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Molecular Mechanisms for the Induction of Peroxidase Activity of the Cytochrome *c*−Cardiolipin Complex

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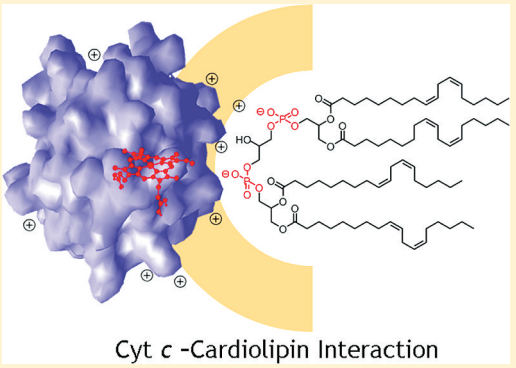
Supporting Information

ABSTRACT: Induction of the peroxidase activity of cytochrome *c* (cyt *c*) by cardiolipin (CL) and H_2O_2 in mitochondria is suggested to be a key event in early apoptosis. Although electrostatic interaction between the positively charged cyt *c* and negatively charged CL is a predominant force behind the formation of a specific cyt *c*−CL complex and sequential induction of the peroxidase activity, molecular mechanisms of hydrophobic interactions involving the fatty acyl chains of CL remain to be investigated. To elucidate the function of the acyl chains, particularly the role of the double bond, we synthesized a variety of CL analogues and examined their peroxidase inducing activity. Irrespective of the number of double bonds in the acyl chains, the peroxidase activity of cyt *c* induced by liposomes composed of 1,2-dioleoyl-*sn*-glycero-3-phosphatidylcholine (DOPC) and a different CL (9:1 molar ratio) was similar, except for that of 1,1′,2,2′-tetrastearoylcardiolipin (TSCL, C18:0)-containing liposomes. The peroxidase inducing activity of TSCL-containing liposomes was 3−4-fold greater than that of other CL-containing liposomes. The peroxidase activity induced by all CL-containing liposomes was much lower at high ionic strengths than that at low ionic strengths because of diminution of the electrostatic interaction. The peroxidase inducing effects of various CL-containing liposomes were related well to their ability to associate with cyt *c*. Thus, our results revealed that at low CL levels, the saturated acyl chain of CL is favorable for the activation of peroxidase activity of CL-bound cyt *c* and the proposed critical role of the double bond is not a general feature of the cyt *c*−CL interaction. The polarity of the membrane surface of TSCL-containing liposomes was slightly, but significantly, lower than that of other CL-containing liposomes, suggesting that the higher activating ability of TSCL-containing liposomes may be due to a reduced level of hydration of the polar head region reflecting tighter packing of the fully saturated acyl chains. Moreover, using CL analogues in which a central glycerol head moiety was modified, we revealed that the natural structure of the head moiety is not critical for the formation of the active cyt *c*−CL complex. The effects of the CL content of the liposomal membrane on the cyt *c*−CL interaction are discussed.



Cardiolipin (CL), a negatively charged phospholipid bearing four fatty acyl chains, is a major phospholipid found in mammalian mitochondria (up to ∼20%) with a multitude of biological functions.[1,2] For example, CL is responsible for regulating the activity of several proteins involved in ATP biosynthesis,[3,4] though the precise molecular mechanism involved remains to be elucidated. CL also associates with members of the apoptotic machinery:[5−7] specific interactions between CL and cytochrome *c* (cyt *c*), Bid, and more recently caspase-8 have now been established. It is becoming apparent that CL is a versatile phospholipid that not only participates in membrane fluidity and the activity of the electron transport chain but also plays a major role in cell signaling through CL−protein interactions.[6,7]

The ability of cyt *c* to elicit distinct physiological functions within mitochondria and in the cytosol is related to the cellular localization of the heme protein.[8] Cyt *c* is a positively charged protein (net charge of +8 at neutral pH), while the inner mitochondrial membrane contains a large fraction of a negatively charged phospholipid, CL. The detachment of cyt *c* from the inner mitochondrial membrane involves a reversible interaction of the protein with the lipid bilayer. Therefore, the nature of the interaction of cyt *c* with the lipid bilayer has been a focus of extensive studies.[9−16]

Kagan and colleagues demonstrated that CL-bound cyt *c* (cyt *c*−CL complex) acts as a peroxidase capable of catalyzing the H_2O_2-dependent peroxidation of CL and the oxidation of CL is a critical step in the release of cyt *c* into the cytosol during the early stages of apoptosis.[17,18] As native cyt *c* having a hexacoordinated heme iron elicits no peroxidase activity, CL must induce structural change (i.e., unfolding) of cyt *c* to form a pentacoordinated heme iron, which facilitates the access of H_2O_2 to the heme iron catalytic site. It is thought that although formation of the cyt *c*−CL complex is primarily driven by electrostatic interaction, CL destabilizes the tertiary structure of cyt *c* through hydrophobic interaction by inserting one of the four acyl chains into a hydrophobic pocket of cyt *c*; that is, one

