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Dual Effect of Heparin on Fe²⁺ Induced Cardiolipin Peroxidation. Implications to Peroxidation of Cytochrome *c* Oxidase Bound Cardiolipin†

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Abstract

The effect of heparin on ferrous iron initiated cardiolipin (CL) peroxidation was studied *in vitro* using either detergent-solubilized CL, liposomal CL, or CL bound to isolated cytochrome *c* oxidase (CcO). Heparin increased both the rate and extent of CL peroxidation for detergent-solubilized CL and for CcO bound CL. The heparin effect was time and concentration-dependent as monitored by the formation of conjugated dienes or thiobarbituric acid reactive species. The results showed great similarity between the effect of heparin and the effect of certain iron chelators, such as ADP, on phospholipid peroxidation. Heparin increased the peroxidation of CcO bound CL only when tertiary butyl hydroperoxide was also present. The enzyme activity of the resulting CcO complex decreased 25 percent, in part due to peroxidation of functionally important CL. In contrast to detergent solubilized CL, peroxidation of liposomal CL was inhibited by heparin suggesting that the effect of heparin and ferrous iron depends on their proximity to the acyl chains of CL.

Keywords

cardiolipin peroxidation; heparin; iron ions; cytochrome *c* oxidase; detergent; liposomes

Introduction

Cardiolipin (CL; 1,3-diphosphatidyl-*sn*-glycerol) is a unique phospholipid of inner mitochondrial membranes and bacterial plasma membranes. It is a well-documented phospholipid required for the function of several inner membrane proteins such as ADP/ATP carrier protein [1,2,3], NADH dehydrogenase [4,5,6], succinate dehydrogenase [7], cytochrome *bc1* [8,9] and cytochrome *c* oxidase [10,11,12], cytochrome *bc1*. The most well characterized mitochondrial complex that depends on CL for its function is the terminal component of the respiratory chain - cytochrome *c* oxidase. Two to four molecules of CL are tightly bound to CcO at specific binding sites in order to maintain its functional and structural integrity [11,12,13]. Removal of the tightly bound CL decreases electron-transport activity by ~ 50% and completely inhibits proton translocation [12,13, Musatov and Robinson, unpublished]. Full electron-transport activity is restored by the addition of exogenous CL, but not by phosphatidylcholine (PC) or phosphatidylethanolamine (PE).

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Furthermore, CL removal destabilizes CcO and specific subunits dissociate in the absence of CL [13]. Protein bound CL is structurally and/or functionally important not only to the individual electron transport complexes, but it also stabilizes the formation of super-complexes between these enzymes. For example, a super-complex of cytochrome *bc*₁ and CcO involves participation of CL [14].

The mitochondrial membrane is characterized by a high rate of oxygen utilization and by the generation of reactive oxygen species, which potentially damage the proteins and lipids of the inner mitochondrial membrane. Cardiolipin is the most highly unsaturated among the mitochondrial inner membrane phospholipids. In animal tissues 80-90 % of the acyl groups are composed of linoleic acid (18:2(n-6)), making CL much more susceptible to oxidative damage than mitochondrial PC or PE. In fact, loss of CcO activity and CL content has been observed in bovine heart submitochondrial particles exposed to ROS [15]. Loss of CL or oxidative damage to CL has been also suggested to be a significant factor in apoptosis, aging and age-related degenerative diseases [16,17,18]. This is true for both bilayer CL that is not protein bound and is presumably free to diffuse within the mitochondrial inner membrane, and CL that is tightly bound to mitochondrial inner membrane proteins. Indeed, it has been confirmed *in vivo* and *in vitro* experiments that peroxidized CL affects function and/or structure of electron-transport complexes [19,20,21,22,23,24]. Age-related decline in mitochondrial electron-transport may, in fact, be due to loss of CL and one current hypothesis proposes that this decline is due to an age-related accumulation of free radicals [23].

Ferrous iron is commonly thought to be an effective initiator of lipid peroxidation. Oxygen is partially reduced by Fe²⁺ giving rise to the superoxide radical, which starts unwanted free-radical reactions. Iron induced lipid peroxidation is not a simple process. Generated superoxide anion can dismutate to form hydrogen peroxide which in the presence of transition metals produces very reactive hydroxyl radicals. Hydroxyl radicals are capable to abstract a hydrogen atom and therefore, to initiate lipid peroxidation. Lipid peroxides, in turn, are able to react with transition metals generating both alkoxyl and peroxy radicals. In addition several important factors have been described which affect both the rate and extent of lipid peroxidation. For example, the presence of chelating agents [25,26], buffers composition [26], the presence of pre-existing hydroperoxides [27], and/or the requirement of Fe³⁺ for the reaction [28].

