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Original Contribution

Contribution of peroxidized cardiolipin to inactivation of bovine heart cytochrome *c* oxidase

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Abstract

The lipid-soluble peroxides, *tert*-butyl hydroperoxide and peroxidized cardiolipin, each react with bovine cytochrome *c* oxidase and cause a loss of electron-transport activity. Coinciding with loss of activity is oxidation of Trp_{19} and Trp_{48} within subunits VIIc and IV, and partial dissociation of subunits VIa and VIIa. *tert*-Butyl hydroperoxide initiates these structural and functional changes of cytochrome *c* oxidase by three mechanisms: (1) radical generation at the binuclear center; (2) direct oxidation of Trp_{19} and Trp_{48}; and (3) peroxidation of bound cardiolipin. All three mechanisms contribute to inactivation since blocking a single mechanism only partially prevents oxidative damage. The first mechanism is similar to that described for hydrogen peroxide [*Biochemistry* **43**:1003–1009; 2004], while the second and third mechanism are unique to organic hydroperoxides. Peroxidized cardiolipin inactivates cytochrome *c* oxidase in the absence of *tert*-butyl hydroperoxide and oxidizes the same tryptophans within the nuclear-encoded subunits. Peroxidized cardiolipin also inactivates cardiolipin-free cytochrome *c* oxidase rather than restoring full activity. Cardiolipin-free cytochrome *c* oxidase, although it does not contain cardiolipin, is still inactivated by *tert*-butyl hydroperoxide, indicating that the other oxidation products contribute to the inactivation of cytochrome *c* oxidase. We conclude that both peroxidized cardiolipin and *tert*-butyl hydroperoxide react with and triggers a cascade of structural alterations within cytochrome *c* oxidase. The summation of these events leads to cytochrome *c* oxidase inactivation.

Keywords: Cytochrome *c* oxidase; *tert*-Butyl hydroperoxide; Inactivation; Subunit modification; Cardiolipin peroxidation

Introduction

The inner mitochondrial membrane is characterized by a high rate of oxygen utilization and generation of reactive oxygen species (ROS). During active respiration, two electron-transport complexes, NADH dehydrogenase (Complex I) and cytochrome bc_1 (Complex III), are the main sites of ROS production [1–3]. Both "leak" electrons and partially reduce oxygen to produce superoxide anions. Subsequent dismutation of superoxide anion generates hydrogen peroxide (H_2O_2) which, in the presence of transition metals, produces highly reactive hydroxyl radicals (HO*U*). When ROS levels overpower the defense mechanisms of the cell, proteins, nucleic acids, and/or phospholipids are irreversibly damaged. The mitochondrial electron-transport complexes are potential targets for damage, since they are either a sources of ROS or they are in close proximity to a site of ROS production. In fact, a number of tryptophans within electron-transport complexes I, III, and IV have been found to be oxidized in samples isolated from "normal" human heart tissue [4]. Apparently, oxidative damage to electron-transport complexes occurs even during normal respiration.

The mitochondrial membrane is also characterized by cardiolipin (CL), a phospholipid much more sensitive than other phospholipids to ROS-induced damage. Any damage to CL would negatively impact the biochemical functions of the

Abbreviations: CcO, bovine heart cytochrome *c* oxidase; *tert*-BOOH, *tert*-butyl hydroperoxide; CL, cardiolipin; CL-OOH, peroxidized CL; ROS, reactive oxygen species; MDA, malondialdehyde; DM, dodecyl maltoside; TBA, thiobarbituric acid; ESI/MS, electrospray ionization mass spectrometry; MALDI-TOF/MS, matrix-assisted laser desorption/ionization time-of-flight spectrometry; HPLC, high-performance liquid chromatography; SDS-PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis.

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inner mitochondrial membrane. It is well-documented that CL is required for the functional activity of many inner membrane protein complexes, e.g., NADH dehydrogenase [5], cytochrome bc_1 [6], cytochrome *c* oxidase [7–9], ATP synthase [10], and the adenine nucleotide transporter [11]. Loss of cardiolipin occurs during ischemia [12,13], during many age-related diseases [14], and during in vitro exposure of submitochondrial particles to ROS, which may explain the loss of mitochondrial function [15,16]. In the case of cytochrome *c* oxidase (CcO), four molecules of CL are tightly bound at specific sites on each monomeric unit, and CL occupancy at these sites is required to maintain functional and structural integrity [8,17].

Exposure of bovine cytochrome *c* oxidase to H_2O_2 is known to oxidize Trp_{19} and Trp_{48} within subunits VIIc and IV, to peroxidize a small amount of bound CL, to cause dissociation of subunits VIa and VIIa, and to inactivate the enzyme [18,19] (the term peroxidized cardiolipin is used throughout this work to describe the mixture of conjugated dienes and various hydroperoxides of linoleic acid, described previously [20]). However, H_2O_2 peroxidizes CL very slowly and, in this case, it is not the primary cause of CcO inactivation [18] and the role of CL peroxidation in ROS-induced CcO inactivation remains unclear. To clarify whether CL peroxidation negatively impacts CcO, we have investigated oxidative damage to CcO induced by *tert*-butyl hydroperoxide (*tert*-BOOH) and by peroxidized cardiolipin (CL-OOH). Because it is a lipid-soluble organic peroxide, *tert*-BOOH more closely resembles endogenous lipid hydroperoxides generated during oxidative stress than H_2O_2. Like lipid hydroperoxides, *tert*-BOOH partitions into the apolar environment surrounding CcO, which places it in close proximity to the hydrocarbon chains of CL. Indeed, *tert*-BOOH effectively peroxidizes CL bound to CcO and, therefore, introduces the additional inactivation factor. The effect of peroxidized CL on CcO was confirmed by direct experiments with exogenous peroxidized CL which, in the absence of any additional peroxides, also reacts with bovine cytochrome *c* oxidase causing a loss of electron-transport activity, oxidizes Trp_{19} and Trp_{48} within subunits VIIc and IV, and induces partial dissociation of subunits VIa and VIIa.