Received: July 3, 2011
Revised: August 7, 2011
Published: August 30, 2011

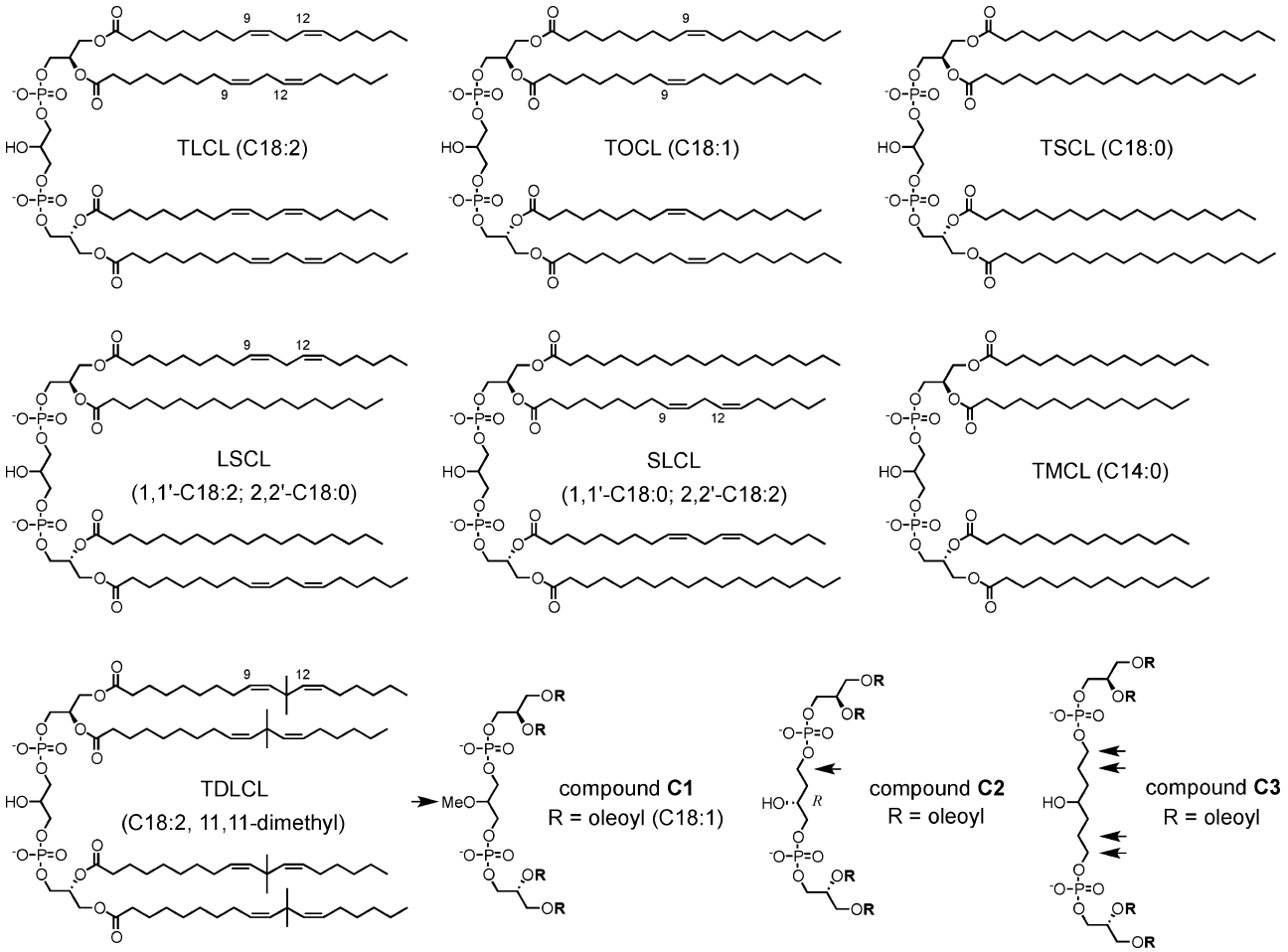


Figure 1. Structures of cardiolipins synthesized in this study. For compounds **C1**−**C3**, the modified position is denoted with an arrow. All compounds were purified as ammonium salts (see the Supporting Information).

of the acyl chains protrudes out of the membrane bilayer to interact with cyt *c*, while the other three acyl chains remain in the membrane bilayer.[18,19] This so-called "lipid-extended" model was originally proposed to explain the binding of acidic phospholipids to cyt *c* by Kinnunen and colleagues,[10] and consequent studies have supported this model.[11−14]

In the formation of the cyt *c*−CL complex and sequential induction of the peroxidase activity of cyt *c*, the presence of a double bond(s) in the acyl chains of CL was suggested to be critical.[17−19] For instance, using liposomes made of phosphatidylcholine and a different CL (at a 1:1 molar ratio), Belikova et al.[18] indicated that the ability to induce the peroxidase activity decreases in the following order: polyunsaturated tetralinoleoyl CL (TLCL, C18:2) ≥ monounsaturated tetraoleoyl CL (TOCL, C18:1) ≫ saturated tetramyristoyl CL (TMCL, C14:0). They also found that the cyt *c*−TOCL complex, but not the cyt *c*−TMCL complex, is remarkably resistant to dissociation at high salt concentrations. They interpreted these observations to mean that a specific hydrophobic interaction arising from the double bond(s) is important for strongly stabilizing the cyt *c*−CL complex. This notion has been widely accepted[5−7] and sometimes taken to mean that CL having saturated fatty acyl chains cannot stimulate the peroxidase activity of cyt *c*, as seen in ref 5. It should, however, be realized that the total number of carbon atoms of the acyl chains, one of the factors determining the physicochemical properties of lipid bilayers,[20,21] differs between TLCL and TOCL (C_{18}) and TMCL (C_{14}) and that the hydrophobicity of unsaturated alkyl chains, in terms of the partition coefficient between an organic solvent and water, is rather less than that of saturated alkyl chains with the same number of carbon atoms.[22,23]

Nantes and colleagues studied the spin states of cyt *c* interacting with different types of lipid membranes by electron paramagnetic resonance spectroscopy.[12] Their study supported the lipid-extended model but raised questions about the role of the double bond in the fatty acyl chains; namely, both saturated and unsaturated acyl chains can insert into the supposed hydrophobic cleft in cyt *c*, but the latter is less efficient than the former because of steric hindrance caused by the bent form. Moreover, in light of the diversity in fatty acid composition of various CLs from different organisms, Schlame questioned the proposed critical role of polyunsaturation of the acyl chains of CL in the formation of a specific cyt *c*−CL complex in mitochondria.[2] Taken together, the contribution of the hydrophobic interaction to the formation of cyt the *c*−CL complex is generally accepted, whereas the molecular mechanisms of the hydrophobic interaction, particularly the role of the double bond, remain to be elucidated.

One possible explanation for the discrepancy in the role of the double bond in the acyl chains may be differences in experimental techniques and/or conditions, including methods for preparing liposomes and lipid compositions. It should also be noted that the previous studies used only natural and/or a

dx.doi.org/10.1021/bi2010202 | *Biochemistry* 2011, 50, 8383−8391

few commercially available CLs; the acyl chains are a mixture of different fatty acids in the former, and chemical variation of the chains is very poor in the latter. Therefore, to explore the molecular mechanisms behind the formation of the cyt *c*−CL complex and induction of the peroxidase activity of cyt *c*, biochemical studies using structurally diverse CL analogues are needed. In this study, we synthesize a series of CL analogues by the procedure recently developed in our laboratory.[24] With numerous CLs in hand, we examined the structural factors required to induce the peroxidase activity of the cyt *c*−CL complex. Our results reveal that the presence of a double bond(s) in the fatty acyl chains is not essential for the induction of peroxide activity of cyt *c*.