The wide spectrum of available iron chelators and the effect of these chelators on lipid peroxidation have been reviewed [25]. Heparin is one of the strongest polyanions that occurs naturally in living organisms and it has a well-known capacity to chelate inorganic ions [29,30]. Therefore, heparin potentially may be involved in lipid peroxidation due to chelation metal ions. Heparin is generally thought to inhibit ferrous ion initiated peroxidation of phospholipids. However, the effect of heparin on lipid peroxidation is not well understood.

Here we describe Fe²⁺ initiated CL peroxidation in the presence or absence of heparin for: 1) detergent-solubilized CL; 2) CL bound to isolated and detergent-solubilized CcO; and 3) liposomal CL. The results demonstrate that heparin greatly increases peroxidation of both CL that is detergent solubilized and CL that is bound to CcO. The enzyme activity of the resulting CcO complex decreases by 25 percent, due at least in part, to peroxidation of functionally important CL. In contrast to detergent solubilized CL, peroxidation of liposomal CL was inhibited by the addition of heparin.

Materials and Methods

Materials

Bovine cardiolipin in chloroform was obtained from Avanti Polar Lipids. Dodecyl maltoside was obtained from Anatrace. Malonaldehyde bis(dimethyl acetal) was from Aldrich. Heparin (from porcine intestinal mucosa, low molecular weight, with av. M. W. ~ 2500), ferrous sulfate, ADP and tertiary butyl hydroperoxide were from Sigma. Fe^{2+} solutions for all experiments were prepared from $\text{FeSO}_4 \times 7\text{H}_2\text{O}$ by dissolving in 0.01 N HCl. The prepared solution was used immediately after preparation. The silicic acid HPLC column (5 μm Radial Pac Resolve Silica cartridge, 0.8 cm $\times$ 10 cm) was purchased from Waters Corporation, Inc. All other chemicals were reagent grade.

Methods

Bovine cytochrome c oxidase—Enzyme was isolated from Keilin-Hartree muscle particles [31] by the method of Fowler et al. [32] as modified by Capaldi and Hayashi [33]. The isolated complex had a molecular enzyme activity of 340 – 370 s^{-1} when assayed spectrophotometrically by following the oxidation of ferrocytochrome *c*. The phospholipid content of the enzyme preparations varied from 10 to 20 mol of P/monomer of CcO. Most of the CL remains tightly bound to isolated CcO [12,13]. All experiments with CcO were done in the presence of 2 mM sodium cholate and 2 mM dodecyl maltoside. This protocol gives a stable dimer of CcO [34]. CcO subunit composition before and after treatment with heparin, Fe^{2+} and/or *tert*-BOOH was analyzed by reversed-phase HPLC [13].

Cardiolipin/dodecyl maltoside mixed micelles—CL in chloroform was dried under nitrogen and the resulting phospholipid film was solubilized in 50 mM Tris- SO_4 buffer, pH 7.4 containing 2 mM of dodecyl maltoside.

Formation of liposomes—CL in chloroform was dried under nitrogen. The resulting lipid film was dispersed in 50 mM Tris- SO_4 buffer, pH 7.4, vortexed and sonicated on ice under nitrogen until clarity.

Measurement of Fe^{2+} oxidation—The remaining Fe^{2+} concentrations were determined by reaction with 1,10-phenanthroline (*o*-phenanthroline) according to Mahler and Elowe [35]. The initial concentration of Fe^{2+} was 50 μM . At various times, 1,10-phenanthroline was added to stop the Fe^{2+} oxidation (the final concentration of 1,10-phenanthroline was 2.5 mM), and the absorbance at 515 nm was measured immediately.