Experimental procedures

Materials

Bovine cardiolipin in chloroform was obtained from Avanti Polar Lipids. Dodecyl maltoside was obtained from Anatrace. Malonaldehyde bis(dimethylacetal) was from Aldrich. Hydrogen peroxide, *tert*-butyl hydroperoxide, and xylenol orange were from Sigma. The silicic acid HPLC column (5-μm Radial Pac Resolve Silica cartridge, 0.8 × 10 cm) was purchased from Waters Corporation, Inc. All other chemicals were reagent grade.

Bovine cytochrome c oxidase

Bovine cytochrome *c* oxidase was isolated from Keilin-Hartree particles by the method of Fowler et al. [21] with modifications described previously [22]. After the final ammonium sulfate precipitation, the oxidase pellet was dissolved in 0.1 M NaH_2PO_4, pH 7.4, buffer, containing 1% sodium cholate, and stored at −70°C at a concentration of approximately 30 mg/mL. To prepare CcO samples in dodecyl maltoside (DM), the stock enzyme (5–10 μM) was solubilized in 20 mM Tris-SO_4 buffer, pH 7.4, containing 2 mM DM and dialyzed versus two changes of the same buffer containing 0.2 mM DM for 12 h at 4°C. HiTrapQ ion-exchange column chromatography was subsequently used to remove small amounts of contaminating cytochrome bc_1 and all phospholipids except for four CL that remain tightly bound to the enzyme [17]. The purity of isolated CcO was verified by UV/VIS-spectroscopy, SDS-PAGE, reversed-phase HPLC, and matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF/MS) as described previously [6,17–19]. CL was extracted from the resulting CcO as previously described [23] and quantified by silicic acid HPLC [24]. The purified complex had a molecular activity of 340–370 s^{-1} when assayed spectrophotometrically by following the pseudo-first-order rate of ferrocytochrome *c* (30 μM) oxidation by 1.8 nM CcO in 50 mM phosphate buffer, pH 7.2, containing 2 mM DM as described by Dale and Robinson [25].

Cardiolipin-depleted cytochrome c oxidase

CcO (~10 μM) solubilized in 20 mM Tris-SO_4 buffer, pH 7.4, containing 10 mM $CaCl_2$ and 2 μM DM was delipidated by phospholipase A_2 (20 μM) at room temperature [17]. The reaction was stopped and phospholipase A_2 was removed by HiTrapQ ion-exchange column chromatography.

Methods

CcO was reacted with either peroxidized CL or *tert*-BOOH at room temperature. The reaction was stopped by removing peroxide using HiTrapQ column ion-exchange chromatography. All experiments with *tert*-BOOH were done in the presence of a catalytic amount of catalase to decompose any H_2O_2 that might be present. Catalase was not inactivated by incubation with 30 mM *tert*-BOOH for 60 min. The amount of "ferryl" and "peroxy" CcO intermediates formed by reaction of CcO with *tert*-BOOH was calculated using $\Delta\varepsilon_{434-412}$ = 65 mM^{-1} cm^{-1} [26]. Quantitative reversed-phase HPLC subunit analysis of control, CL-OOH, or *tert*-BOOH-reacted CcO was performed as described previously [18]. Cyanide-inhibited CcO was prepared by reacting CcO with 5 mM KCN for 18 h at 4°C. Formation of the cyanide-CcO complex was confirmed by spectroscopic analysis.

Cardiolipin peroxidation

Two pools of CL were used: (1) commercially available cardiolipin from Avanti Polar Lipids and (2) CL bound to isolated and detergent-solubilized CcO. Commercially available cardiolipin was peroxidized by incubation of 100–140 μM CL, solubilized in 20 mM Tris-SO_4, pH 7.4, buffer containing 2 mM DM, with 0.5 mM H_2O_2 (or 20–30 mM *tert*-BOOH) and 50 μM

Fe_2SO_4 for 30 min at room temperature [27]. CL was extracted, dried under N_2, and solubilized in the same buffer or ethanol. CL bound to CcO (10 μM CcO), solubilized in 20 mM Tris-SO_4 buffer, pH 7.4, containing 2 mM DM, was peroxidized by a reaction with either 0.5 mM H_2O_2 or 20–30 mM *tert*-BOOH at room temperature for 30 and 60 min, respectively. Peroxides were removed by HTQ anion-exchange chromatography. CL was extracted from peroxide-treated enzymes, dried under N_2, and solubilized in 20 mM Tris-SO_4 buffer, pH 7.4, containing 2 mM DM. The percentile of peroxidized CL was estimated spectrophotometrically and using the FOX2 method (see below).

Lipid peroxidation assays

Conjugated dienes determination. The extent of lipid peroxidation was determined by the generation of conjugated dienes as described by Buege and Aust [28]. Absorption spectra are recorded after extraction of cardiolipin by the method of Bligh and Dyer [23], drying the extracted CL under N_2, and dissolving it in ethanol. The amount of conjugated dienes was calculated using $\varepsilon_{234} = 25.2\ mM^{-1}\ cm^{-1}$ [28].