EXPERIMENTAL PROCEDURES

Materials. Horse heart cyt *c* (type C7752, >95%) was purchased from Sigma-Aldrich (St. Louis, MO). 1,2-Dioleoyl-*sn*-glycero-3-phosphatidylcholine (DOPC) was obtained from NOF Corp. (Tokyo, Japan). Aminophenyl fluorescein (APF) was purchased from Sekisui Medical Co., Ltd. (Tokyo, Japan). Amplex Red (AR) and *N*-(5-dimethylaminonaphthalene-1-sulfonyl)-1,2-dihexadecanoyl-*sn*-glycero-3-phosphoethanolamine triethylammonium salt (DHPE) were purchased from Invitrogen Co. (Carlsbad, CA).

Synthesis of Various Cardiolipins. The CLs synthesized in this study are shown in Figure 1. The synthetic procedures for these CLs and the spectral data are given as Supporting Information. The name of each CL is represented in Abbreviations. The CL analogues, in which the central glycerol skeleton was modified, were termed compounds **C1**−**C3**.

Preparation of Liposomes. Liposomes (large unilamellar vesicles) made of DOPC alone or a CL/DOPC mixture (at a 1:9, 2:8, or 1:1 molar ratio) were prepared by the extrusion method.[25,26] Stock solutions of DOPC and CL in a methanol/chloroform (1:3, v/v) solution were mixed in the required proportions. A thin lipid film obtained by evaporating organic solvents and leaving them under vacuum overnight to remove the residual organic solvents was hydrated with a HEPES buffer [10 mM HEPES and 100 μM DTPA (pH 7.4)] and vortexed. After five rounds of freezing and thawing under an Ar atmosphere, the lipid suspension was extruded through a 100 nm pore size polycarbonate filter using a LiposoFast device (Avestin, Ottawa, ON). For the binding reaction with cyt *c*, sucrose-loaded liposomes were prepared by the same method in a HEPES buffer [10 mM HEPES, 100 μM DTPA, and 48 mM sucrose (pH 7.4)]. The particles scarcely varied in diameter with lipid composition, and the mean diameter, determined from dynamic light scattering measurements (Photal FPAR-1000, Otsuka Electronic, Osaka, Japan), was ~110 nm. The concentration of DOPC in liposomal preparations was determined using an enzyme assay kit for choline[26] (Wako, Osaka, Japan).

Determination of the Peroxidase Activity of Cyt *c*. The peroxidase activity of cyt *c* based on the H_2O_2-induced oxidation of APF was determined by measuring the fluorescence of fluorescein, an oxidation product of APF.[27] Cyt *c* (1.0 μM) was incubated with DOCP or CL/DOPC liposomes (450 μM choline, equivalent to 50 μM CL) in 2.0 mL of reaction buffer [10 mM HEPES and 100 μM DTPA (pH 7.4)] for 10 min at 25 °C. The peroxidase reaction was started with the addition of APF (10 μM) and H_2O_2 (100 μM) and conducted for 10 min. After a 1−2 min lag, depending on the CL, the reaction rate was linear in the time interval. Fluorescence was measured with a Hitachi RF-5000 spectrophotometer with excitation and emission wavelengths of 490 and 515 nm, respectively (excitation and emission slit width of 5 nm). The concentration of peroxidized APF was estimated from the fluorescent intensity according to the procedure described in ref 27.

The peroxidase activity was also determined using AR (10 μM) as a substrate with excitation and emission wavelengths of 535 and 585 nm, respectively. When AR was used, the concentration of H_2O_2 was reduced (50 μM) to prevent the transformation of resorufin, an oxidation product of AR, into less fluorescent product(s).[28]

Binding of Cyt *c* to Liposomes. Cyt *c* (1.0 μM) and sucrose-loaded liposomes (225 μM choline, equivalent to 25 μM CL) were incubated in 1.0 mL of reaction buffer [10 mM HEPES, 100 μM DTPA, and 25 mM KCl (pH 7.4)] at 25 °C for 10 min using a 1.5 mL polycarbonate centrifuge tube. The mixture was centrifuged at 60000*g* for 1 h at 25 °C using a Beckman Optima MAX-E instrument. Sucrose-loaded liposomes were not under osmotic stress because of the presence of 25 mM KCl in the binding buffer.[29] Immediately after the ultracentrifugation, 50 μL of the supernatant was subjected to a high-performance liquid chromatography (HPLC) analysis of cyt *c* using a BIO wide pore C18 column (4.6 mm× 150 mm) (Supelco, Bellefonte, PA).[30] A linear gradient increasing from 20 to 60% acetonitrile in water over a 15 min period was employed at a flow rate of 1.0 mL/min with monitoring at 393 nm.[30] Both the 20 and 60% acetonitrile solutions contained 100 mM KCl and 0.1% trifluoroacetic acid (v/v). The binding affinity of cyt *c* for liposomes was expressed as an index, $B_{L/S}$, defined as the number of moles of bound cyt *c* per milligram of DOPC per mole of unbound cyt *c* per milliliter of supernatant.

Alternatively, we also measured the amount of cyt *c* in both the supernatants and the liposomal pellets by 12.5% sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS−PAGE). The liposomal pellets were suspended in 120 μL of binding buffer and solubilized via addition of 4× Laemmli's sample buffer (40 μL). Cyt *c* on the gel was visualized by silver staining and quantified by exposure to an imaging plate (BAS-MS2040, Fuji Film, Tokyo, Japan) and digitalization ("Multi Gauge" software, Fuji Film). Nevertheless, we did not employ this method for estimation of the $B_{L/S}$ value because of relatively large errors in quantifying the digital image over a high cyt *c* concentration range.

The separation of sucrose-loaded liposomes from the supernatant was verified by both liquid chromatography−mass spectroscopy analysis and choline detection, and less than 5% of total DOPC was present in the supernatant fractions after ultracentrifugation. To determine the level of nonspecific binding of cyt *c* to the walls of the centrifuge tubes, we conducted the incubation and centrifugation under the same experimental conditions without liposomes. The nonspecific binding accounted for ~10% of the total cyt *c* added to the binding reaction mixture.