Lipid Peroxidation Assay—1) *Absorption spectra*. The extent of lipid peroxidation was estimated by the appearance of conjugated dienes, as described by Buege and Aust [36]. Conjugated double bond structure was confirmed by the presence of sharp negative peaks in second derivative spectra at 234 nm and 243 nm [37]. The amount of conjugated dienes was calculated using $\epsilon_{234} = 25.2 \text{ mM}^{-1} \text{ cm}^{-1}$ [36]. Absorption spectra of detergent solubilized CL were recorded directly in the solution; formation of conjugated dienes in liposomes and CL bound to CcO were monitored after chloroform/methanol/water extraction of cardiolipin [38]. Extraction of CL did not affect CL peroxidation. CL extracted from CcO was analyzed by silicic acid HPLC as described recently [39]. CL eluted from the silicic acid column was monitored at 208 nm. Digital absorbance data were collected and spectra were analyzed for the presence of conjugated dienes by Millennium 2010 software version 4.0. 2) *Thiobarbituric Acid Assay*. Samples were incubated with or without Fe^{2+} (with or without heparin) in 0.1 mL of reaction mixture, were treated with 0.5 mL of 2.8 % (w/v) trichloroacetic acid and 0.5 mL of 1% 2-thiobarbituric acid in 0.05 N NaOH and then were boiled for 20 min. After cooling absorbance of the solution was measured at 534 nm. The

concentration of thiobarbituric acid reactive substances in the samples was determined from a calibration curve with MDA as a standard. MDA was prepared by acid treatment (0.05 N HCl) of MDA bis(dimethylacetal). The concentration of MDA was determined at 267 nm using $\epsilon = 31500 \text{ M}^{-1}\text{cm}^{-1}$ [40].

Results

Heparin enhances Fe^{2+} initiated peroxidation of detergent solubilized cardiolipin

Heart CL contains 80-90% linoleic acid making CL very susceptible to peroxidation. The peroxidation of linoleic acid resulted in rearrangement of the non-conjugated double bonds to conjugated double bonds (conjugated dienes). Such structural reorganization leads to strong increase in ultraviolet absorption or presence of sharp negative peaks in second derivative spectra at 234 nm and 243 nm. Incubation of CL in detergent solution for 30 min at room temperature did not affect second derivative absorption spectra (Figure 1, panel a, spectrum 1). A slight increase in the amplitudes of negative peaks in the second derivative spectrum at 234 nm and 243 nm was observed in the absence of ferrous iron after 30 min of CL incubation with 100 μM of heparin (Figure 1, panel a, spectrum 2). Formation of conjugated dienes was detected after incubation of CL with 50 μM of Fe^{2+} (Figure 1, panel a, spectrum 3). Conjugated dienes were greatly increased by heparin addition to Fe^{2+} initiated peroxidation. (Figure 1, panel a, spectra 4-9). The heparin effect was time and concentration-dependent. Increasing the molar ratio of heparin to CL was accompanied by increased diene conjugation. It should be mentioned that commercial bovine heart CL may contain oxidation products [41]. In our experiments, a very small amount of peroxidized CL was detected, but this “pre-existing” peroxidized CL was present in the same amount in all of our experiments and did not affect our study.

The kinetics of CL peroxidation initiated by Fe^{2+} in buffer containing dodecyl maltoside with and without heparin were monitored at 234 nm. The change of absorbance with respect to time was clearly triphasic in the presence of heparin and biphasic without heparin (Figure 1, panel b). The amplitude of the first “fast” phases was almost the same and did not depend on the presence of heparin. In fact the rate constants of these first fast phases quite similar $1.52 \times 10^{-2} \text{ s}^{-1}$ and $1.6 \times 10^{-2} \text{ s}^{-1}$ with and without heparin, respectively. However, careful analysis of the kinetics showed that the first phase lasted 1 min in the presence of heparin and ~2 min without heparin. In the presence of heparin, a “slow” phase continued beyond 60 min. The amount of peroxidized CL was calculated using $\epsilon_{234} = 25.2 \text{ mM}^{-1} \text{ cm}^{-1}$ after 60 min [36]. Without heparin only 39 μM of CL was peroxidized (29 % of total amount of CL). However, when heparin was present more than 75 μM of CL (56 % of total) was found in form of conjugated dienes.

Lipid peroxidation was also assayed by the presence of thiobarbituric acid reactive substances. Aliquots of incubation mixture, containing 2.7 μM CL and iron with or without heparin, were removed after 30 min of incubation at room temperature and analyzed as described under Materials and Method. In the absence of heparin, there was 0.016 μM of TBA reactive substance; in the presence of heparin and iron for 30 min incubation, there was 0.031 μM of TBA reactive substance.