Lipid hydroperoxide assay. The amount of CL hydroperoxides was measured using the ferrous ion oxidation in the presence of xylenol orange (FOX2 method) according to Nourooz-Zadeh [29]. The FOX2 reagent was composed of xylenol orange (100 μM), butylated hydroxytoluene (4.4 mM), sulfuric acid (25 mM), and ammonium ferrous sulfate (250 μM). CL extracted from control and *tert*-BOOH treated CcO was solubilized in 100 μL of methanol and incubated with 900 μL of FOX2 reagent at room temperature for 30 min. The amount of CL-OOH was calculated using $\varepsilon_{560} = 4.5 \times 10^4\ M^{-1}\ cm^{-1}$ [29]. The validity of the extinction coefficient was confirmed using known concentrations of hydrogen peroxide.

Thiobarbituric acid assay method. CcO was incubated with or without *tert*-BOOH in 0.1 mL of the reaction mixture, 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 boiled for 20 min. After cooling, the 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 using $\varepsilon_{267} = 31500\ M^{-1}\ cm^{-1}$ [30].

Mass spectrometry

Matrix-assisted laser desorption ionization time-of-flight and HPLC-electrospray ionization tandem mass spectrometry analysis (ESI/MS/MS) of CcO subunits were described recently in detail [18,31]. Matrices (dissolved in 50% acetonitrile/0.1% trifluoroacetic acid) included sinapinic acid (50 mg/mL)/dodecyl maltoside (2 mg/mL) for intact subunits and 2,5-dihydroxybenzoic acid (50 mg/mL) for tryptic digests. Spectra represent the average of 100 laser shots.

Results

Inactivation of cytochrome c oxidase by lipid-soluble peroxidized cardiolipin or tert-butyl hydroperoxide

Contribution of CL peroxidation in the inactivation of CcO was confirmed by exposing CcO to peroxidized CL. Both CL-rich and CL-free CcO are inactivated by peroxidized CL (Fig. 1A; Fig. 2). Exposure of CL-rich CcO to peroxidized CL results in up to 30–40% inactivation. With CL-free CcO, the situation is more complicated. Incubation of CL-free CcO with unmodified CL (CL either extracted from control CcO or purchased from Avanti Polar Lipids) as expected restores full enzymatic activity. Exposure of CL-free CcO to increasing amounts of peroxidized CL has the opposite effect; up to a 40% decrease in activity with all of the CL containing conjugated dienes. A similar inhibition effect is observed with *tert*-BOOH. Fifty percent of CcO electron-transport activity is lost during

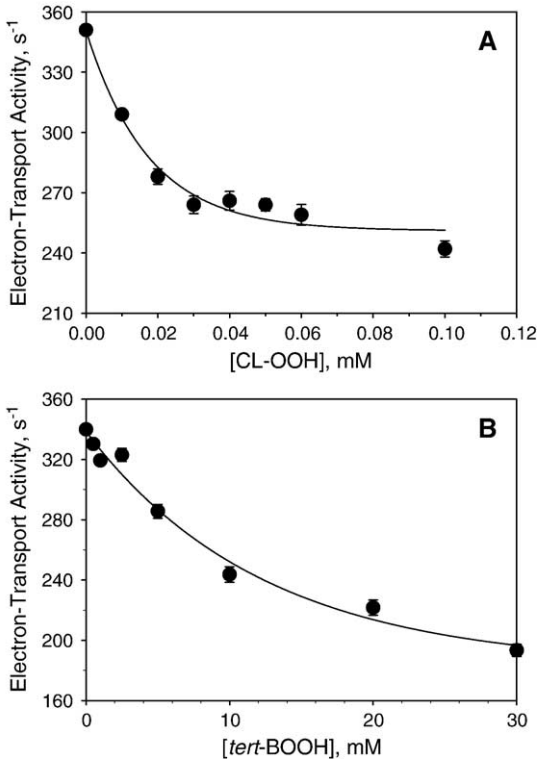

Fig. 1. Inactivation of cytochrome *c* oxidase by peroxidized cardiolipin (A) and *tert*-butyl hydroperoxide (B). Electron-transport activity of CcO was measured as a function of either CL-OOH or *tert*-BOOH concentration after removal of excess of peroxides using HiTrapQ ion-exchange column chromatography. (A) CcO (5 μM) in 20 mM Tris-SO_4 buffer, pH 7.2, containing 2 mM dodecyl maltoside was incubated for 30 min at room temperature with various concentrations of CL-OOH. (B) CcO (5μM) in 20 mM Tris-SO_4 buffer, pH 7.2, containing 50 nM catalase and 2 mM dodecyl maltoside was incubated for 60 min at room temperature with various concentrations of *tert*-BOOH. Activity was measured spectrophotometrically as described under Experimental procedures. Data were fitted to a single-exponential decay (solid line).