Determination of the Polarity of the Liposomal Membrane Surface. To determine the polarity of the aqueous interface of liposomal membranes, we prepared liposomes containing a lipid fluorescent probe (DHPE), which possesses a dansyl chromophore in the polar headgroup, by the method described above (9:1:0.1 DOPC:CL:DHPE molar ratio). The maximum emission wavelength (λ_{max}) of DHPE around 530 nm was measured at 25 °C using a Hitachi RF-5000 spectrophotometer with excitation at 336 nm

dx.doi.org/10.1021/bi2010202 | *Biochemistry* 2011, 50, 8383−8391

(excitation and emission slit widths of 5 nm). The liposomal concentration was 450 μM DOPC (equivalent to 5.0 μM DHPE) in 2.5 mL of a buffer [10 mM HEPES and 100 μM DTPA (pH 7.4)].

RESULTS

Activation of the Peroxidase Activity of Cyt *c* by CL-Containing Liposomes. The interaction between cyt *c* and CL-containing liposomes becomes complicated as the content of CL greatly increases. For instance, the reversibility of the interaction diminishes significantly with an increasing CL content primarily because of formation of nonlamellar structure in liposomes.[9,10,13] Therefore, we fixed the CL:DOPC molar ratio, unless otherwise noted, at 1:9 to prevent difficulty in interpreting the experimental data. To make the lipid composition identical to that in the studies of Kagan and colleagues,[17−19] we used DOPC as a phosphatidylcholine.

The peroxidase activity of cyt *c* activated by DOPC or different CL/DOPC liposomes was determined at a cyt *c*:DOPC ratio of 1:450 (1:50 cyt *c*:CL ratio) using APF as a peroxidizable substrate, which has greater resistance to autoxidation than the widely used AR and 2,7-dichlorodihydrofluorescein (DCF).[27] All measurements were performed in at least triplicate using independently prepared liposomes. The peroxidase activity in the presence of DOPC liposomes was identical to that of soluble cyt *c*, indicating that liposomes composed of a neutral lipid alone have little activating ability (Figure 2A). Compared to DOPC liposomes, all CL-containing liposomes efficiently activated the peroxidase activity. When the activation was compared among CLs having the same number of carbon atoms (C_{18}), the ability of saturated TSCL (18:0) was found to be markedly better than that of unsaturated TOCL (C18:1) and TLCL (C18:2). The superior efficiency of TSCL to TOCL was also confirmed at different cyt *c*:CL ratios: 5 μM cyt *c* versus 450 μM DOPC (1:10 cyt *c*:CL) and 1 μM cyt *c* versus 900 μM DOPC (1:100 cyt *c*:CL) (data not shown). The level of activation by a saturated, but shorter, TMCL (C14:0) was significantly lower than that by TSCL and comparable to that by TOCL and TLCL. When AR was used as a substrate in place of APF, a similar tendency was observed; the activation efficiency of TSCL was significantly superior to that of TLCL and TOCL (Figure 2B).

To examine the effect of CL content, we also determined the peroxidase inducing ability of TSCL, TOCL, TLCL, and TMCL with CL/DOPC (2:8) liposomes under the same experimental conditions using APF as a substrate. The advantage of saturated TSCL over other CLs was confirmed with the liposomes containing 20 mol % CL (Figure 2C). When the corrected peroxidase activity (by subtracting the activity of DOPC liposomes from an apparent total activity of CL-containing liposomes) was compared between the CL/DOPC (1:9) and CL/DOPC (2:8) systems, the difference between TSCL and other CLs was diminished with an increase in CL content (panel A vs panel C of Figure 2). Taken together, our results clearly indicate that if the total number of carbon atoms is the same, the saturated acyl chain of CL is favorable for the induction of peroxide activity of the cyt *c*−CL complex and the presence of a double bond is not critical. The effects of CL content on the interaction between cyt *c* and CL-containing liposomes will be discussed later.

Effects of Modification of the Linoleoyl (C18:2) Chain. We synthesized TDLCL (Figure 1) for several reasons. First, if the presence of a double bond(s) is critical to the peroxidase activity of cyt *c*, specific interactions (both steric and electronic) between the double bond(s) and its supposed hydrophobic counterpart(s) in cyt *c* would occur. To check the steric hindrance around the double bonds, TDLCL possessing two methyl groups at the C11 position should be useful. Second, as polyunsaturated TLCL is much more readily peroxidized by the activated cyt *c* than TOCL and TSCL,[19] we cannot exclude the possibility that the amount of intact TLCL in TLCL/DOPC liposomes is diminished by self-peroxidation under the experimental conditions. To prevent the peroxidation of TLCL, the highly reactive methylene sandwiched by two double bonds was masked by dimethylation in TDLCL.

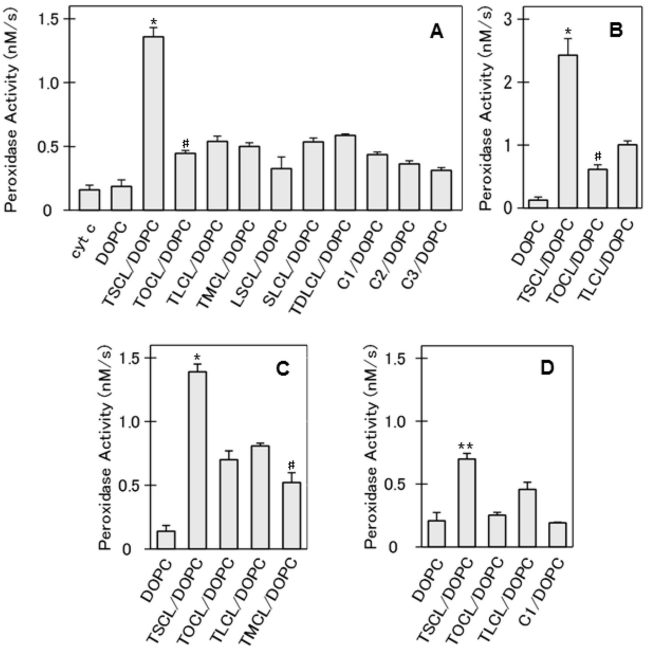


Figure 2. Peroxidase activity of cyt *c* induced by DOPC or different CL/DOPC liposomes. (A) Cyt *c* (1.0 μM) was incubated with DOCP or CL/DOPC (1:9 molar ratio) liposomes (450 μM choline, equivalent to 50 μM CL) in 2.0 mL of reaction buffer [10 mM HEPES and 100 μM DTPA (pH 7.4)] for 10 min at 25 °C. The peroxidase reaction was started by the addition of APF (10 μM) and H_2O_2 (100 μM) and conducted for 10 min. As a reference, the peroxidase activity of soluble cyt *c* without liposomes is shown. (B) Same experimental conditions as described for panel A, except that AR (10 μM) in place of APF was used as a peroxidizable substrate and the concentration of H_2O_2 was reduced to 50 μM to prevent the transformation of resorufin into less fluorescent product(s).[28] (C) Same experimental conditions as described for panel A, except that CL/DOPC (2:8 molar ratio) liposomes were used (400 μM choline, equivalent to 100 μM CL). (D) Same experimental conditions as described for panel A, except that the pH of the reaction buffer was 6.4. Bars show the mean ± standard deviation of three independent measurements: $^{\#}P < 0.01$ vs DOPC liposomes, $^{*}P < 0.01$ vs TOCL/DOPC liposomes, and $^{**}P < 0.01$ vs TLCL/DOPC liposomes.

