectroscopically different states of bound cyt *c* at pH 7 and 4. During the experiments, however, we found that the EPR intensity of the spin label attached to cysteine-102 decayed rapidly when cyt *c* was allowed to interact aerobically with a membrane that contained acidic phospholipids. A similar observation was made some 20 years ago by Brown and Wüthrich,[10] who used horse heart cyt *c* with a spin label attached to methionine-65. Kinetic properties are rather complicated but a few things about the phenomenon are obvious from their work: electrostatic interaction between positively charged cyt *c* and a negatively charged membrane is required; both a lipid oxidation product, if pre-existent, and oxygen are involved; and the spin label on cyt *c* as well as that inside the membrane is destroyed. The lipid oxidation product, a reactive species that destroyed the spin labels, may be lipid hydroperoxides which undergo homolytic scission by the heme. In addition, Goñi and coworkers[11,12] included the binding of cyt *c* to the membrane and the presence of cyt *c* in the oxidized state as the requirements for the membrane oxidation. Other heme proteins are also known to

cause lipid oxidation[13,14] and these oxidation processes may have important biological implications such as oxidative damage and aging.[15]

Yeast cyt *c* spin labeled at cysteine-102 has been useful in elucidating details of the translocation of cyt *c* across a membrane bilayer.[9,16] As is clear in the present work, it also is a sensitive probe for a reactive species generated by the cyt *c*-membrane interaction. Decrease in the intensity of an EPR spectrum was measured to monitor the degradation of the spin label attached to either cyt *c* or to a fatty acid embedded in the membrane. We found that the decay rate of the spin labels largely depends on pH, corresponding to a different mode of interaction. A doubly unsaturated fatty acid chain was required for a fast destruction of the spin label. Association of cyt *c* with negatively charged membranes sequestered acidic phospholipids leading to membrane oxidation. Unlike phosphatidylcholine (PC), phosphatidylethanolamine (PE) was able to destroy the spin label very efficiently despite its electrical neutrality. Interestingly KCl slowed down the process suggesting involvement of an electrostatic interaction.

Experimental

Materials. Egg PC, beef heart CL, tetrastearoyl CL, distearoyl phosphatidylglycerol (PG), dioleoyl PG, dilinoleoyl PG, distearoyl PE, and dilinoleoyl PE were purchased from Avanti Polar Lipids (Alabaster, AL). (1-Oxyl-2,2,5,5-tetramethylpyrroline-3-methyl)-methanethiosulfonate (MTSSL), a thiol-specific spin-label, was obtained from Reanal (Budapest, Hungary). 16-Doxylstearate was from Aldrich (Milwakee, WI). 1-Palmitoyl-2-[6-(pyren-1-yl)hexanoyl]-sn-glycero-3-phosphocholine (PPHPC), a pyrene-labeled PC, was from Molecular Probes (Eugene, OR). All other chemicals including 5,5'-dimethyl-1-pyrroline-N-oxide (DMPO) and yeast cytochrome *c* were from Sigma (St. Louis, MO).

Preparation of spin-labeled cyt c. Yeast cyt *c* was spin labeled with MTSSL[17] as previously described.[9] Briefly, 30 mg of yeast cyt *c* was dissolved in 5 mL of 10 mM potassium phosphate, pH 7.0 and treated with a 2-fold molar excess of dithiothreitol to dissociate any disulfide-bridged dimers. The solution was incubated for 1 h at room temperature under nitrogen and then excess dithiothreitol was removed by a small Sephadex G-15 column. A 1.5 fold molar excess of MTSSL in ethanol (final ethanol concentration <1 %) was added to cyt c and the mixture was incubated for 2 hr at room temperature to label the cysteine residue at position 102. Unreacted spin-label was removed by gel fitration on a Sephadex G-15 column.