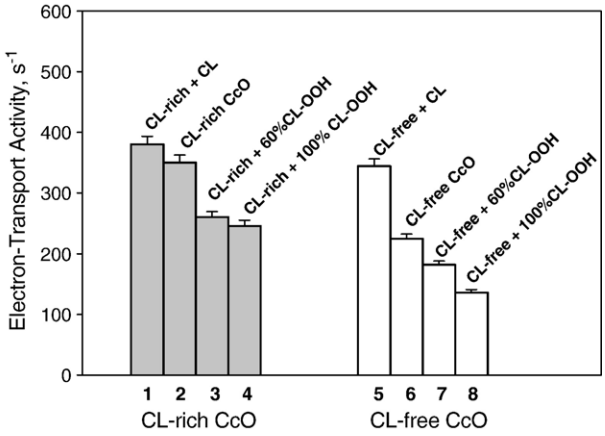

Fig. 2. Inhibition of cardiolipin-rich and cardiolipin-free cytochrome *c* oxidase by peroxidized cardiolipin. The electron-transport activity of CcO was measured after its incubation with CL which contained different amounts of peroxidized CL. Cardiolipin-rich CcO (filled bars). Sample 1: activity of CL-rich CcO after addition of CL. Sample 2: activity of CL-rich CcO. Sample 3: activity of CL-rich CcO after incubation with 60% peroxidized CL (CL from Avanti Polar Lipids peroxidized by *tert*-BOOH). Sample 4: activity of CL-rich CcO after incubation with 100% peroxidized CL (CL extracted from CcO after peroxidation by *tert*-BOOH). Cardiolipin-free CcO (unfilled bars). Sample 1: activity of CL-free CcO after incubation with CL. Sample 2: activity of CL-free CcO. Sample 3: activity of CL-free CcO after incubation with 60% peroxidized CL (CL from Avanti Polar Lipids peroxidized by *tert*-BOOH). Sample 4: activity of CL-depleted CcO after incubation with 100% peroxidized CL (CL extracted from CcO after peroxidation by *tert*-BOOH). In each assay, 0.25 μM CcO (solubilized in 50 mM phosphate buffer, pH 7.2, containing 20% glycerol, 1 mM EDTA, and 1 mg/mL Triton X-100) was incubated with 7.5 μM CL for 5 min prior to dilution 1500-fold into the assay buffer (30 μM reduced cytochrome *c*, 2 mM dodecyl maltoside, 50 mM phosphate, pH 7.2). Assays were done in triplicate and have an accuracy of ± 3.5%.

incubation with 30 mM *tert*-BOOH for 60 min at room temperature, pH 7.2 (Fig. 1B). The loss of enzymatic activity exhibits pseudo-first-order kinetics. Inactivation of detergent-solubilized CcO in the present work is in good agreement with ~45% of CcO activity loss described by Chen et al. in isolated mitochondria treated with *tert*-BOOH [32]. CL-free CcO is also inactivated by *tert*-BOOH, but to a lesser extent than CL-rich CcO. CL-free CcO has 60% of the activity of CL-rich CcO prior to the addition of *tert*-BOOH (Fig. 2). The activity decreases further upon addition of *tert*-BOOH. However, the extent of activity loss is 10–15% less than that obtained with CL-rich CcO. CcO bound CL must have a significant role in the inactivation process. Visible spectral changes are also induced by *tert*-BOOH in both types of oxidase that are characteristic of the peroxy and ferryl forms (Fig. 3). However, *tert*-BOOH is ineffective in producing these intermediates; e.g., 30 mM *tert*-BOOH induces less than half the $\Delta A_{435-412}$ produced by 0.5 mM H_2O_2.

Modification of cytochrome c oxidase nuclear-encoded subunits by lipid-soluble peroxides

Both reversed-phase HPLC subunit analysis and MALDI-TOF mass spectrometry indicate that only two nuclear-encoded subunits are modified by either *tert*-BOOH or CL-OOH. Two new RP-HPLC elution peaks are produced by the reaction of *tert*-BOOH with CcO (Fig. 4, middle chromatogram). These products elute with the same retention times as H_2O_2-modified subunits VIIc and IV [18]. MALDI-TOF/MS analysis indicates that each subunit VIIc and IV is modified by insertion of a single oxygen atom (Fig. 5B). Based on the area under the new individual RP-HPLC peaks, 36–40% of subunit VIIc and 10–15% of subunit IV are modified in 60 min by 30 mM *tert*-BOOH. These percentages are about half those produced by H_2O_2. Coincident with these subunit modifications is the dissociation of ~33% of subunit VIIa and ~25% of subunit VIa, which are once again about half the amount dissociated during the reaction of CcO with H_2O_2. These structural changes are not caused entirely by free radical generation at the binuclear center of CcO since cyanide inhibition of CcO does not completely prevent *tert*-BOOH-induced oxidation of Trp_{19} and Trp_{48} or the dissociation of VIa and VIIa (Table 1). In contrast to *tert*-BOOH, peroxidized CL was proven to be exceptionally effective for modifying CcO subunits VIIc and IV (Fig. 4, lower chromatogram). Up to 40% of subunit VIIc was modified by the reaction of CcO with only 40 μM CL-OOH (Table 1). This concentration is 100–1000 times lower than the concentrations of H_2O_2 or *tert*-BOOH used to modify these subunits to the similar extent (Table 1). Again, MALDI-TOF/MS analysis confirms that each subunit is modified by insertion of a single oxygen atom (Fig. 4C). Localization of