As shown in Figure 2A, the peroxidase inducing ability of TDLCL/DOPC liposomes (1:9) was almost identical to that of TLCL/DOPC liposomes, indicating that the steric hindrance around the double bonds in linoleoyl chains does not hamper the formation of an active cyt *c*−CL complex. It is also likely that self-peroxidation of TLCL is not a reason for its poor ability compared to that of TSCL.

Positional Specificity of the Function of Acyl Chains. In the so-called lipid-extended model for the cyt *c*−CL

dx.doi.org/10.1021/bi2010202 | *Biochemistry* 2011, 50, 8383−8391

complex, the positional specificity of the function of acyl chains (*sn*-1 or *sn*-2) has not been fully investigated. Using 1-palmitoyl-2-oleyl-*sn*-glycerophosphoglycerol (POPG) as a model of CL, Tuominen et al.[11] suggested that the *sn*-2 acyl chain of POPG would protrude out of the lipid bilayer and associate with a supposed hydrophobic pocket in cyt *c*. This idea was based on a conformational prediction of phospholipids; the *sn*-2 acyl chain may be more readily adopted for the extended conformation than the *sn*-1 chain.[31] It was also largely based on the fact that specific hydrophobic interaction with the *sn*-2 acyl chain of phospholipids is a major determinant for the binding of annexin to lipid membranes.[32]

To investigate the positional specificity of the function of stearoyl chains in TSCL regarding its high peroxidase inducing activity, we synthesized LSCL and SLCL, which have both stearoyl (C18:0) and linoleoyl (C18:2) acyl chains, but in opposite positions at the *sn*-1 and *sn*-2 sites (see Figure 1). SLCL was slightly superior to LSCL, whereas both were much weaker than TSCL (Figure 2A). It is therefore likely that the strong activating effect of TSCL can be attributed to the presence of stearoyl chains at both positions.

Effects of Modification of the Polar Headgroup of CL. The electrostatic interaction between cyt *c* and CL, the predominant force initiating the formation of the cyt *c*−CL complex, has been closely examined using different techniques.[10−15,33] Despite some contradictions in the details, a charged patch of basic residues in cyt *c*, with Lys72, Lys73, Lys86, and Lys87 being the most frequently identified ones,[12,14,15] was thought to be critical for the electrostatic interaction. However, to the best of our knowledge, no study has examined the effect of structural changes in the polar head moiety of CL on the formation of the cyt *c*−CL complex. Therefore, to investigate whether the chemical structure of the polar head moiety of CL is critical for the association with cyt *c*, we synthesized three CL analogues fixing all acyl chains with an oleoyl group (compounds **C1**−**C3** in Figure 1). The central glycerol OH group of TOCL was masked by methylation to abolish the hydrogen bond donating ability of **C1**. The central glycerol skeleton was asymmetrically elongated by one methylene unit in **C2**. In **C3**, the glycerol skeleton was symmetrically elongated by two methylene units, for a total of four methylene units. We here chose TOCL as a reference, given that the effects of structural modifications might be amplified or even masked by using TSCL that exhibits high peroxidase inducing activity.

The peroxidase inducing effect of **C1** was almost the same as that of TOCL (Figure 2A), indicating that the central OH group is not essential for activating the peroxidase activity. This result strongly suggests that hydrogen bonding between the OH group and cyt *c* (intermolecular) or the phosphate group (intramolecular) is not important for activation. The activities of **C2** and **C3** decreased considerably compared to that of TOCL but were still higher than that of liposomes with DOPC alone. Thus, structural modifications of the central glycerol skeleton did not result in a critical loss of activity, indicating that this moiety is not recognized in a strict sense by cyt *c*.

Effects of pH on the Peroxidase Inducing Activity. pH is one of the factors that modulate the binding of cyt *c* to CL-containing liposomes as well as the peroxidase activity of unfolded cyt *c*.[13,34] To investigate the effect of pH on the peroxidase activity of CL-bound cyt *c*, we determined the activity at pH 6.4 using CL/DOPC (1:9) liposomes and compared it with the activity determined at pH 7.4. Prior to the experiments, we confirmed that an increase in vesicle turbidity, which results from cyt *c*-mediated fusion of vesicles at low pH (<7),[35] dose not occur under our experimental conditions (1:50 cyt *c*:CL ratio).

The peroxidase inducing activity of all CL-containing liposomes decreased significantly with a decrease in pH; the activity of TOCL- and **C1**-containing liposomes was almost the same as that of liposomes with DOPC alone (Figure 2D). Nonetheless, the superiority of fully saturated TSCL over other CLs was still maintained. Given that the extent of ionization of the phosphate group of CL incorporated into liposomal membranes changes drastically over the pH range of 6−8,[13] the significant decrease in activity may be primarily due to pronounced diminution of an ionized form of the phosphate group responsible for the electrostatic interaction with cyt *c*.

Effects of Ionic Strength on the Peroxidase Inducing Activity. To further characterize the electrostatic and hydrophobic interactions between cyt *c* and CL-containing liposomes, we studied the effects of different ionic strengths on the peroxidase inducing activity. The electrostatic interaction between cyt *c* and CL is known to be remarkably reduced in a high-ionic strength buffer.[9,13,18] The procedure used to vary ionic strength was adopted from ref 18 to facilitate comparisons. When cyt *c* and CL/DOPC (1:9) liposomes were mixed in a high-ionic strength buffer containing 1.0 M KCl, the Fe−S(Met80)-associated 695 nm band of cyt *c* remained unchanged (data not shown) and the level of peroxidase activity was essentially as low as that of DOPC liposomes (Figure 3, second set of bars). As a reference, the peroxidase activity measured at low ionic strengths (taken from Figure 2A) is presented in the first set of bars. These results indicate that the electrostatic interaction is absolutely necessary to initiate the formation of an active cyt *c*−CL complex.