Preparation of liposomes. Liposomes were prepared by an extrusion method.[18] Lipids (about 50 mg) of desired composition were dissolved in chloroform, dried under nitrogen and evacuated for 2 h. Resulting lipid film was hydrated in 20 mM 2-[N-morpholino]ethanesulfonic acid (MES) at pH 4 or 7 containing 0.1 mM EDTA at room temperature and subjected to 5 cycles of freezing and thawing. The resulting suspension was extruded through a polycarbonate filter (100 nm pore size) with a LiposoFast homogenizer (Avestin, Ottawa, Canada). Compositions were calculated on the basis of fatty acid chains: one mol of CL corresponded to two mol of other phospholipids. Therefore the composition of CL-containing liposomes was 20 : 80 CL : PC in terms of fatty acid chains and that of other phospholipids was 20 : 80 in terms of phosphate. For liposomes containing spin-labeled fatty acid embedded in the membrane bilayer, 0.12 mol% 16-doxylstearate was mixed with other phospholipids in chloroform at the biginning of the liposome preparation and followed the above procedures.

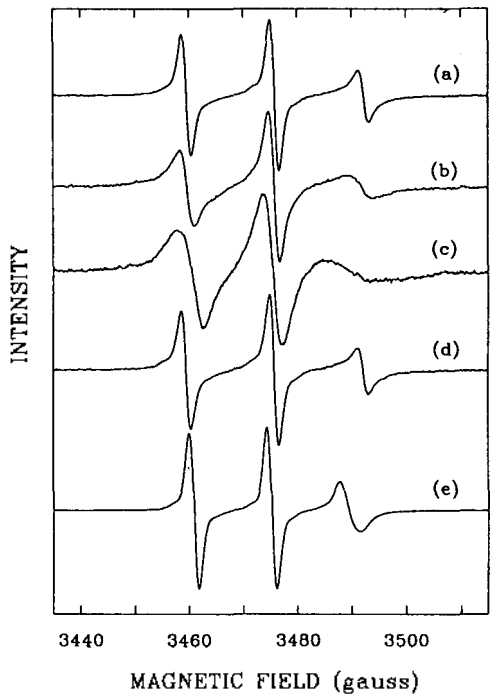

Figure 1. EPR spectra of spin-labeled yeast cyt *c* incubated with liposomes containing (a) 100% egg PC, (b) 10 mol% beef heart CL at pH 7 and (c) at pH 4, (d) 20 mol% PE, and (e) 0.12 mol% 16-doxylstearate. Samples were in 10 mM MES and pH was 7 unless otherwise stated. For comparison, the intensity was normalized with respect to the center line ($\Delta m_I=0$). Spectroscopic conditions: microwave frequency, 9.76 GHz; microwave power, 64 mW; modulation frequency, 100 kHz.

Fluorescence measurements of cyt c binding. Binding of cyt *c* to the membrane was estimated according to a published method.[19] Same liposomes as those used in the EPR experiments were prepared in the presence of 1 mol% PPHPC. To 25 μM (total phospholipid concentration) of PPHPC-containing liposomes in 20 mM MES (pH 7.0) with 0.1 mM EDTA, aliquots of cyt *c* were added and the fluorescence intensity was measured at 394 nm on a SLM-Aminco AB-2 spectrofluorometer. Excitation wavelength was 344 nm. Emission at 394 nm was absorbed by the heme of cyt *c* *via* a F rster-type enrgy transfer mechanism when cyt *c* binds to the membrane.

EPR spectroscopy. EPR measurements were performed at room temperature on a Bruker ER-200 X-band spectrometer. 30 μM of spin-labeled cyt *c* was added to a liposomal suspension and the mixture was immediately transferred to a quartz flat cell and the spectra were scanned successively to follow the destruction of the spin label. Spectral con-