Complete Fe^{2+} oxidation also characterizes the first phase. The rate of ferrous iron oxidation was analyzed with α -phenanthroline. Figure 2 (panel a) shows, that in the presence of heparin, Fe^{2+} is oxidized within the first minute (filled triangles). This is the exact time of the first “fast” phase (Figure 1, panel b). In the absence of heparin, the rate of oxidation is slower and Fe^{2+} is oxidized within the first 4 minutes (Figure 2, panel a, filled squares). Therefore, the “slow” phase of CL peroxidation in the presence of heparin could be due to the effect of the heparin- Fe^{3+} complex. Indeed, addition of Fe^{3+} to dodecyl maltoside

solubilized CL in the presence of heparin also initiated diene conjugation in a time-dependent manner (Figure 2, panel **b**). Moreover, the fit of the Fe^{3+} induced CL peroxidation (Figure 1, curve 3) indicates that the rate is very close to that for the “slow” phase (between 20 and 60 min) of Fe^{2+} induced CL peroxidation in presence of heparin (Figure 1, curve 2). Both occur with rates of approximately $1.2 \times 10^{-4} \text{ s}^{-1}$. This suggests that the complex of heparin with Fe^{3+} slowly, but evidently, peroxidizes CL. This is in contrast to the experiment performed without heparin (Figure 1, curve 1). Therefore, the major role of heparin is most likely involving of Fe^{3+} in peroxidation of CL. No effect of Fe^{3+} alone on CL peroxidation was found in the absence of heparin (data not shown).

The results, taken together, showed great similarity between the effect of heparin and the effect of certain iron chelators on phospholipid peroxidation described previously [42,43]. In fact, the addition of ADP – Fe^{2+} to detergent-solubilized CL also enhances the formation of conjugated dienes (Figure 3). However, at the same molar concentration, the effect of ADP is only half as great as that of heparin (compare spectra 3 and 5 on Figure 3). It should be noted that such comparison must be taken with a great precaution. ADP binds iron in 1:1 complex with the binding constant of 10^6 [25]. According to Stevic et al. [44] the number of binding sites for the binding of heparin to Fe^{3+} at low pH is up to 8.3/mg, however actual maximum binding capacity (S_{max}) is much lower (2 to 5) [44]. Moreover, the data can be different for Fe^{2+} , but binding constant is not known. The addition of Fe^{2+} to CL, in the presence of equal amounts of ADP and heparin, produces spectral changes reflecting only the heparin induced effect (Figure 3, spectrum 4).

Heparin inhibits Fe^{2+} initiated CL peroxidation in liposomes

In contrast to the results obtained with detergent-solubilized CL, incubation of liposomes with heparin inhibits the CL peroxidation initiated by Fe^{2+} (Figure 4). These results are similar to those when heparin was added to Fe^{2+} or Cu^{2+} initiated peroxidation of liposomes formed from phosphatidylcholine [45]. The rate of Fe^{2+} oxidation is extremely low in liposomal suspensions and is not affected by the absence or presence of heparin (Figure 2, panel **a**). The same slow rate of Fe^{2+} oxidation is observed in the absence of CL, i.e. in 50 mM Tris- SO_4 buffer, pH 7.4 with or without dodecyl maltoside (data not shown). This low rate of Fe^{2+} oxidation in Tris buffer at neutral pH is in good agreement with data previously published by Yang and Chasteen [46].

Peroxidation of CL bound to isolated and detergent-solubilized CcO

The reduction potential of $\text{Fe}^{3+}/\text{Fe}^{2+}$ in solution at neutral pH is about +110 mV [47]. Therefore, it is thermodynamically possible that a pool of Fe^{2+} added to CcO may be spent on reduction of the redox-centers of CcO, but not on the initiation of CL peroxidation. In fact, analysis of the oxidation rate shows that about 50 % of Fe^{2+} oxidizes immediately (less than 30 sec) in the presence or absence of heparin (Figure 2, panel **a**, empty diamonds). Addition of 50 μM Fe^{2+} , with or without heparin, does not initiate peroxidation of CL bound to CcO (data not shown). However, peroxidation of CL bound to CcO is greatly stimulated by addition of *tert*-BOOH. CcO (5 μM) solubilized in 50 mM Tris- SO_4 buffer with 2 mM DM and 2 mM Na-cholate was incubated in the presence of 5 mM *tert*-BOOH, 50 μM Fe^{2+} with and without heparin. After 60 min incubation at room temperature, CL was extracted with chloroform/methanol/water [38] and purified by HPLC. CL extracted from CcO elutes from the silicic acid column as a single peak (Fig. 5, panel **a**). This chromatogram is similar to data reported previously [48,39]. As shown in Figure 5, CL extracted from either CcO (solid line), CcO incubated with Fe^{2+} and *tert*-BOOH (dotted line) or CcO incubated with Fe^{2+} , *tert*-BOOH and heparin (dashed line) show almost identical elution profiles at 208 nm. However, absorbance spectra taken from distinct points of the chromatogram (Figure 5, panel **a**) indicates the presence of conjugated dienes (Figure 5, panels **b-d**). Greater