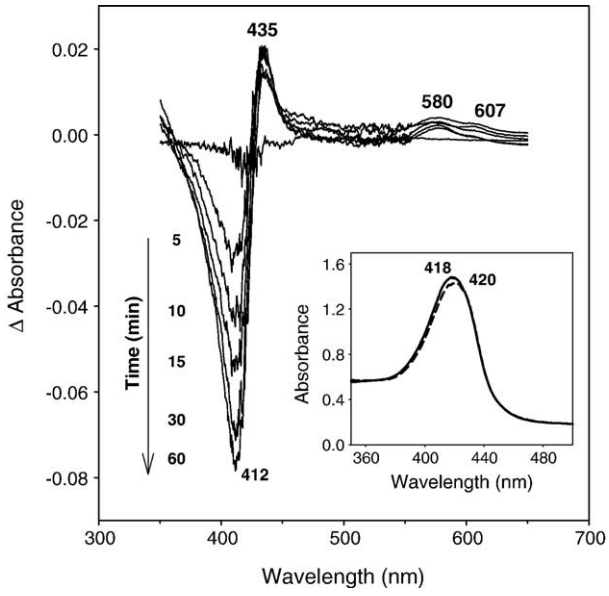

Fig. 3. Time-dependent spectral changes in bovine cytochrome *c* oxidase induced by *tert*-butyl hydroperoxide. Main panel: Difference spectra for peroxide-treated minus untreated CcO after reaction with 30 mM *tert*-BOOH for 5, 10, 15, 30, and 60 min at room temperature. Reaction conditions were 10 μM CcO solubilized in 20 mM Tris-SO_4 buffer, pH 7.2, containing 2 mM dodecyl maltoside, 50 nM catalase, and 50 units/mL of MnSOD. Inset: Absolute Soret spectrum of CcO before (solid line) and after (dashed line) reaction with *tert*-BOOH for 60 min. The Soret maximum for the *tert*-BOOH-treated enzyme is red-shifted by 2 nm with respect to the oxidized enzyme. Inhibition of CcO by KCN prevented the *tert*-BOOH-induced spectral change.

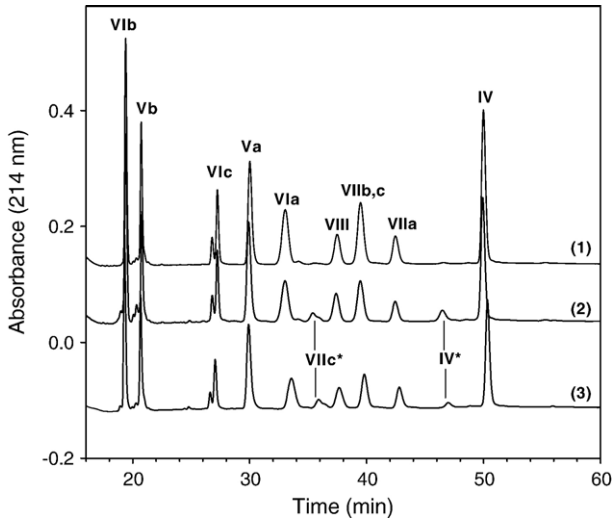

Fig. 4. Detection of organic peroxide-induced modification of CcO subunits by quantitative reversed-phase HPLC. Upper chromatogram (1): elution profile for the nuclear-encoded subunits prior to the reaction of CcO with peroxides. Middle chromatogram (2): elution profile for CcO the nuclear-encoded subunits after reaction of 10 μM CcO with 30 mM *tert*-BOOH for 60 min. at room temperature (excess *tert*-BOOH was first removed by HiTrapQ anion-exchange chromatography). Lower chromatogram (3): elution profile for the CcO nuclear-encoded subunits after reaction of 10 μM CcO with 50 μM of peroxidized CL. In each analysis, 0.5 nmol of CcO (1% trifluoroacetic acid in water) was injected onto the HPLC column. The two additional elution peaks obtained with the tert-BOOH-treated CcO (each labeled with an asterisk) were identified as modified subunit VIIc and subunit IV by MALDI-TOF/MS and ESI/MS (refer to Results and [18,19]).

the sites of oxygen addition was unambiguously identified by analysis of tryptic peptides by MALDI-TOF/MS and HPLC-ESI/MS/MS. Indication that Trp_{19} within subunit VIIc and Trp_{48} within subunit IV are oxidized was obtained by analysis of MS/MS data by the Mascot software package (Matrix Science). Assignments for the collision-induced dissociation fragment ions in the spectra, obtained from the tryptic peptides, derived from these two subunits confirm that the site of oxygen addition is Trp_{19} in subunit VIIc and Trp_{48} in subunit IV.

Peroxidation of cardiolipin bound to cytochrome c oxidase by tert-butyl hydroperoxide

All of the CL bound to CcO can be peroxidized by *tert*-BOOH as evidenced by the conversion of its nonconjugated double bonds into conjugated dienes. For example, 96% of the CL bound to CcO contains conjugated dienes after exposure of CcO to 30 mM *tert*-BOOH for 60 min at room temperature (Fig. 6). The *tert*-BOOH-induced CL peroxidation was also confirmed by FOX2 assay, suggesting the generation of cardiolipin hydroperoxide. Blocking the binuclear center of CcO with cyanide decreases the formation of these conjugated dienes to approximately half of that generated in the absence of cyanide (Table 1). This indicates that at least two pathways exist for CL peroxidation. The incomplete effect of cyanide is in sharp contrast to the complete blocking of conjugated dienes formation when CcO is exposed to hydrogen peroxide [18].

Small amounts of TBA-reactive substances are also generated in a time-dependent manner (Fig. 7) consistent with alkyl chain cleavage during prolonged exposure to *tert*-BOOH. However, the amount of MDA generated by *tert*-BOOH is very small; i.e., only 0.2 μM MDA is generated from 12 to16 μM CL.