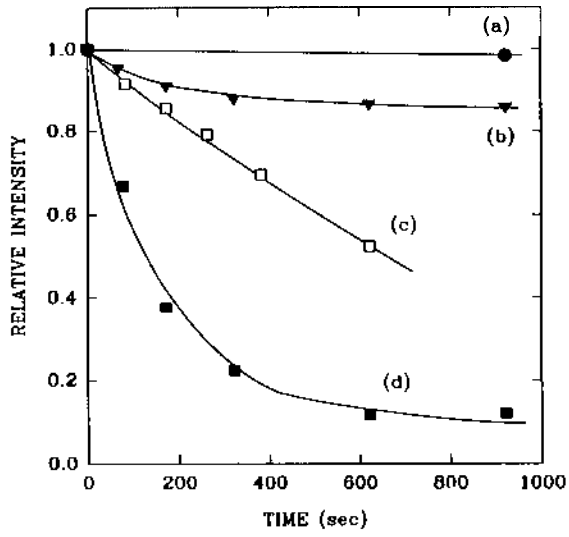

Figure 2. Decay of the spin label attached to cyt *c* as measured by the EPR intensity of the center line. Intensity was referenced to that of cyt *c* interacting with the liposomes containing 100% saturated fatty acid chains, *i.e.*, dipalmitoyl PC for egg PC and tetrastearoyl CL for beef heart CL. (a) Cyt *c* in buffer, (b) incubated with 100% PC, with beef heart CL-containing liposomes (c) in the presence and (d) in the absence of 200 mM KCl.

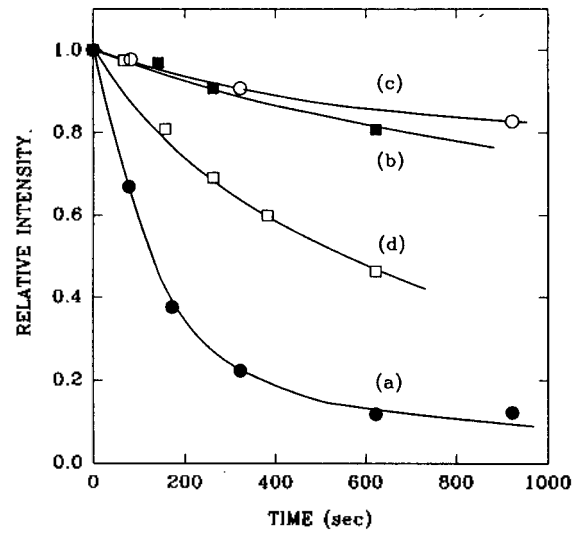

Figure 3. Decrease in the EPR intensity of the spin-labeled cyt *c* and 16-doxylstearate after incubation of cyt *c* with acidic membranes. In case of 16-doxylstearate, intensity was referenced to that of 16-doxylstearate in liposomes without cyt *c*. (a) Spin-labeled cyt *c* incubated with beef heart CL-containing liposomes at pH 7 and (b) at pH 4, (c) unlabeled cyt *c* incubated with beef heart CL-containing liposomes (0.12 mol% 16-doxylstearate included) at pH 7 and (d) at pH 4.

ditions: microwave frequency 9.76 GHz, microwave power 64 mW, modulation 100 kHz. When calculating relative intensities, the intensity of spin-labeled cyt *c* bound to liposomes containing correspondining phospholipids with saturated fatty acid chains (tetrastearoyl CL, distearoyl PG, and distearoyl PE) was set to 1. Destruction of the spin label by these phospholipids was negligible. All the experiments were carried out at an ambient temperature.

Results

The EPR spectra of spin labeled cyt *c* interacting with 100% PC (Figure 1a), with CL-containing liposomes at pH 7 (Figure 1b) and at pH 4 (Figure 1c), and with PE-containing liposomes (Figure 1d) were shown in Figure 1. As previously reported,[9] the spin label attached to cyt *c* became motionally restricted upon binding to CL-containing liposomes. Liposomes containing 100% PC or 20 mol% PE did not show such motional restriction suggesting that cyt *c* did not bind to the membrane. 16-Doxylstearate gave a typical EPR spectrum of a membrane-embedded spin label (Figure 1e). Interaction of 16-doxylstearate-containing liposomes with unlabeled cyt *c* did not alter the EPR spectrum of 16-doxylstearate. At pH 4, cyt *c* bound to CL-containing liposomes gave a very broad spectrum. However, cyt *c* induced aggregation that was almost fully reversed by increasing pH. We measured the intensity of the center lines ($\Delta m_I = 0$) to follow the decay of the spin labels.

