Published in final edited form as:

J Biol Inorg Chem. 2013 January ; 18(1): 137–144. doi:10.1007/s00775-012-0958-0.

Elucidating the mechanism of ferrocytochrome *c* heme disruption by peroxidized cardiolipin

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Abstract

The interaction of peroxidized cardiolipin with ferrocytochrome *c* induces two kinetically and chemically distinct processes. The first is a rapid oxidation of ferrocytochrome *c*, followed by a second, slower, irreversible disruption of heme *c*. The oxidation of ferrocytochrome *c* by CLOOH is explained by the Fenton-type reaction. Heme scission is a consequence of the radical mediated reactions initiated by the interaction of ferric heme iron with cardiolipin hydroperoxides. Simultaneously with the heme *c* disruption, generation of hydroxyl radical is detected by EPR spectroscopy using the spin trapping technique. The resulting apocytochrome *c* sediments as a heterogeneous mixture of high aggregates, as judged by sedimentation analysis. Both the oxidative and the destructive processes were suppressed by non-ionic detergents and/or high ionic strength. The mechanism for the generating of radicals and the heme rupture is presented.

Keywords

ferrocytochrome *c*; peroxidized cardiolipin; heme destruction; hydroxyl radical; aggregation

Introduction

Cardiolipin (CL, diphosphatidylglycerol) is a unique phospholipid having four acyl groups and two negative charges. It is found exclusively in the mitochondrial inner membrane and bacterial plasma membranes. Two populations of CL exist within the inner mitochondrial membrane: the first CL population is tightly bound to mitochondrial inner membrane proteins, such as cytochrome *c* oxidase [1,2], cytochrome *bc*₁ complex [3,4,5], Complex I [4], ATP/ADP translocase [6], F₀F₁ ATP synthase [7], phosphate carrier [8], and succinate dehydrogenase [9]; the second CL population is not bound to proteins and is free to diffuse within the bilayer of the mitochondrial inner membrane. It is generally accepted that in addition to helping to maintain structural integrity of the inner mitochondrial membrane, the bilayer CL can also bind cytochrome *c*. CL contains a much higher percentage of unsaturated to saturated fatty acid residues than do other mitochondrial phospholipids (80 – 90% linoleic acid). Therefore, CL is much more susceptible to oxidative damage than mitochondrial phosphatidylcholine (PC) or phosphatidylethanolamine (PE).

Cytochrome *c* is a small globular protein located in the mitochondrial intermembrane space where its main function is to shuttle electrons from cytochrome *bc*₁ to cytochrome *c* oxidase

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during respiration. Cytochrome *c* has also catalase/peroxidase-like activity, which can be part of the protecting mechanism against oxidative damage in mitochondria [10]. The next line of defense against oxidative damage is the reactivity of cytochrome *c* with superoxide that has also technological applications for the construction of biosensors [11]. In addition, cytochrome *c* has a significant role in the activation of programmed cell death. Release of cytochrome *c* from the mitochondria to the cytosol is a key step in the induction of the apoptotic cascade that leads to programmed cell death [12,13]. The apoptotic activity of cytochrome *c* can also be controlled via interaction with antiapoptotic proteins [14]. In mitochondria cytochrome *c* is bound to the inner membrane primarily by CL. Peroxidation of bilayer CL would be expected to generate a high local concentration of lipid peroxide near the cytochrome *c* binding site. Indeed, multiple studies point to the role of oxidative modification of CL resulting in loss of molecular interaction between CL and cytochrome *c*, however, the precise mechanism of this reaction is not known [15,16].

In this study, we have used absorption spectroscopy and EPR to probe the effect of CL oxidative modification on its interaction with the reduced form of cytochrome *c* *in vitro*. The resulting modified protein was also analyzed by sedimentation velocity analysis. Our data reveal that the reaction of ferrocytochrome *c* with peroxidized bovine cardiolipin results in a two phase process: first, is the oxidation of ferrocytochrome *c*, and second, is the heme *c* rupture. Moreover, in contrast to untreated protein or unmodified CL treated cyt *c* which sediments as a homogeneous species, CLOOH treated cytochrome *c* sediments as a very heterogeneous mixture of high aggregates. The mechanism of the reaction of peroxidized cardiolipin with ferrocytochrome *c* is discussed.