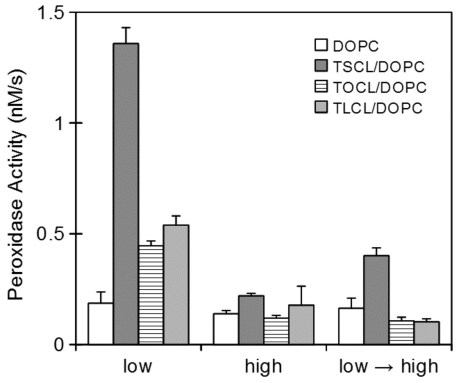


Figure 3. Peroxidase activity of cyt *c*−CL complexes at different ionic strengths. For the first set of bars, the peroxidase activity was taken from Figure 2A. For the second set of bars, the same reaction conditions as in Figure 2A were used with 1.0 M KCl. For the third set of bars, KCl was added to give a final concentration of 1.0 M after incubation of cyt *c* with liposomes for 10 min under the same reaction conditions as in Figure 2A. Bars show means ± the standard deviation of three independent measurements.

When KCl was added to give a final concentration of 1.0 M after the cyt *c*−CL complex had formed, the peroxidase activities of all CL-containing liposomes remained significantly lower than those measured in a low-ionic strength buffer, indicating almost complete reversibility of the association of cyt *c* and CL-containing liposomes. This result is in line with an earlier study,[9] which showed the dissociation of cyt *c* from CL-

dx.doi.org/10.1021/bi2010202 | *Biochemistry* 2011, 50, 8383−8391

containing liposomes by increasing KCl concentrations to be almost complete when the CL content was less than ~20%. It should be mentioned that irrespective of ionic strength, the peroxidase activity induced by TSCL/DOPC liposomes was higher than that induced by TLCL/DOPC, TOCL/DOPC, or DOPC liposomes.

Inconsistent with our results, Belikova et al.[18] reported that the cyt *c*−TOCL complex formed with TOCL/DOPC (1:1) liposomes, but not the cyt *c*−TMCL complex, is considerably resistant to addition of KCl (final concentration, 1.0 M) after the formation of the cyt *c*−CL complex; 60−70% of the peroxidase activity before the addition of KCl was retained (see Figure 6 of ref 18). A possible explanation for this discrepancy will be discussed later.

Binding of Cyt *c* to CL-Containing Liposomes. The binding affinity of cyt *c* for CL-containing liposomes (1:9 CL:DOPC) was measured by sedimenting sucrose-loaded liposomes and measuring the amount of soluble (nonbound) cyt *c* in the supernatant. The sedimentation method has several advantages over other methods such as surface plasmon resonance,[36] but a problem that arises with this method is nonspecific binding of proteins to the walls of the centrifuge tube, particularly hydrophobic proteins. While the level of nonspecific binding of cyt *c* was relatively low (~10%) in our case, a correction was made for loss of the protein tentatively assuming that 10% of total cyt *c* binds to the centrifuge tube. The affinity, in terms of the index $B_{L/S}$, is given in Table 1.

Table 1. Binding Affinities ($B_{L/S}$) of Cyt *c* for Different Liposomes

liposomal composition[a]	$B_{L/S}$[b]
DOPC alone	2.1 ± 0.6
TSCL/DOPC	>470[c]
TOCL/DOPC	58 ± 15
TLCL/DOPC	170 ± 44
TMCL/DOPC	66 ± 20
LSCL/DOPC	11 ± 2.0
SLCL/DOPC	43 ± 6.6

[a]For CL-containing liposomes, a 1:9 CL:DOPC molar ratio was used. [b]$B_{L/S}$ represents the number of moles of bound cyt *c* per milligram of DOPC per mole of unbound cyt *c* per milliliter of supernatant. The values are means (±standard deviation) of three independent experiments. [c]Because more than 99% of total cyt *c* (after the correction for nonspecific binding to the centrifuge tube) bound to TSCL/DOPC liposome pellets, the concentration of cyt *c* in the supernatant was too low to allow accurate measurement by HPLC.

Because more than 99% of cyt *c* (after the correction for nonspecific binding) bound to TSCL/DOPC liposomal pellets, the concentration of cyt *c* in the supernatant was too low for an accurate measurement by HPLC; hence, $B_{L/S}$ could not be estimated. Compared to CL-containing liposomes, liposomes with DOPC alone scarcely associated with cyt *c*, consistent with previous studies.[9,37] This result indicates that the presence of negatively charged CL is critical to the binding of cyt *c* to liposomal membranes. Notably, TSCL/DOPC liposomes, which exhibited the most efficient peroxidase inducing effect, showed the greatest affinity among CL-containing liposomes.

We also confirmed a similar variation in the binding affinity by conducting an SDS−PAGE-based analysis of cyt *c* in both the supernatants and the liposomal pellets (Figure 4); the variation in intensity ratios of silver staining (pellets vs supernatants) was comparable to that of the $B_{L/S}$ values. In particular, no significant binding of cyt *c* to liposomes with DOPC alone was observed, and the concentration of cyt *c* in the supernatant for TSCL/DOPC liposomes was the lowest among the results for CL-containing liposomes.

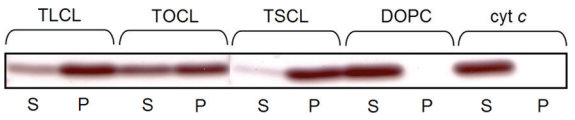


Figure 4. Binding of cytochrome *c* to various liposomes. Cyt *c* (1.0 μM) was incubated with sucrose-loaded CL-containing liposomes (225 μM choline, equivalent to 25 μM CL) or without liposomes (control) and centrifuged as described in Experimental Procedurse. Aliquots (20 μL) of the solubilized pellets (P) and supernatants (S) were subjected to 12.5% SDS−PAGE and visualized by silver staining. Data are representative of three independent experiments.

Variations in the peroxidase inducing ability (Figure 2) and binding affinity ($B_{L/S}$) are compared in Figure S1 of the Supporting Information. The two attributes were closely related; the greater the affinity, the better the peroxidase inducing effect. This result indicates that the peroxidase inducing activity of CL-containing liposomes is primarily decided by their ability to associate with cyt *c*.