The spin label attached to cyt *c* was very stable when added to a buffer (Figure 2a) or to liposomes that contain 100% PC (Figure 2b). A rapid decay of the spin label was observed when cyt *c* was allowed to interact with liposomes containing 10 mol% beef heart CL (Figure 2d). The intensity

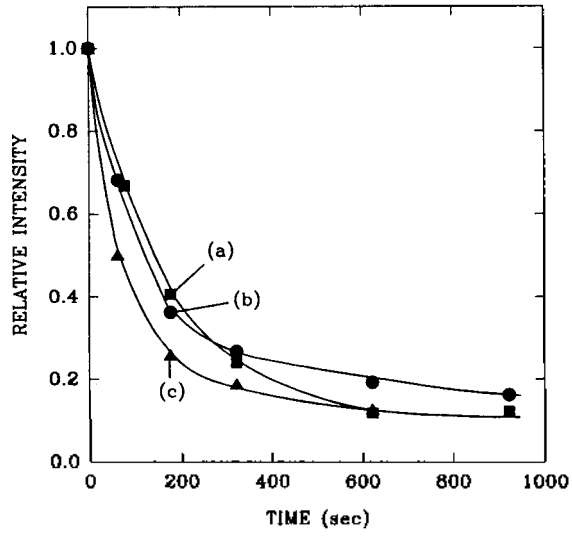

Figure 4. Destruction of the spin label attached to cyt *c* by the interaction with liposomes containing (a) 10 mol% beef heart CL, (b) 20 mol% dilinoleoyl PG, and (c) 20 mol% dilinoleoyl PS.

of spin-labeled cyt *c* bound to tetrastearoyl CL, a CL with saturated fatty acid chains, did not decrease and was set to 1 in the calculation of relative intensities. 200 mM KCl retarded the decay but was not able to completely stop the process (Figure 2c).

When 16-doxylstearate was incorporated in the CL-containing liposomes and the liposomes were allowed to interact with unlabeled cyt *c* at pH 7, a slow decay of the EPR inten-

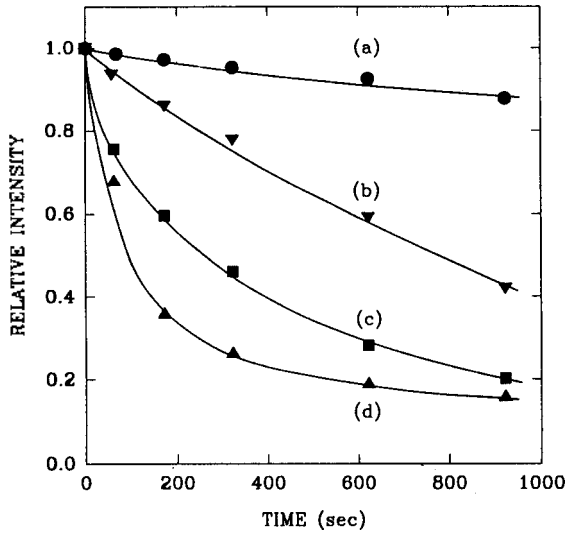

Figure 5. Destruction of the spin label attached to cyt *c* by the interaction with liposomes containing 20 mol% of (a) distearoyl PG, (b) dioleoyl PG, (c) 1-palmitoyl-2-linoleoyl PG, and (d) dilinoleoyl PG.

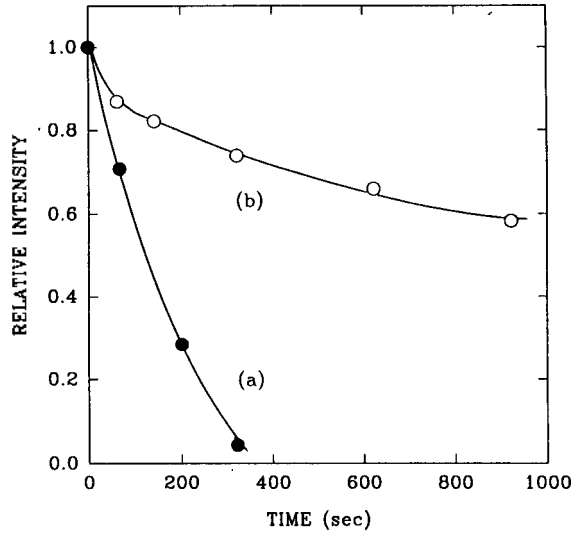

Figure 6. Destruction of the spin label attached to cyt *c* by the interaction with liposomes containing 20 mol% dilinoleoyl PE (a) in the absence and (b) in the presence of 200 mM KCl.

sity of 16-doxylstearate was observed (Figure 3b). When the same experiment was repeated at pH 4, the decay of 16-doxylstearate became very fast (Figure 3d). Interestingly, the intensity of the spin label attached to cyt *c* decreased very slowly (Figure 3c) at pH 4, *i.e.*, the spin label embedded in the membrane was destroyed at a greater rate at pH 4 but destruction of the spin label on cyt *c* was faster at pH 7.