Materials and Methods

Materials

Bovine cardiolipin (1,3-diphosphatidyl-*sn*-glycerol) and phosphatidylcholine (1,2-dioleoyl-*sn*-glycero-3-phosphocholine) in chloroform or lyophilized were obtained from Avanti Polar Lipids. Dodecyl maltoside was from Anatrace. Horse heart cytochrome *c* (95% purity), sodium dithionite and hydrogen peroxide were from Sigma. The reduced form of cytochrome *c* was prepared with excess of sodium dithionite, followed by gel filtration (Sephadex PD-10, Amersham Biosciences, Inc.). Ferri- and ferrocytochrome *c* concentrations were determined using $\epsilon_{410}(\text{ox}) = 106 \text{ mM}^{-1}\text{cm}^{-1}$ and $\epsilon_{550}(\text{red}) = 27.7 \text{ mM}^{-1}\text{cm}^{-1}$, respectively [17]. A Spin trap α -(4-pyridyl-1-oxide) N-tert-butyl nitron (POBN) of high purity was obtained from Alexis Biochemicals. All other chemicals were reagent grade.

Cardiolipin peroxidation—Commercially available cardiolipin was peroxidized essentially as reported by Parinandi [18] with a minor modification. Briefly, CL in chloroform was dried under nitrogen and the resulting film was suspended by vortexing in 0.15 M NaCl, pH adjusted to 7.4 with NaHCO_3 for 3 min followed by sonication using a Branson model 250 sonifier. Finally, air was slowly blown through the resulting suspension at 25 °C for 2–4 hours. To remove salt peroxidized CL was extracted using chloroform/methanol/water extraction [19], dried under N_2 , and dissolved in chloroform. The extent of lipid peroxidation was determined by the generation of conjugated dienes as described by Buege and Aust using $\epsilon_{234} = 25.2 \text{ mM}^{-1}\text{cm}^{-1}$ [20]. Visible spectra were collected using an SLM Aminco 3000 Diode Array spectrophotometer.

Liposomes preparation—The phospholipids in chloroform were dried under nitrogen, and the resulting film was suspended by vortexing in 20 mM Tris-Cl, 1 mM EDTA buffer, pH 7.4. The mixture was clarified by sonication for a few minutes on ice under nitrogen

using a Branson model 250 sonifier. Substitution of 20 mM Tris-Cl buffer for 20 mM phosphate buffer or omitting EDTA did not affect the results.

EPR spectroscopy—All EPR samples were prepared in 20 mM Tris-Cl buffer, pH 7.4. The reaction was initiated by the addition of CLOOH (167 μ M) to the mixture of ferrocytochrome *c* (10 μ M) with POBN (48 mM) and then the sample was transferred into the capillary tube for the EPR measurements.

EPR spectra were recorded with a Varian E 6 spectrometer at 25°C. Conditions of measurements: modulation amplitude 1 G; microwave power 10 mW; frequency 9.225 GHz; time constant 1 and scan time 4 minutes.

Analytical ultracentrifugation—Sedimentation velocity studies were performed in an Optima XL-I Beckman analytical ultracentrifuge in a four-hole AnTi-60 rotor. All samples were analyzed in 25 mM phosphate buffer, 1 mM EDTA pH 7.2 buffers with or without 25 μ M cardiolipin or peroxidized cardiolipin after 30 min incubation at 25°C. The rotor speed was 50000 rpm. Data were collected at 25 °C using optical absorption detection at 280 nm. The resulting data were analyzed by the method of van Holde-Weischet [21] using the UltraScan II version 9.3 data analysis software developed by Dr. B. Demeler (Department of Biochemistry, University of Texas Health Sciences Center). The experimental data were corrected for buffer density and viscosity. The effect of cardiolipin density and viscosity, however, was not considered, therefore, obtained sedimentation values for cytochrome *c* in presence of cardiolipin micelles are presented as observed sedimentation coefficients.