absorbance at ~234 nm, reflecting a higher amount of oxidation products, was observed with CL extracted from CcO incubated with Fe^{2+} ions and heparin. To determine the amount of peroxidized CL the phospholipids that eluted from the silicic acid column were collected and analyzed spectrophotometrically. Using the extinction coefficient for conjugated dienes it was found that without heparin about 0.75 – 1.5 μM (5-10 % of total CL) is peroxidized while in presence of heparin the amount of peroxidized CL is doubled and depending on CcO preparation varied from 1.5 – 3 μM (10 to 20 % of total CL). Thiobarbituric acid assay confirmed the higher amount of TBA reactive substances in the sample of CL extracted from CcO incubated in the presence of *tert*-BOOH, heparin and iron ions. In this sample, there was 0.2 μM of TBA reactive substances compared with 0.143 μM in the sample incubated with *tert*-BOOH and Fe^{2+} , but without heparin and 0.06 μM of TBA reactive substances in the control sample (CcO with or without Fe^{2+} ions). It should be noted that CL remained tightly bound to CcO and was unreleased from the enzyme during incubation. This was confirmed by comparing the phosphorus content of the control and *tert*-BOOH plus iron (with or without heparin) – treated CcO (data not shown). It should be also noted that neither Fe^{2+} , heparin, low concentration of *tert*-BOOH (5 mM) or combination of all three components did not perturb subunits structure of CcO as it was revealed by reversed phase HPLC (data not shown).

The next set of experiments was designed to investigate the effect of *tert*-BOOH, iron ions and/or heparin, upon CcO electron transfer activity (Figure 6). CcO electron transfer activity was measured spectrophotometrically as a first-order reaction. CcO (5 μM) was incubated for 60 min with 5 mM *tert*-BOOH, 50 μM Fe^{2+} with or without 100 μM heparin. Enzyme was diluted 3000 times and molecular activity measured as the rate of ferrocytochrome *c* oxidation. Ferrous iron is capable to partially reduce CcO and therefore can interfere with the measurement of CcO activity. To eliminate this effect the assay buffer containing all components was always briefly, but intensively vortexed, in order to saturate a solution with the air and to keep CcO oxidized. Then the reaction was started by addition of cytochrome c^{2+} . Molecular activity of CcO was not affected by incubation with heparin or Fe^{2+} alone and was ~ 370 s^{-1} (Figure 6). CcO was inactivated by 5 mM *tert*-BOOH. However, the extent of activity loss was at least 10 % less than that obtained with *tert*-BOOH plus heparin and iron ions. Incubation of CcO with *tert*-BOOH, iron ions and heparin added together, decrease activity by ~ 25 % ($\pm 2.6\%$ of control).

Discussion

The ferrous ion is commonly accepted as an effective initiator of lipid peroxidation. In aqueous phase, under aerobic conditions, Fe^{2+} can react with dioxygen and give rise to the formation of the superoxide anion and other reactive oxygen species. The efficiency of this process depends on a few key factors, especially on the presence of iron-chelates [for a review see 25]. For instance, ADP, ATP or EDTA strongly affects both the rate and extent of lipid peroxidation. However, depending on the nature of the chelator and experimental conditions, the rate and extent of lipid peroxidation is either increased or decreased [42].

Heparin is a linear polyanion with a very high density of negative charge and a strong ability to interact with many different molecules including inorganic ions, amines, peptides and proteins [29,49,50,51,52]. The complexing of ferrous ions by heparin has also been shown [30]. The ability of heparin to inhibit the Fe^{2+} -initiated peroxidation of linolenic acid was first demonstrated by Ross et al [53]. Albertini, R. et al. have shown that heparin inhibits Fe^{2+} or Cu^{2+} initiated peroxidation of phosphatidylcholine liposomes [45]. On the basis of liposome size and the presence or absence of heparin, the authors concluded that heparin interacts with phospholipids to make them less susceptible to lipid peroxidation [45].