Requirement of unsaturated fatty acid chains for the phenomenon was illustrated in Figure 4. Liposomes containing beef heart CL, dilinoleoyl PG, and dilinoleoyl phosphatidylserine (PS) destroyed the spin label on cyt *c* with nearly equal efficiency. Approximately 90% of the fatty acid chains in beef heart CL was linoleic acid. Therefore, as far as acidic phospholipids are concerned, the structure of headgroup was not an important factor that determine the rate of destruction. When the fatty acid chains were fully saturated, the spin label on cyt *c* as well as that on 16-doxylstearate was not destroyed at all by the cyt *c*-membrane interaction (data not shown). Since the acidic phospholipids (CL, PG, and PS) were in a matrix of egg PC which contained large amount of doubly unsaturated fatty acids, it can be concluded that only the fatty acids of an acidic phospholipid that interacts with cyt *c* need be unsaturated. In other words, liposomes containing acidic phospholipids with saturated fatty acids (e.g. tetrastearoyl CL or dipalmitoyl PG) never destroyed the spin labels regardless of the unsaturation level of the fatty acids in PC matrix.

In Figure 5, four PG's with different level of unsaturation were compared for the efficiency of destroying the spin label on cyt *c*. As expected, dilinoleoyl PG was the most effective. Dioleoyl PG destroyed the spin label on a bound cyt *c* at a much lower rate than dilinoleoyl PG. Interstingly, PG with one palmitic (16 : 0) and one linoleic (18 : 2) acid was much more effective than that with dioleoyl (18 : 1). Therefore a doubly unsaturated fatty acid chain is required for a fast

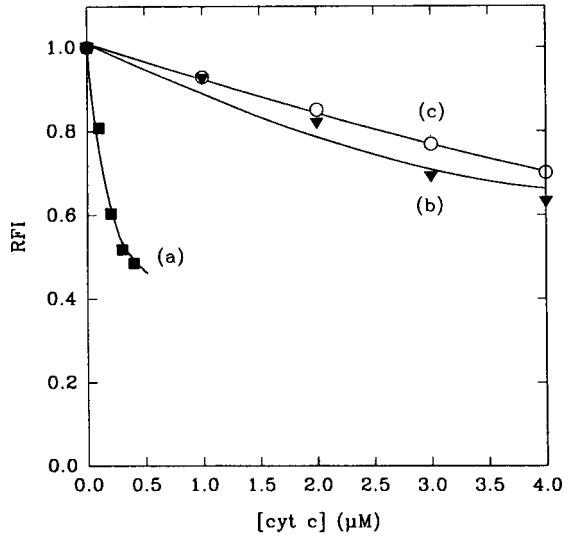

Figure 7. Quenching of pyrene fluorescence by the heme of cyt *c*. PPHPC (1mol%) was incorporated in the liposomes containing (a) 10 mol% beef heart CL, (b) 20 mol% dilinoleoyl PE, and (c) 20 mol% dipalmitoyl PC. Aliquots of cyt *c* solution were added to a liposome suspension (25 μM in phospholipid concentration) and the pyrene moiety was excited at 344 nm. Relative fluorescence intensity (RFI) was measured at 394 nm. As cyt *c* binds to the membrane, the heme absorbs the fluorescence of the pyrene moiety by a F rster-type energy transfer.

destruction of the spin label by the cyt *c*-membrane interaction.