Results

CLOOH-induced oxidation and disruption of heme *c*

Blue-shifted Soret band and intensity decreases were observed immediately after mixing of CLOOH with cyt c^{2+} (Fig. 1). The first spectrum recorded ~10 s following initiation of the reaction is characteristic of ferricytochrome *c*. A comparison of the spectrum of cyt c^{3+} with the spectrum of cyt c^{2+} reacted with CLOOH for 10 s confirms that the two spectra are nearly identical. Such a CLOOH generated form of cyt *c* is fully reducible by Na-ascorbate suggesting that heme *c* remains intact.

Oxidation of cyt c^{2+} is followed by a slower disappearance of the heme *c* absorption (Fig. 1). For example, the Soret absorption band of 2.3 μ M cyt c^{2+} decreased about 83% after incubation with 25 μ M CLOOH for 30 min at 25°C (Fig. 1 panel C). Addition of Na-ascorbate to CLOOH treated cyt *c* (>10 s) does not restore a Soret maximum at 414 nm suggesting an irreversible loss of heme *c* reducibility (Fig. 2). The extent of the heme *c* destruction was time – and concentration–dependent. Oxidation of heme *c* and heme *c* rupture were also observed if cyt c^{2+} was added to CLOOH-DOPC liposomes (1:1 w/w), however the extent of heme *c* disruption was about 30% lower than in the case of pure CLOOH micelles. It should be mentioned that without CL or CLOOH cyt c^{2+} is stable in solution. With or without detergent and/or salt, it did not show measurably altered visible absorption spectra for at least 30 min of observation at 25°C.

To establish if the disruption of heme *c* is affected by redox state of cytochrome *c*, the reactions of CLOOH with cyt c^{2+} and cyt c^{3+} were compared. Under identical experimental conditions, the reactions did not differ in either the rate or the amount of destroyed heme *c* (data not shown).

The extent of the heme *c* destruction was affected by oxygen. If the reaction of 9.5 μ M cyt c^{2+} with 100 μ M CLOOH was performed in argon saturated buffer, the extent of heme *c*

degradation increased but the rate of degradation ($k = 0.014 \text{ s}^{-1}$) was unchanged compared with the reaction in air saturated buffer (data not shown).

Both CLOOH induced oxidation and heme *c* disruption were completely prevented if the same experiments were performed in the presence of 2 mM nonionic detergents, such as dodecyl maltoside, Triton X-100 or Tween-20, suggesting solubilization of CL liposomes, formation of detergent-CL micells and elimination of CL-cyt c^{2+} hydrophobic interaction. In addition, the interaction of CLOOH with cyt c^{2+} was ionic strength dependent. When cyt c^{2+} and CLOOH containing vesicles were mixed in buffers with increased NaCl (20–150 mM), CLOOH initiated heme *c* rupture disappeared, although oxidation of cyt c^{2+} was still observed. Complete inhibition of oxidation and degradation of heme *c* was achieved at a NaCl concentration greater than 0.5 M NaCl (Figure 3).

Reaction of unmodified CL with cyt c^{2+} at neutral pH produces absorption spectral changes in the Soret region similar to those described previously [22,23,24] and explained by either oxidation of cyt c^{2+} due to generation of monoepoxide of linoleic acid [22] or conformational changes of cytochrome *c* [24]. The Soret maximum of cyt c^{2+} was blue-shifted from an initial value of 414.0 nm to 410.5 nm after 30 minutes incubation with 25 μM CL. The maximum absorbance intensity in Soret area is decreased by about 10%. The intensity decrease was also observed at 550 nm. Both absolute and difference spectral changes indicate the oxidation of cyt c^{2+} . The spectral changes were fully reversible. Addition of 5 mM Na-ascorbate resulted in complete recovery of the initial absolute spectrum of reduced cytochrome *c*.