The inhibitory effect of heparin on cardiolipin liposomes peroxidation has also been observed in our experiments. However, CL is acidic and negatively charged. Thus, interaction of an extremely negative charged heparin with a strongly negative charged and acidic CL is very unlikely. Moreover, heparin effectively increases peroxidation of detergent solubilized CL. The effect is time and concentration-dependent. Concentrations of heparin lower than Fe^{2+} or higher than Fe^{2+} stimulate CL peroxidation. The increase of conjugated diene formation and TBA reactive substances correlates with the rate of ferrous iron oxidation. Our results demonstrate that in the presence of heparin, Fe^{3+} ions can extend peroxidation of detergent-solubilized CL initiated by Fe^{2+} ions (refer to Figure 2).

Observation that heparin stimulates iron-initiated CL peroxidation in detergent solution, but inhibits CL peroxidation in liposomes, probably reflects the fact that Fe^{2+} reacts with dioxygen favored ROS formation and CL peroxidation only when the reaction is accomplished near acyl chains. Therefore, our results are in good agreement with data obtained by Tien et al. [42] of a similar effect of EDTA on peroxidation of detergent solubilized and liposomal phospholipids. Our results also support a so-called “site-specific” mechanism, which suggests that molecules closer to the sites of ROS formation result in greater peroxidative damage [54]. Solubilization of CL with nonionic detergent, such as dodecyl maltoside, certainly allows better access to fatty acid chains, i.e. to the site of radical generation. Site-specific lipid oxidation has also been observed in the lipid peroxidation of linoleic acid in positively charged tetradecyltrimethylammonium bromide micelles and discussed in detail [55].

Finally, we investigated the effect of heparin on peroxidation of CL tightly bound to CcO that had been isolated and detergent solubilized. Localization of CcO in the inner mitochondrial membrane near a ROS source, e.g. Complex I and III, makes this enzyme a possible target for ROS attack. It is likely that ROS (superoxide radical, hydroxyl radical, hydrogen peroxide) may affect directly (via modification of protein) or indirectly (via modification of CL bound to the enzyme) the structure and/or function of CcO. Two to four molecules of CL per monomer of CcO are tightly bound to bovine CcO and these molecules greatly affect the catalytic activity of CcO [12,13]. Removal of all CL leads to lower electron-transport activity (~50 %) that is reversible and restored only by exogenous CL [12,13]. Cleavage of CL by PLA_2 also dissociates subunits VIa and VIb from the CcO [13]. Therefore, it is very likely that CL modification may also affect the functional and/or structural integrity of CcO. In fact, isolated bovine heart CcO is inactivated by the lipid peroxidation product 4-hydroxy-2-nonenal via adducts formation with subunits VIII and/or VIIc [56]. Moreover, exposure of CcO to peroxidized CL modifies the enzyme subunits and decreases the activity of both CL-rich and CL-depleted CcO [21]. Decreased CcO activity and CL content have been observed by Paradies et al. [15] in intact bovine heart mitochondria and submitochondrial particles exposed to ROS. In the present work we have used an *in vitro* model of isolated and purified CcO containing tightly bound CL. The CL was extracted and analyzed after 30-60 min of CcO incubation with Fe^{2+} ions in the presence or absence of heparin. The absorption spectroscopy and TBA assays both showed that Fe^{2+} , with or without heparin, is not an effective initiator of CL peroxidation when the CL is bound to CcO. Bovine CcO contains 4 redox-active metal sites including 2 heme *a* (cytochromes *a* and *a*₃) and 2 copper sites (Cu_A and Cu_B) [57]. Electrons from a reductant are transferred via Cu_A and cytochrome *a* to the binuclear center of cytochrome *a*₃ and Cu_B . The apparent midpoint redox-potential of CcO's two heme components is about 230 mV and 380 mV at neutral pH [57]. The reduction potential of $\text{Fe}^{3+}/\text{Fe}^{2+}$ in solution at neutral pH is about +110 mV [47] and may be even lower if Fe^{2+} is chelated. Therefore, part of the 50 μM Fe^{2+} added to CcO may be spent on reduction of the redox-centers of CcO, and not on initiation of CL peroxidation. Indeed, as we described in the Result section, about 50 % of Fe^{2+} is oxidized immediately in the presence of CcO and partial heme reduction is observed