Discussion

Lipid-soluble peroxide, *tert*-BOOH, and polar, water-soluble peroxide, H_2O_2, have a number of similarities and differences in the way that they damage CcO. Both inactivate CcO, react with the binuclear center to form the peroxy and ferryl intermediates, oxidize Trp_{19} of subunit VIIc and Trp_{48} of subunit IV, and directly or indirectly cause the dissociation of subunits VIa and VIIa. However, the mechanism by which these products are formed is quite different. Hydrogen peroxide effectively reacts with the binuclear center of CcO and must do so to produce any of the above-noted effects [18]. Blocking the binuclear center with cyanide completely prevents all oxidative damage to CcO by H_2O_2. On the other hand, *tert*-BOOH reacts poorly with the binuclear center and oxidation of the two tryptophans and dissociation of subunits VIa and VIIa occurs even if CcO is first inhibited with cyanide. Furthermore, CL is resistant to peroxidation by H_2O_2, but is quite susceptible to peroxidation by *tert*-BOOH. Therefore, even though the products of peroxidation are similar, the mechanism of oxidative damage is quite different; i.e., a second pathway must exist for both tryptophan oxidation and CL peroxidation by *tert*-BOOH. The reaction of *tert*-BOOH with heme-containing proteins resulted in the generation of peroxyl, alkoxyl, and protein-derived radicals [33–35]. These radicals are believed to induce protein modification and/or phospholipid peroxidation. It is generally accepted that heme is the initial site for the reaction of peroxides with heme-containing proteins. Clearly, *tert*-BOOH does not have to first react with the binuclear center. Considering the fact that cyanide inhibits generation of *tert*-BOOH-induced peroxyl radicals, but does not completely inhibits generation of alkoxyl radicals [33], the second mechanism most likely involves direct reaction of alkoxyl radicals with Trp_{19} and Trp_{48} and also CL. Such a direct reaction mechanism is reasonable since *tert*-BOOH partitions into the apolar environment surrounding CcO, which places it in close proximity to the two tryptophans and protein-bound CL.

The other major difference between *tert*-BOOH and H_2O_2-induced damage to CcO is the involvement of CL peroxidation. CL bound to CcO is effectively peroxidized by *tert*-BOOH and the resulting mixture of products contributes to the loss of electron-transport activity. Because this process cannot occur with CL-free CcO, this form of the enzyme is less sensitive to *tert*-BOOH than CL-rich CcO. Furthermore, peroxidized CL very effectively (at very low concentrations) induced modification of nuclear-encoded subunits in the absence of peroxide. Peroxidized CL reacts with and inhibits both CL-rich and CL-free CcO which illustrates the potential danger of forming CL

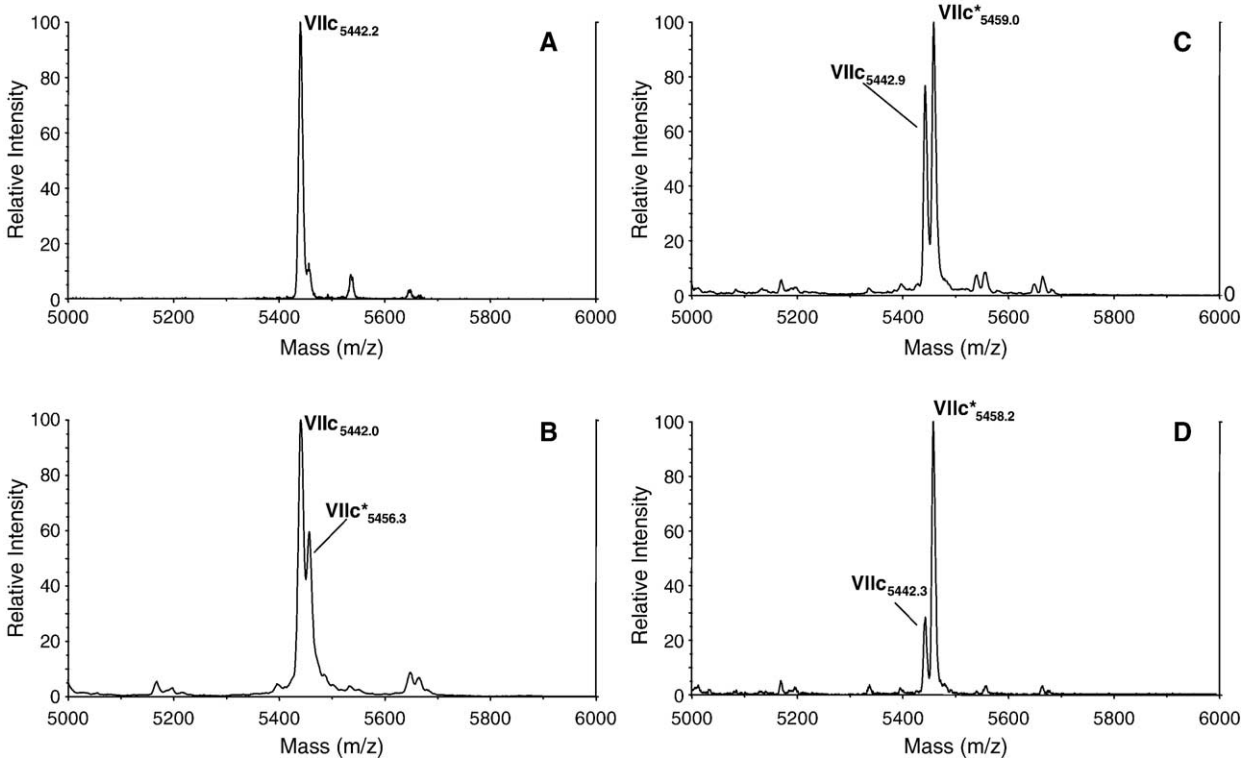

Fig. 5. Comparison of MALDI-TOF mass spectrum of subunit VIIc before and after exposure to peroxides. (A) MALDI-TOF/MS of CcO subunit VIIc before exposure to organic peroxides. (B) MALDI-TOF/MS of CcO subunit VIIc after exposure to 30 mM *tert*-BOOH for 60 min. (C) MALDI-TOF/MS of CcO VIIc after exposure to 0.05 mM CL-OOH for 60 min. (D) MALDI-TOF/ MS of CcO subunit VIIc after exposure to 0.5 mM H_2O_2 for 30 min. In each case, the excess of peroxide was removed by anion-exchange chromatography, and ~15 pmol spotted on the MALDI target using dihydroxybenzoic acid as the matrix. Each spectrum represents an average of 100 laser shots.