Kinetic analysis and EPR spectroscopy study of the reaction of CLOOH with Cyt c^{2+}

Upon mixing of reduced cytochrome *c* with CLOOH the protein is oxidized in less than 10 sec. Oxidation is followed by disappearance of the heme absorbance (Fig. 4b). The loss of heme absorption occurs in two phases described by rate constants $k_1 = 8.10^{-3} \text{ s}^{-1}$ (contribution 83%) and $k_2 = 2.10^{-4} \text{ s}^{-1}$ (17%). Simultaneously with the heme *c* destruction, observed by absorption spectroscopy, the radical formation was detected using the POBN spin trap EPR spectroscopy. The EPR spectra clearly exhibits rising and the falling phases of POBN adduct formation within the time of observation (Fig. 4a). The fit of dependence of the EPR amplitude indicates that the rate of the rising phase is very close to that for the fast phase of loss absorption of heme (Fig. 4b). Both occur with rates $\sim 8.10^{-3} \text{ s}^{-1}$.

A six-line EPR spectrum results from hyperfine coupling of nitrogen, $A_N = 14.5 \text{ G}$ and hydrogen, $A_H = 1.5 \text{ G}$ (Fig. 4a). The magnitudes of these splitting are not identical but are close to values for OH• adduct to POBN in benzene, where the corresponding splittings were 14.5 and 1.8 [25]. In the more polar solvent water, the values for nitrogen are larger with values between 14.93 and 14.97. However, A_H in water is decreased to a value of 1.68 [25]. This imperfect fit of observed spin adduct parameters with the tabulated data can be reconciled by the assumption that OH.POBN adduct is located in the hydrophobic core of CLOOH vesicles. In control EPR measurements made on pure spin trap POBN (58 mM), reduced cytochrome *c* (10 μM) with POBN (58 mM) and finally CLOOH (167 μM) with POBN (48 mM) the EPR signal was not observed or developed (data not shown).

Apoprotein aggregation

Cyt c^{2+} in low ionic strength buffer sediments as a mono-disperse homogeneous species with $s_{20,W}$ of 1.8 S (Fig. 5). The homogenous sedimentation behavior of cyt *c* was unaffected by treatment of protein with unmodified CL. However, cyt *c* was a very heterogeneous mixture of high molecular weights aggregates when sedimentation velocity

was performed after reaction with CLOOH. Observed sedimentation coefficients varied from 1.8 to 15 S, indicating the aggregation of protein.

Discussion

The reaction of ferrocyanochrome *c* with peroxidized CL was examined using absorption, EPR spectroscopy and sedimentation velocity analysis. In the presence of peroxidized CL, ferrocyanochrome *c* is oxidized with subsequent destruction of the heme *c*.

Oxidation of ferrocyanochrome *c* and scission of the heme ring

The initial rapid oxidation of CLOOH-reacted ferrocyanochrome *c* is implicated by both the spectroscopic analysis and kinetic data. Two lines of evidence suggest that the obtained form of cytochrome *c* is indeed oxidized protein. First, it is the optical spectrum of cytochrome *c* exposed shortly (approximately 10 s) to CLOOH. This spectrum is nearly identical to that of the native untreated ferricytochrome *c*. Also the presence of characteristic to $\text{cyt } c^{3+}$ the charge transfer band at ~700 nm indicates the unmodified six-coordination ferric iron following short exposure to CLOOH (data not shown). Additionally, this exposed cytochrome *c* is fully reducible by either Na-ascorbate or Na-dithionite.

Second, evidence supporting the initial formation of oxidized cytochrome *c* by CLOOH that is equivalent to the native ferric protein, comes from a comparison of the rates of heme destruction. Under the same experimental conditions both the rates and the final amount of damaged heme were found identical in the reaction of native $\text{cyt } c^{3+}$ and $\text{cyt } c^{2+}$ with CLOOH. If the oxidized protein produced at the beginning of the interaction of $\text{cyt } c^{2+}$ with the CLOOH were not identical to the native $\text{cyt } c^{3+}$ then the rates of heme degradation would have shown some disparity. However, this is not a case and both forms of $\text{cyt } c^{3+}$ displayed equal reactivity towards to CLOOH.

The cleavage of heme *c* induced by the CLOOH is demonstrated by the irreversible loss of the UV-Vis absorbance of the protein and by significant changes in the protein aggregation state. The absence of heme *c* degradation in experiments when $\text{cyt } c^{2+}$ reacts with unmodified cardiolipin indicates the initiation of the reaction by CL hydroperoxides.