Cyt *c* did not bind to liposomes of 100% PC. Nor was the spin label distroyed by the cyt *c*-membrane interaction. Therefore it was suggested that the binding of cyt *c* to the membrane is essential for the generation of a reactive species that destroys the spin label. An interesting case was the liposomes containing 20 mol% dilinoleoyl PE. PE is a

major, neutral lipid of the inner mitochondrial membrane. When cyt *c* was incubated with PE-containing liposomes, a very rapid decay of the spin label was observed (Figure 6a). Like other acidic phospholipids, PE with saturated fatty acid chains did not destroyed the spin label. The EPR spectrum of spin-labeled cyt *c* interacting with PE was very different from that interacting with acidic phospholipids (compare Figures 1b and 1d): there was no indication of the cyt *c* binding to PE-containing liposomes. In order to confirm that cyt *c* does not bind to PE, we measured the efficiency of resonance energy transfer from a pyrene moiety attached to a fatty acid chain to the heme of cyt *c*.[19] Binding causes a decrease in the fluorescence intensity of pyrene due to efficient quenching by the heme of bound cyt *c*. The method has been shown to be very convenient in assessing the cyt *c* binding. As shown in Figure 7, CL-containing liposomes were able to bind cyt *c* very efficiently (Figure 7a) whereas liposomes containing 100% PC or 20 mol% PE did not bind cyt *c* as evidenced by a poor quenching of the pyrene fluorescence. It is remarkable, however, that the destruction of the spin label was largely retarded in the presence of 200 mM KCl (Figure 6b).

It was not easy to identify the reactive species that destroyed the spin label. In the case of acidic phospholipids, addition of KCl at high concentration partially inhibited the decay of the spin label demonstrating that an electrostatic interaction between cyt *c* and the membrane is required for the process. A slow decay, however, persisted even at a relatively high concentration (e.g. 200 mM) of KCl. Anaerobiosis or addition of cyanide also slowed down the process indicating the heme of cyt *c* and oxygen were involved in the process. These results are consistent with those of Brown and Wüthrich.[10] Optical absorption spectra were recorded on the samples that were used in the EPR experiment. Neither Soret- nor Q-band absorption of the heme was perturbed by addition of liposomes at pH 7. This suggests that the bound cyt *c* is in six-coordinated, ferric configuration. At pH 4, however, the Soret maximum shifted to a shorter wavelength with a concomitant decrease in the intensity, corresponding to a large alteration in the coordination state (data not shown).

A spin-trapping experiment was carried out with DMPO to identify the reactive species that destroyed the spin labels. When cyt *c* was incubated with freshly prepared liposomes at the concentrations used in the decay experiment, no significant amount of radical species was trapped by DMPO. A small signal corresponding to hydroxyl radical adduct of DMPO was detected, however, when cyt *c* at a higher concentration (150 μM) was allowed to interact with liposomes that were aged aerobically to increase peroxidation of the fatty acid chains (data not shown). It is not clear at the moment if this hydroxyl radical is responsible for the decay of the spin label although it is possible that a small amount of hydroxyl radical was indeed produced under the conditions used in the decay experiments.

Discussion

Heme proteins are known to cause membrane oxidation,[11~14] whose consequences include inactivation of the membrane proteins[21,22] and a damage to membrane integrity.[5,14] Oxidation of the inner mitochondrial membrane by cyt *c* in particular has been studied extensively because cyt *c* interacts with the inner mitochondrial membrane[1] and mitochondria are under an oxidative stress.[15] The mechanisms of the protein-membrane interaction and concurrent membrane oxidation are not well understood on a molecular level. In the case of cyt *c*, the initiation reaction is probably the heme-catalyzed homolytic scission of preformed hydroperoxides.[11,23] This is in accord with our observation that aged liposomes, known to have a higher hydroperoxide content, destroyed the spin labels more efficiently than freshly prepared liposomes. Cyt *c* also catalyzes the formation of new lipid hydroperoxides. Goñi *et al.*[11] proposed that singlet oxygen may also be involved in the membrane oxidation as a reaction intermediate.