hydroperoxide at the surface of CcO. Lastly, peroxidized CL is not able to restore any activity to CL-free CcO as intact CL; it either does not bind or is incapable of stabilizing the same interactions as undamaged CL. Peroxidation of CL may also explain the dissociation of subunits VIa and VIIa. Endogenous CL, which is bound near each of these subunits, stabilizes their association with CcO (Fig. 8). CL peroxidation may weaken these interactions to cause their dissociation from the complex.

Table 1
Cytochrome *c* oxidase modification caused by *tert*-BOOH, CL-OOH, or H_2O_2

Subunit and CL modification	30 mM *tert*-BOOH		0.04 mM CL-OOH		0.5 mM H_2O_2 [a]	
	–KCN	+KCN	–KCN	+KCN	–KCN	+KCN
IV	10	7	5	5	20	0
VIa	25	0	20	22	55	0
VIIa	33	18	12	13	55	0
VIIc	36	11	40	32	70	0
Peroxidation of CL bound to CcO	100	64	N/D	N/D	20	0
Inhibition	40–50	N/D	35–40	N/D	70–80	N/D

Data are expressed as a percentile of control. Percentile of modified subunits was calculated using the HPLC peak area. Percentile of peroxidized CL was estimated spectrophotometrically and calculated using a molar extinction coefficient of 25.2 mM^{-1} cm^{-1} at 234 nm [27]. Percentage of *tert*-BOOH or CL-OOH-induced inhibition is calculated using electron-transport activity data of peroxide-treated and untreated (100% activity) CcO. Cyanide-inhibited CcO was prepared by reaction of 10 μM CcO with 5 mM KCN for 18 h at 4°C. Formation of the cyanide-CcO complex was confirmed by spectroscopic analysis. Activity of cyanide-inhibited CcO was not determined (N/D).

[a] Data for H_2O_2-induced CcO modification, except the formation of CL conjugated dienes, were published recently [19].

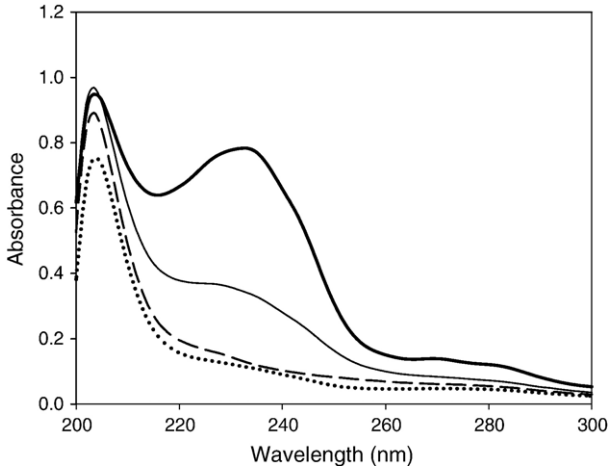

Fig. 6. Inhibition of cytochrome *c* oxidase by cyanide partially blocks *tert*-butyl hydroperoxide-induced peroxidation of bound cardiolipin. Peroxidation of CcO bound cardiolipin was determined from the increase in absorbance at 234 nm for phospholipids extracted and dissolved in ethanol. Ultraviolet absorption spectra are shown for cardiolipin extracted from (1) unmodified cytochrome *c* oxidase (dotted line); (2) cyanide inhibited CcO (dashed line); (3) CcO reacted with 30 mM *tert*-BOOH for 30 min (thick line); and (4) cyanide inhibited CcO after reaction with 30 mM *tert*-BOOH for 30 min (thin line). In each case, CL was extracted from 8 nmol of CcO.

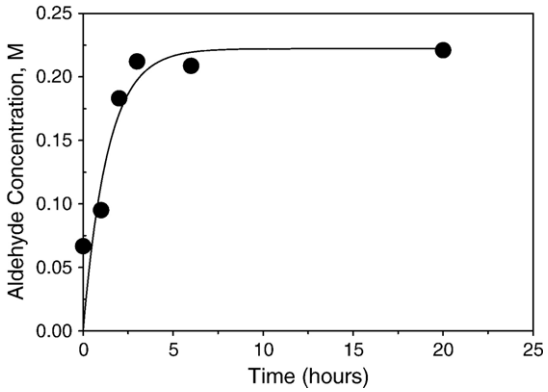

Fig. 7. Aldehyde generation by *tert*-butyl hydroperoxide in the cardiolipin bound to cytochrome *c* oxidase. The concentration of aldehydes (thiobarbituric acid-reactive substances) was determined in CL extracted from 8 nmol of CcO before and after reaction with *tert*-BOOH. TBA-reactive substances were quantified as described under Experimental procedures using malondialdehyde as a standard. Solid line is the nonlinear regression fit to a single exponential.