The interaction of CLOOH with $\text{cyt } c^{2+}$ results in the generation of hydroxyl radicals ($\text{OH}\cdot$) as demonstrated by the EPR data. Both EPR and absorption spectroscopy kinetic data indicate that the effect of CLOOH on $\text{cyt } c^{2+}$ consists of two stages: a rapid oxidation of $\text{cyt } c^{2+}$ followed by a slower biphasic destruction of $\text{cyt } c^{3+}$, described by the two rate constants. The oxidation of ferrocyanochrome *c* by CLOOH can be explained by the Fenton-type reaction [26]:

Unfortunately, the hydroxyl radicals produced by this reaction were not detected by EPR since the first EPR data were acquired 30 sec after initiation of the reaction making detection of the short-lived $\text{OH}\cdot$ impossible. It is reasonable to assume that as soon as $\text{OH}\cdot$ radicals are generated by the Fenton reaction they immediately attack the oxidized heme *c* resulting in heme disruption which is accompanying by the release of non-heme Fe^{3+} . Therefore, EPR detected hydroxyl radicals are most likely generated by the Haber-Weiss mechanism using released Fe^{3+} as a catalyst [27]:

Good correlation of rates of OH• production and the first phase of heme *c* rupture ($k_1 = 8 \cdot 10^{-3} \text{ s}^{-1}$) suggests that hydroxyl radicals are mostly responsible for heme destruction. The hydroxyl radical generation and therefore, heme *c* destruction, were also registered during the second slower phase with $k_2 = 2 \cdot 10^{-4} \text{ s}^{-1}$. The question, however, remains whether hydroxyl radical induced damage is the only mechanism of heme *c* rupture. An additional and plausible mechanism of heme *c* degradation is based on generation of ferryl iron and either alkoxyl or peroxy radical.

CLO• radicals are further involved in the release of OH• by the reaction:

The generation of an alkoxyl or peroxy radical during the reaction of fatty acids with heme-proteins and their destructive effect is well known [28,29,30,31,32]. Moreover, similar mechanisms, based on formation of Fe^{4+} (Fe^{5+}) forms, have been proposed for heme *c* destruction by *tert*-butyl hydroperoxide [33] and degradation of cytochrome *P*-450 [32].

The enhancement of heme degradation in the presence of O_2 can be understood on the basis of propagation of peroxidation of lipids by radical:

The reactions above lead to the increase in concentration of hydroperoxides that further promote the heme scission to larger extent than that occurring under anaerobic conditions.

Neither sodium ascorbate nor dithionite can reduce the CLOOH modified cytochrome *c*. Peroxidized cardiolipin is effective even at very low concentrations. Nearly 80 – 90 % of heme *c* destruction occurs with a molar ratio of CLOOH to cyt c^{2+} of 10 – 20. These concentrations of CLOOH are ~ 100 times lower than the concentrations of *tert*-BOOH that is required to bleach ferricytochrome *c* to a similar extent [34].

The reaction of CLOOH with cyt c^{2+} is in sharp contrast with the effect of H_2O_2 on ferrocyanochrome *c*. Reaction of cyt c^{2+} with 5–100 μM H_2O_2 is characterized by a very slow rate of heme *c* oxidation with no evidence of heme *c* destruction. For example, treatment of 3.5 μM cyt c^{2+} with 50 μM H_2O_2 for 30 min resulted in less than a 5–7% decrease of absorbance in the α and γ spectral bands which were fully reversible by addition of Na-ascorbate (data not shown).