(data not shown). Thus, the actual concentration of Fe^{2+} , which can induce peroxidation, is much lower than that in experiments with pure detergent solubilized CL. The second possible explanation for the inefficiency of Fe^{2+} at initiating peroxidation of CL bound to CcO is the localization of CL. Most likely, CL is located either in the large cavity between the concave surfaces of the two monomeric units [58] or near subunits VIIa and VIIc [13,59]. In any case, tightly bound CL's which are functionally or structurally important, are not localized on the surface of CcO and, therefore, are not easily accessible especially in dimeric CcO. In fact, it was shown that CcO bound CL is resistant to peroxidation by hydrogen peroxide, but quite susceptible to peroxidation by *tert*-BOOH [21,60]. Higher efficiency of *tert*-BOOH was explained by its hydrophobic nature allowing the lipid hydroperoxide places it in close proximity to the hydrocarbon chains of CL [21]. Coincident with peroxidation of bound CL is activity loss and modification of nuclear-encoded subunits [21]. However, significant effects of *tert*-BOOH were observed only at high (20-30 mM) concentration of lipid-soluble peroxide. For example, peroxidation of CcO bound CL was observed when the enzyme was reacted with 30 mM *tert*-BOOH and significant inactivation was also detected at high *tert*-BOOH concentration [21]. In present work we have shown, that CL in CcO is peroxidized in the presence of heparin, Fe^{2+} and relatively low concentration of *tert*-BOOH. Despite the fact, that CL is tightly bound to the enzyme, the peroxidation of CL follows the same scenario as CL in detergent solution and its peroxidation increases in the presence of heparin. Moreover, electron-transfer activity of CcO decreases up to 25 % when the enzyme is incubated with Fe^{2+} , heparin and 5 mM *tert*-BOOH. Therefore, we concluded that heparin significantly increases CL peroxidation in presence of Fe^{2+} and *tert*-BOOH, and that inactivation of CcO at least partially, is due to cardiolipin peroxidation. Presented data, however, also strongly suggest that CcO is quite resistant to oxidative damage even in the presence of high concentrations of ROS. One reasonable explanation of this phenomenon is the existence of numerous defense mechanisms which effectively prevent and/or reduce the effect of ROS [18]. It's reasonable to assume that one of these mechanisms is also involved in decreasing of the effect of Fe^{2+} - heparin induced peroxidation of CL bound to CcO.

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Abbreviations

CL	cardiolipin
CcO	cytochrome <i>c</i> oxidase
ROS	reactive oxygen species
ATP	adenosine triphosphate
PC	phosphatidylcholine
PE	phosphatidylethanolamine
ADP	adenosine diphosphate
EDTA	ethylenediaminetetraacetic acid
TBA	thiobarbituric acid
MDA	malodialdehyde

DM	dodecyl maltoside
Na-cholate	sodium cholate
tert-BOOH	tertiary butyl hydroperoxide
HPLC	high performance liquid chromatography

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Figure 1. Fe^{2+} initiated peroxidation of detergent-solubilized cardiolipin as a function of heparin concentration (upper panel) and time (lower panel)

Upper Panel: Cardiolipin ($134\ \mu\text{M}$) was solubilized in 1 mL of 50 mM Tris- SO_4 buffer pH 7.4, containing of 2 mM of dodecyl maltoside. Different concentrations of heparin were added and peroxidation was started by addition of $50\ \mu\text{M}\ \text{Fe}^{2+}$. Spectra were taken after 30 min. of incubation at room temperature: (1) CL; (2) CL + $100\ \mu\text{M}$ heparin; (3) CL + Fe; (4) CL + Fe + $25\ \mu\text{M}$ heparin; (5) CL + Fe + $50\ \mu\text{M}$ heparin; (6) CL + Fe + $70\ \mu\text{M}$ heparin; (7) CL + Fe + $100\ \mu\text{M}$ heparin; (8) CL + Fe + $140\ \mu\text{M}$ heparin; (9) CL + Fe + $150\ \mu\text{M}$ heparin. Lower Panel: Peroxidation was initiated by addition of $50\ \mu\text{M}$ of Fe^{2+} or $50\ \mu\text{M}$ of Fe^{3+} . Concentration of heparin was $0\ \mu\text{M}$ (curve 1) and $100\ \mu\text{M}$ (curve 2 and curve 3).