CL hydroperoxide is probably responsible for the *tert*-BOOH-induced damage to CcO and loss of enzymatic activity. Peroxidation of phospholipids produces a complicated mixture of initial products including conjugated dienes, hydroperoxides, and a variety of secondary products such as PL-derived aldehydes [20]. We have direct evidence that *tert*-BOOH generates each conjugated dienes (Fig. 6), hydroperoxides, and CL-derived aldehydes (Fig. 7), but only CL hydroperoxides are likely to be responsible for the activity loss. Conjugated dienes could not, by themselves, directly oxidize tryptophan while the concentration of *tert*-BOOH-generated lipid aldehydes (<200 nM) is too small to account for the 30–50% inhibition of CcO (e.g., >100 μM MDA is required for 50% inactivation of CcO; Musatov and Robinson, unpublished). We conclude that CL hydroperoxides are likely to be the damaging species. CL peroxidation could damage CcO in one of two ways; (1) it could oxidize amino acids near the CL binding sites; and (2) it could interfere with structural and functional functions of bound CL. At least one of the CL binding sites is located in close proximity to subunit VIIc; therefore, CL hydroperoxide may react with and damage Trp_{19} within this subunit. The final result would be similar to oxidation of Trp_{19} by radical migration from the binuclear center of CcO. Alternatively, peroxidation of CL may destabilize essential subunit interactions with CcO. Four molecules of CL are bound at specific sites and are absolutely required for maintenance of structural integrity and retention of full enzymatic activity [8,17]. Removal of these CL leads to partial inhibition of electron-transport activity, complete loss of proton translocation activity, and destabilization of subunit interactions within CcO, particularly subunits VIa, VIb, and VIIa [17]. If peroxidized CL is incapable of maintaining these subunit interactions, the result would be dissociation of these subunits, which does occur during *tert*-BOOH-induced damage.

Perhaps the most surprising result, considering that the mechanisms of oxidative damage are different, is that the same sites within CcO are damaged by H_2O_2, *tert*-BOOH, or CL-OOH. As we suggested based upon the previous H_2O_2 study [18,19], Trp_{19} and Trp_{48} must be especially susceptible to peroxide-induced oxidation; i.e., they are “stable sinks” for reactive free radicals. The unusual sensitivity of at least one of these tryptophans is supported by the fact that oxidized Trp_{48} has been detected even in normal human heart mitochondria [4].

We conclude that lipid-soluble peroxide, e.g., *tert*-BOOH, can react with and modify CcO function by three distinct mechanisms. First, *tert*-BOOH can react with the binuclear

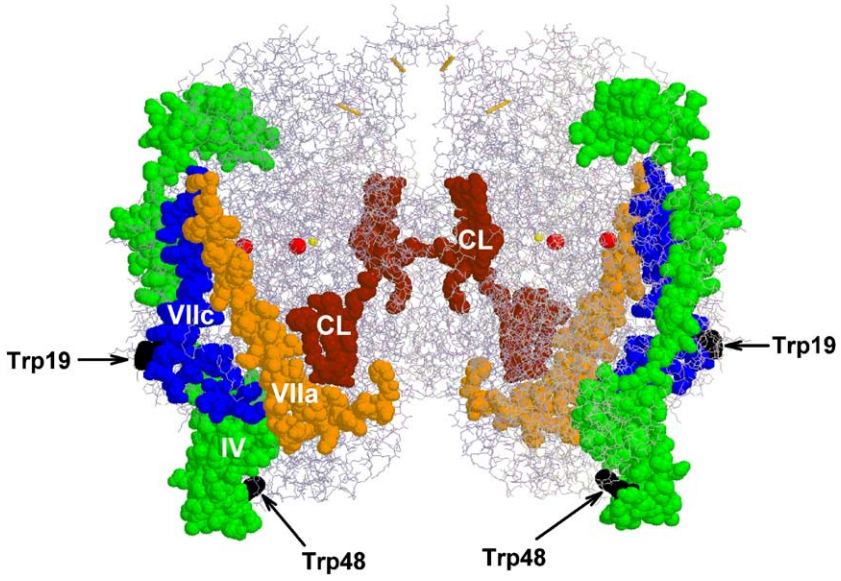

Fig. 8. Location of oxidized Trp19, Trp48, and bound cardiolipin within the 3-dimensional structure of dimeric cytochrome *c* oxidase. Trp19 within subunit VIIc and Trp48 within subunit IV are shown black; the two CL bound per monomer of CcO that were detected in the crystal structure are shown in brown. The color scheme for three CcO subunits altered by *tert*-BOOH are VIIa (gold), VIIc (blue), IV (green). The iron atoms of cytochrome *a* and a_3 are red. All other subunits are shown in gray wireframe. Figure was prepared using atomic coordinates of CcO in the Protein Bank (PDB ID code 1v54 [36]). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

center and generate catalytic intermediates and free radicals, which migrate through subunit I to oxidize Trp_{19} and Trp_{48} of subunits VIIc and IV, respectively. Bound CL, which is located in close proximity to these tryptophans, is also peroxidized by the migrating free radicals. Second, *tert*-BOOH directly reacts with both tryptophan residues and bound CL. The end result is similar to the first mechanism, but these reactions occur even if the binuclear center is blocked by cyanide. Third, *tert*-BOOH peroxidizes the CL bound to CcO and the resulting CL peroxidation products inactive CcO. In the latter case, inactivation may be caused by CL-hydroperoxide-mediated peroxidation of key amino acid residues, or the destabilization of functionally important subunit interactions. It is, therefore, reasonable to speculate that each of these processes also could occur in vivo when the local concentration of ROS near the inner membrane surface becomes very high. The end result would be decreased cytochrome *c* oxidase activity and loss of mitochondrial electron-transport function.

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