The effectiveness of CLOOH can be explained by exceptionally specific nature of interaction with cytochrome *c*. According to extended lipid conformational model [35] one acyl chain of the CL penetrates into hydrophobic channel of cyt *c*; therefore, in the case of

peroxidized cardiolipin a peroxy group would be localized in close proximity to heme *c* (Figure 6). Moreover, a recent model of the cytochrome *c* – CL complex suggests the insertion of two acyl chains of the CL into the hydrophobic channel of the same cytochrome *c* molecule [36]

Binding of unmodified CL involves both hydrophobic and ionic interactions [35,37,38,39,40]. Oxidatively modified CL is also capable of binding to cyt *c*, although the binding is not as strong as with non-oxidized CL [41,42]. Our data also suggest that the binding of CLOOH to cyt c^{2+} involves both hydrophobic and ionic interactions. Addition of detergent or increase of ionic strength results in a decrease or elimination of the CLOOH effect on cyt c^{2+} . The significance of a hydrophobic interaction has been stressed by Kapetanaki et al [43], in which addition of the non-ionic detergent Triton X-100 completely abolished the interaction of carbon monoxide with cyt c^{2+} -CL complex. Peroxidized CL-induced inhibition of both oxidation and heme *c* bleaching by nonionic detergents is very likely due to solubilization of lipid vesicles. This solubilization results in the dispersion of CL into the small detergent micelles. We assume that in these mixed micelles CL is more thermodynamically stable relative to the pure CL vesicles. The relative stabilization or stronger binding of CL in lipid-detergent micelles may be a reason for the inability of lipid to reach the hydrophobic pocket of cytochrome *c* and to initiate the oxidative/bleaching reactions. The stabilization of CL in micelles may have an origin in the change of energy of interaction between acyl chains, in the change of the electrostatic repulsion between negatively charged CL polar heads or in the combination of both of these factors. Clearly, we can expect that in micelles, having the smaller radius comparing to the lipid vesicles, the distance between the charged CL heads should be enlarged. Consequently, the electrostatic repulsion between heads of CL is decreased and the stability of lipids in micellar aggregates should be enhanced. Our results also agree with the observation that unmodified CL triggers spectral changes within ferrocycytochrome *c* [22,23,24] reflecting the oxidation of reduced cytochrome *c* (refer to *Results Section*).

The possible effect of CL polymorphism or structural reorganization of CL in aqueous solvents should also be considered. Pure CL or CL-phospholipid mixtures in aqueous solvents are organized in typical unilamellar liposomes [44]. However, under certain conditions CL is capable to form the inverted hexagonal phase (H_{II}) [45,46]. For example the Ca^{2+} , Mg^{2+} and Ba^{2+} salts induce well-defined bilayer-hexagonal (H_{II}) transitions [47]. More importantly this transition can be also triggered by cytochrome *c* [48]. Therefore, in addition to structural alterations of cytochrome *c* it is likely that concurrent perturbation of CL liposomes can facilitate the interaction between the acyl chain of lipid and the hydrophobic pocket of cyt c^{2+} .

Heme *c* degradation also caused significant changes in the aggregation state of cytochrome *c*. Cyt c^{2+} before or after exposure to unmodified CL sediments as homogeneous species (sedimentation coefficient of about 1.8 S). However, when cyt c^{2+} is reacted with CLOOH, it sediments as highly heterogeneous species with sedimentation coefficients varying from 1.8 to 15 S. The aggregation of cytochrome *c* is most likely initiated by exposure of hydrophobic sites on cytochrome *c* by the radical induced formation of crosslinks between the individual proteins.

In conclusion our results demonstrate that peroxidized cardiolipin rapidly oxidizes ferrocycytochrome *c* followed by a slower biphasic degradation of heme *c* together with aggregation of the protein. Although these results were obtained *in vitro* we envision that such effects are relevant to *in vivo* exposure of cytochrome *c* to peroxidized CL in mitochondria, although this assumption remains to be examined.

Acknowledgments

This work was supported by grants from the National Institute of Health (NIH GM024795 for Neal C. Robinson and NIH GM0843348 for Marian Fabian), and from European Union Structural Fund (ESF 26110230061). Sedimentation velocity analyses were performed in the Center for Analytical Ultracentrifugation of Macromolecular Assemblies of the University of Texas Health Science Center at San Antonio. The authors thank Virgil Schirf for technical assistance. The authors also thank Dr. Neal C. Robinson (UTHSCSA) for his invaluable discussions regarding these data and for editorial help in preparing the manuscript.