Figure 2. Rate of oxidation of Fe^{2+} (upper panel) and the effect of ferric iron on detergent-solubilized cardiolipin in the presence of heparin (lower panel)

Upper panel: Ferrous iron was added to 1) dodecyl maltoside-solubilized CL with heparin (black filled squares) and without heparin (empty squares); 2) CL liposomes with heparin (black filled circles) and without heparin (empty circles); and 3) dodecyl maltoside-solubilized CcO (empty diamonds are for both measurements with or without heparin). The initial concentration of Fe^{2+} was $50 \mu\text{M}$; heparin was $100 \mu\text{M}$ and CcO was $5 \mu\text{M}$. Absorbance at 515 nm was measured immediately after addition of 2.5 mM 1,10-phenanthroline. To determine the absorbance, which reflects 100% amount of Fe^{2+} , 1,10-phenanthroline was added in control sample before addition of ferrous ions. Lower panel: Cardiolipin ($67 \mu\text{M}$) was solubilized in 1 mL of 50 mM Tris- SO_4 buffer pH 7.4 containing 1 mg/mL of dodecyl maltoside. Concentration of heparin was $100 \mu\text{M}$ (spectrum 1). Reaction was initiated by addition of $50 \mu\text{M}$ Fe^{3+} . Spectra 2, 3, 4 and 5 were taken after 0 min, 1 min, 5 min and 30 min, respectively.

Figure 3. Effect of ADP and heparin on Fe^{2+} -initiated detergent-solubilized CL peroxidation
Cardiolipin ($134 \mu\text{M}$) was solubilized in 1 mL of 50 mM Tris- SO_4 buffer pH 7.4 containing 1 mg/mL of dodecyl maltoside. Peroxidation was initiated by addition of $50 \mu\text{M}$ Fe^{2+} . Spectra were taken after 30 min of incubation at room temperature: (1) CL; (2) CL+Fe; (3) CL +Fe+ $100 \mu\text{M}$ ADP; (4) CL +Fe+ $100 \mu\text{M}$ ADP+ $100 \mu\text{M}$ heparin (solid line); (5) CL + Fe + $100 \mu\text{M}$ heparin (dotted line).

Figure 4. Heparin inhibits Fe^{2+} induced peroxidation of cardiolipin in liposomes
Liposomes were made from 134 μM CL as described in Material and Method. Peroxidation was initiated by addition of 50 μM of Fe^{2+} . After 30 min incubation at room temperature cardiolipin was extracted by methanol – chloroform, dried under nitrogen and dissolved in 0.5 mL of ethanol. (1) CL; (2) CL + Fe + 100 μM heparin; (3) CL + Fe

Figure 5. Silicic acid HPLC and spectral analysis of cardiolipin extracted from cytochrome *c* oxidase

Upper panel – silicic acid HPLC chromatogram. The numbers (I, II and III) designate the randomly chosen areas where spectra were analyzed. Lower panels (b, c and d) represent spectra extracted from the randomly chosen areas (I, II and III) of silicic acid HPLC. Solid line is CL extracted from 5 μM CcO; dotted line is CL extracted from 5 μM CcO incubated with 5 mM *tert*-BOOH and 50 μM Fe^{2+} ; dashed line is CL extracted from 5 μM CcO incubated with 5 mM *tert*-BOOH, 50 μM Fe^{2+} and 100 μM heparin.

Figure 6. Inactivation of CcO electron transport activity by *tert*-BOOH, ferrous ion with and without heparin

CcO (5 μ M) solubilized in 50 mM Tris-SO₄ buffer, pH 7.4, containing 2 mM dodecyl maltoside and 2 mM sodium cholate was incubated for 60 min at room temperature with: (1) no additions; (2) 5 mM *tert*-BOOH $\pm$ 100 μ M heparin ; (3) 5 mM *tert*-BOOH + 50 μ M Fe²⁺; (4) 5 mM *tert*-BOOH + 50 μ M Fe²⁺ + 100 μ M heparin; (5) 50 μ M Fe²⁺ ; (6) CcO + 50 μ M Fe²⁺ + 100 μ M heparin. The reaction was stopped by dilution and the activity measured spectrophotometrically with reduced cytochrome *c* as a substrate. These results are representative of three independent experiments. The error in determination of molecular activity was $\pm 2.6\%$.