Polarity of the Membrane Surface of CL-Containing Liposomes. To gain insight into the pronounced difference in both the peroxidase inducing ability and the binding affinity for cyt *c* of TSCL- and other CL-containing liposomes, we determined the polarity of the aqueous interfacial region of each liposomal preparation using a lipid fluorescent probe possessing a dansyl chromophore in the polar headgroup (DHPE).[38] The λ_{max} was averaged from triplicate experiments using independently prepared liposomes. The λ_{max} of DHPE incorporated into liposomes with DOPC alone was 525 ± 1 nm, which is between that measured in methanol and in a methanol/water mixture (1:1, v/v).[38] The λ_{max} in TSCL/DOPC liposomes was 525 ± 1 nm. For other CL-containing liposomes (TLCL/DOPC, TOCL/DOPC, and TMCL/DOPC), λ_{max} values were essentially the same (528 ± 1 nm), indicating that the maximal wavelength in TSCL/DOPC liposomes is slightly shorter than that of other CL-containing liposomes. When the fluorescence properties of this fluorophore are taken into account,[38,39] these results indicate that the polarity around the dansyl chromophore is slightly lower in TSCL/DOPC liposomes than in other CL-containing liposomes.

DISCUSSION

Studying the molecular mechanisms behind the formation of a specific cyt *c*−CL complex and sequential activation of the peroxidase activity of CL-bound cyt *c* is important for elucidating the events occurring in mitochondria during the early stages of apoptosis. Kagan and colleagues showed that TOCL (also TLCL)-containing liposomes (1:1 CL:DOPC) induce the peroxidase activity of cyt *c* much more efficiently than saturated TMCL and that the cyt *c*−TOCL complex is remarkably resistant to dissociation at high salt concentrations.[17,18] On the basis of these findings, they concluded that the presence of a double bond(s) in the acyl chain is critical for a strong hydrophobic interaction with cyt *c* and the induction of peroxide activity. While this notion has been widely accepted, several studies seem to contradict it.[2,12] An aim of our work was to examine whether their idea is a general

feature of cyt *c*−CL interaction under different experimental conditions.

Our results indicated that at low CL levels (10 or 20 mol %), the saturated acyl chain of CL is favorable for the formation of the cyt *c*−CL complex and the induction of peroxide activity; hence, the proposed critical role of the double bond[17,18] is not a general feature of the cyt *c*−CL interaction. The peroxidase inducing effects of different CL-containing liposomes were well related to their ability to associate with cyt *c*. Moreover, the association of cyt *c* with CL-containing liposomes was almost completely reversed when the ionic strength was changed. If the presence of a double bond(s) is critical for the formation of an active cyt *c*−CL complex, specific interactions between the double bond and hydrophobic counterparts in cyt *c* would occur. However, it is difficult to imagine such specific interactions occurring because TDLCL- and TLCL-containing liposomes elicited the same level of activation.

Thus, some discrepancies concerning the role of a double bond exist between our work and the works of Kagan and colleagues.[17−19] To further elucidate the molecular mechanisms responsible for the induction of peroxidase activity of CL-bound cyt *c*, a discussion of possible causes of the discrepancies is invaluable. It is well established that the formation of the cyt *c*−CL complex, perturbation of the cyt *c* structure, and appearance of the peroxidase activity depend on many factors such as ionic strength, cyt *c*:CL ratio, acyl chain composition, and pH.[9,13,16,18] With this in mind, there are two points to be made before the discrepancies are discussed. First, Kagan and his colleagues prepared "unilamellar liposomes" by the sonication method,[17−19] but this method affords both multi- and unilamellar liposomes with various vesicle sizes.[40] Judging from their reports, they did not arrange size heterogeneity in the population through appropriate practices. Therefore, their liposomal preparations might be a mixture of different sized multi- and unilamellar liposomes. To complicate matters, the physicochemical properties as well as the size of the vesicles might change with lipid composition. Second, there are seemingly conflicting results in the papers by Kagan and colleagues.[17−19] For instance, the peroxidase activity of cyt *c* induced by TMCL/DOPC (1:1) liposomes was reported to be less than ∼5% of that induced by TOCL/DOPC liposomes in ref 17, whereas it was as high as 30−40% in ref 18. In addition, it might be difficult to reconcile the data presented in ref 18; namely, the peroxidase inducing effects of different CLs (TMCL, TOCL, and TLCL) are not directly proportional to the effects upon disruption of the Fe−S(Met_{80}) bond to form a pentacoordinated heme iron, which is a prerequisite for the binding of H_2O_2 to the heme iron catalytic site. Differences in the experimental conditions employed in refs 17−19 (depending on the aim of each experiment), such as the cyt *c*:CL ratio and H_2O_2 concentration, probably explain the conflicting data.

Perhaps the most significant difference between the earlier studies[17−19] and our research is the amount of CL in the liposomes; the CL:DOPC molar ratios were 1:1 and 1:9, respectively. It is noteworthy that the reversibility of the formation of the cyt *c*−CL complex is drastically diminished with an increasing amount of CL in liposomes because of the formation of nonlamellar structure[10,41−43] and/or an equilibrium shift toward a protonated form of the phosphate groups of CL due to an increase in negative charge density, thus favoring the hydrophobic interactions, including hydrogen bonding.[9,13,15] Accordingly, the effect of the CL content of liposomes should be considered.

Therefore, we investigated the peroxidase inducing effect of CLs using liposomes composed of CL and DOPC at a 1:1 molar ratio. A comparison of saturated TSCL (C18:0) with unsaturated TOCL (C18:1) or TLCL (C18:2) is ideal for addressing the role of the double bond in the acyl chains, but we could not prepare TSCL/DOPC (1:1) liposomes because of enormously high pressure during the extrusion operation, which we had not experienced in the preparation of CL/DOPC (1:9) liposomes. This is probably due to the fairly large sizes of TSCL/DOPC (1:1) liposomes (or aggregation) after the freezing−thawing cycles. Because of this difficulty along with the fact that Kagan and colleagues compared the peroxidase induction between TMCL (C14:0) and TOCL or TLCL,[17−19] we used TMCL/DOPC (1:1) liposomes in place of TSCL/DOPC (1:1) liposomes. As shown in Figure 5, the peroxidase