Abbreviations

ROS	reactive oxygen species
cyt c^{3+}	ferricytochrome <i>c</i>
cyt c^{2+}	ferrocytochrome <i>c</i>
CL	cardiolipin (diphosphatidylglycerol)
CLOOH	cardiolipin hydroperoxide or peroxidized cardiolipin
DM	dodecyl maltoside
POBN	α -(4-pyridyl-1-oxide) N-tert-butyl nitron

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Figure 1. Concentration- and time-dependent effect of peroxidized cardiolipin on ferrocytochrome *c* heme disruption

Absolute spectra of ferrocytochrome *c* were taken before incubation (dotted lines), after incubation with peroxidized cardiolipin for 30 min at room temperature (dashed lines), and after addition of 5 mM Na-ascorbate (thick solid lines). Panels (c) and (d) also demonstrate spectra taken 0.25, 1, 2, 3, 5, 10 and 15 min after an addition of CLOOH to ferrocytochrome *c*. (thin black lines). The initial concentration of cytochrome c^{2+} was 2.9 μM (panel (a) and (b)) and 2.3 μM (panel (c) and (d)). The concentrations of peroxidized cardiolipin were 5, 10, 25, and 50 μM in panels (a), (b), (c), and (d), respectively. Base lines were taken on peroxidized cardiolipin in 20 mM Tris-Cl, 1 mM EDTA, buffer pH 7.4.

Figure 2. Reducibility of peroxidized cardiolipin -treated ferrocytochrome *c*

Ferrocytochrome *c* was treated with 0, 1, 5, 10, 25 or 50 μM peroxidized cardiolipin for 30 min at 25C°. After incubation of ferrocytochrome *c* with peroxidized cardiolipin for 30 min at 25C° reducibility of heme *c* was determined from the optical spectra with addition of 5 mM Na-ascorbate using $\epsilon_{550}(\text{red-ox}) = 21 \text{ mM}^{-1}\text{cm}^{-1}$.

Figure 3. Ionic strength - dependent spectral changes of ferrocytochrome *c* in presence of peroxidized cardiolipin

Upper panel: Time dependent spectral changes of 2.5 μM ferrocytochrome *c* reacted with 25 μM peroxidized cardiolipin in 20 mM Tris-Cl buffer pH 7.4, containing 1 mM EDTA and 150 mM NaCl. *Lower panel:* Ionic strength and time dependent changes of absorbance monitored at 414 nm during the interaction of cytochrome *c* (2.5 μM) with peroxidized cardiolipin (25 μM) in the absence (solid circles) and in the presence of various concentration of NaCl: 20 mM (open circles), 60 mM (solid squares), 100 mM (open squares), 150 mM (solid up triangles), 500 mM (open up triangles) and 1 M (solid down triangles) NaCl.

Figure 4. EPR spectra and kinetics of formation of POBN radical adducts during reaction of reduced cytochrome *c* with peroxidized cardiolipin

(a) The EPR spectra following the reaction of 8.3 μM reduced cytochrome *c*, 167 μM peroxidized cardiolipin in the presence of 48 mM spin trap POBN. Spectra were continually collected with a scan time of 4 minutes at 25 °C. Recording was started at 30, 270 and 2670 sec following the addition of peroxidized cardiolipin to the sample. (b) The dependencies of EPR amplitude of the single line (○) and the optical absorbance at the Soret band (●) on the reaction time. Optical measurements were performed using the same concentrations as those in the EPR measurements, but without spin trap. Symbols – experimental data. Dotted lines – two exponential fits.

Figure 5. Aggregation of peroxidized cardiolipin -treated ferrocytochrome *c* analyzed by sedimentation velocity

Ferrocytochrome *c* aggregation state was analyzed after 30 min of incubation at 25C° in 25 mM phosphate buffer, 1 mM EDTA pH 7.2 buffer containing no additions (black triangles), containing 25 μM cardiolipin (grey squares), or containing 25 μM peroxidized cardiolipin (open circles).

Figure 6. Model of cytochrome *c* –peroxidized cardiolipin complex

Insertion of a monohydroperoxides CL acyl chain (black color) into the hydrophobic pocket of cytochrome *c* surrounding by Tyr97 and Phe10. The heme is in white, and the iron ion is shown as a dark orange sphere. The protein structure was visualized with PDB ID: 1CRC