Wüthrich and Brown[10] used spin-labeled cyt *c* to monitor generation of a reactive species that is responsible for the cyt *c*-catalyzed membrane oxidation. Focusing on the electrostatic interaction between positively charged cyt *c* and negatively charged inner mitochondrial membrane, they found that the binding of cyt *c* to CL-containing liposomes results in destruction of the spin label on cyt *c*. We extended the experiments to other lipids and pH. A fatty acid chain with two double bonds (e.g. linoleic acid) was much more efficient than that with no or one double bond. Goñi *et al.*[11] performed a chromatographic analysis of the fatty acid composition before and after incubation of cyt *c* with asolectin. A severe loss of linoleic acid (18 : 2) was observed without a significant change in the composition of other phospholipids. This is consistent with our results suggesting that linoleic acid among other fatty acids is very reactive in producing oxidants by the cyt *c*-membrane interaction. The composition of the liposomes was 80 mol% of PC and 20 mol% (in terms of fatty acid chains) of PG or CL. Linoleic acid and arachidonic acid, which are prone to oxidation, comprises about 20% of the total fatty acids in egg PC used in the experiment. When acidic phospholipids in the liposomes contained only saturated fatty acids, there was no decay of the spin label although the remaining PC contained linoleic acid. This strongly suggests that interaction of cyt *c* and acidic membranes sequesters acidic phospholipids so that cyt *c* is not in direct contact with PC molecules. In this respect, it is interesting to note a model proposed by Rytömaa and Kinnunen[8] in which a fatty acid chain of the acidic phospholipid is accomodated inside the hydrophobic crevice of cyt *c*. This may explain why an unsaturated fatty acid chain is required in the acidic phospholipids that interact with cyt *c*, not in the PC molecules. Involvement of the heme in the process may also be accounted for by the model. Validity of the model, however, awaits further experimental tests.

At pH 4, oxidation of the spin label attached to cyt *c* was largely retarded. Instead that of 16-doxylstearate was very rapid. The opposite was true for the process at pH 7. The heme may be in direct contact with the membrane interior as the fraction of 5-coordinate heme increases at pH 4.[24] We also observed a large change in the optical absorption spectrum that suggests a transition from a six-coordinate state to a five- or four-coordinate state. Fast oxidation of 16-doxylstearate and slow oxidation of the spin label on cyt

c at low pH can be explained by the location of the heme in the membrane as well as the higher reactivity with oxygen of the five- or four-coordinated heme than the six-coordinate heme.

PC and PE are the two major components of the inner mitochondrial membrane. Both are electrically neutral so that they do not bind cyt *c* as shown in Figure 7. Liposomes containing 100% PC, irrespective of its fatty acids composition, did not generate reactive species *via* interaction with cyt *c*. Incubation of cyt *c* with PE-containing liposomes, on the other hand, destroyed the spin label on cyt *c* at a rate even greater than those for acidic phospholipids. The results can not be explained by the same mechanism as that for acidic phospholipids because the fluorescence experiment indicates that the binding of cyt *c* to the membrane is not significant. It is even more intriguing that high concentration of KCl inhibits the process suggesting the interaction may be electrostatic in nature (Figure 6). Enhanced oxidation of membrane by inclusion of PE was also observed in hemosomes,[13] which consists of hemoglobin sandwiched between the membranes of multilamellar liposomes. However, the situation is very different from that in the present study. Since KCl inhibited the destruction of the spin label, it is likely that an electrostatic interaction is responsible for the process. The interaction, however, has to be characterized by a large dissociation constant to rationalize the low binding affinity of cyt *c* to the membrane. It is possible, however, that a cyt *c* molecule stays in the transiently bound state long enough to undergo oxidation: *i.e.*, the on-rate may be relatively large for a small association constant, which is simply a ratio of the on-rate and off-rate. It is not clear how the substitution of H in the amino group of PE by CH3 in that of PC makes such a large difference in the membrane oxidation. Therefore the above interpretation has to be considered tentative.

In summary, binding of cyt *c* to liposomes containing acidic phospholipids efficiently destroyed the spin labels. The bound cyt *c* seemed to sequester the acidic phospholipids. Membrane oxidation was efficient in the order linoleic≫ oleic>stearic acid. At pH 4, decay of the spin label on cyt *c* was very slow but that on stearic acid was fast, presumably due to a direct contact of the heme with the membrane interior. PE also caused rapid membrane oxidation despite its electrical neutrality that prevents cyt *c* from binding to the membrane. Addition of KCl, however, inhibited the process indicating that the electrostatic interaction was at least partially responsible for the decay of the spin labels.

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