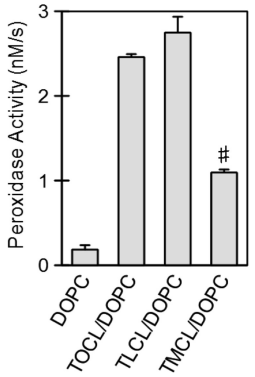


Figure 5. Peroxidase activity of cyt *c* induced by liposomes composed of CL and DOPC at a 1:1 molar ratio. Cyt *c* (1.0 μM) was incubated with CL/DOPC liposomes (250 μM choline) in 2.0 mL of reaction buffer [10 mM HEPES and 100 μM DTPA (pH 7.4)] for 10 min at 25 °C. The peroxidase reaction was started by the addition of APF (10 μM) and H_2O_2 (100 μM) and conducted for 10 min. As a reference, the peroxidase activity induced by liposomes with DOPC alone is shown. Bars show the means ± standard deviation of three independent measurements. $^{\#}P < 0.01$ vs TOCL/DOPC liposomes.

inducing activity of TMCL was much weaker than that of TOCL and TLCL, as reported by Kagan's group.[17−19] Thus, we indeed confirmed a favorable role for the double bond(s) under the particular experimental conditions, where the molar fraction of CL (50%) was considerably higher than that characteristic of the inner mitochondrial membrane (up to ∼20%). It might be difficult to clarify the reason for the poor activity of TMCL because besides the absence of a double bond, the total number of carbon atoms in the acyl chains differs between TMCL and TOCL or TLCL, and the molecular organization of the lipid bilayer containing a large amount of CL changes in a complicated manner.[10,41−43] All things considered, it should be noted that the manner of the interaction between cyt *c* and CL-containing liposomes varies in a complicated way depending on the CL content.

To explain the pronounced difference in peroxidase inducing ability between TSCL- and other CL-containing liposomes, we compared the polarity of the aqueous interfacial region of the liposomal membranes using a fluorescent lipid, DHPE. It is worth noting that the polarity of TSCL/DOPC liposomes is slightly, but significantly, lower that that of other CL-containing liposomes. In light of the effects of unsaturation (or saturation) of phospholipid acyl chains on the organization of the membrane−water interface revealed by X-ray scattering and

dx.doi.org/10.1021/bi2010202 | *Biochemistry* 2011, 50, 8383−8391

molecular dynamics simulation studies,[20,21] this result must be reasonable because the incorporation of a fully saturated TSCL enhances acyl chain packing and consequently reduces the level of hydration of the interfacial region of liposomes. According to Coulomb's law, a lower dielectric constant of the interfacial environment of TSCL/DOPC liposomes due to a reduced level of hydration might be favorable for the electrostatic interaction between cyt *c* and the phosphate group of CL.

Using unique CL derivatives in which the central glycerol skeleton was modified, we revealed that the natural structure of the central moiety is not critical for the formation of an active cyt *c*−CL complex. Two interpretations are possible for this finding. First, if there are two particular counterparts in cyt *c* for the two phosphate groups, the central glycerol moiety, including the phosphate groups, might be able to flexibly adjust its geometry (conformation) to interact with them. Second, taking into consideration the fact that even a simple anionic phospholipid (1,2-dioleoyl-*sn*-glycero-3-phosphate) elicits high peroxidase inducing activity comparable to that of TOCL,[44] we found that the highly restricted geometry of the two phosphates may not be inherently required for the binding to cyt *c*. In spite of detailed studies of the electrostatic interaction between cyt *c* and CL,[10−15] the counterpart residues in cyt *c* have not yet been identified; hence, the two possibilities cannot be ruled either in or out at present.

We here investigated the peroxidation of artificial substrates by CL-bound cyt *c*, but not the peroxidizability of CLs. From a chemical point of view, polyunsaturated acyl chains such as a linoleoyl chain are much more peroxidizable in the presence of reactive oxygen species than monounsaturated and saturated acyl chains.[2,45] Because of this property, TLCL can be much more self-peroxided by CL-bound cyt *c* than TOCL.[19] Our study therefore does not rule out the possibility that peroxidized products of polyunsaturated CLs (both chemically and enzymatically) are key compounds for the detachment of cyt *c* from the mammalian inner mitochondrial membrane.[17,46,47]

In conclusion, we synthesized a series of CL analogues to examine the molecular mechanisms responsible for activating the peroxidase activity of CL-bound cyt *c* using various CL/DOPC liposomes. Our results revealed that if the total number of carbon atoms is the same, the saturated acyl chain of CL is favorable for the activation at low CL levels. The peroxidase inducing effect of various CLs is primarily determined by their ability to associate with cyt *c*. While the electrostatic interaction is a major force in the formation of a specific cyt *c*−CL complex, the structure of the central glycerol head of CL is not necessarily recognized in a strict sense by cyt *c*. Thus, our results reveal new information about the interaction between cyt *c* and CL.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Synthesis of cardiolipins and Figure S1. This material is available free of charge via the Internet at http://pubs.acs.org.

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Funding

This work was supported by a Grant-in-aid for Scientific Research (Grants 20380068 and 23380064 to H.M.) and a Grant-in-aid for Young Scientists (Grant 22780103 to M.A.) from the Japan Society for the Promotion of Science.

■ ACKNOWLEDGMENTS

We thank Dr. Minoru Nakano (Graduate School of Pharmaceutical Science, Kyoto University) for advice on the preparation of large unilamellar vesicles and the use of Photal FPAR-1000. We also thank Dr. Naoko Yoshinaga (Graduate School of Agriculture, Kyoto University) for HRMS analyses.

■ ABBREVIATIONS

APF, aminophenylfluorescein {2-[6-(4-aminophenoxy)-3*H*-xanthen-3-on-9-yl]benzoic acid}; AR, Amplex Red; CL, cardiolipin; cyt *c*, cytochrome *c*; DHPE, *N*-(5-dimethylamino-naphthalene-1-sulfonyl)-1,2-dihexadecanoyl-*sn*-glycero-3-phosphoethanolamine triethylammonium salt; DOPC, 1,2-dioleoyl-*sn*-glycero-3-phosphatidylcholine; LSCL, 1,1′-dilinoleoyl-2,2′-distearylcardiolipin; SLCL, 2,2′-dilinoleoyl-1,1′-distearylcardiolipin; TDLCL, 1,1′,2,2′-tetrakis(11,11-dimethyllinoleoyl)-cardiolipin; TMCL, 1,1′,2,2′-tetramyristoylcardiolipin; TLCL, 1,1′,2,2′-tetralinoleoylcardiolipin; TOCL, 1,1′,2,2′-tetraoleoyl-cardiolipin; TSCL, 1,1′,2,2′-tetrastearylcardiolipin; λ_{max}, maximum emission wavelength.

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