Mechanism in the reaction of cytochrome *c* oxidase with organic hydroperoxides: an ESR spin-trapping investigation

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Organic hydroperoxides are of great utility in probing the reaction mechanism and the toxicological consequences of lipid peroxidation. In the present study, ESR spin-trapping was employed to investigate the peroxidation of mitochondrial cytochrome *c* oxidase (CcO) with *t*-butyl hydroperoxide (*t*-BuOOH) and cumene hydroperoxide (CumOOH). The spin trap 5,5-dimethyl-1-pyrroline *N*-oxide (DMPO) was used to detect the radical species formed from the reaction of CcO with *t*-BuOOH. The presence of *t*-BuOOH-derived alkoxyl radical (*t*-BuO[•]) as the primary radical indicates reductive scission of the O–O bond by CcO. The ESR signal of DMPO/*t*-Bu can be partially abolished by cyanide, implying that the reductive cleavage involved the haem *a*₃Cu_B binuclear site of CcO. A nitroso spin trap, 2-methyl-2-nitrosopropane (MNP), was used to detect and identify radical species from the reaction of CcO with CumOOH. In addition to the *t*-BuOOH-derived methyl, hydroxymethyl and tertiary carbon-centred radicals, a protein-derived radical was detected. The intensity of the ESR signal from the protein radical increased with the CumOOH concentration at low CumOOH/CcO ratios, with maximal intensity at a ratio of 100 mol of CumOOH/mol of CcO. The immobilized protein radical adduct of MNP was stable and persistent after dialysis; it was also resistant to proteolytic digestion, suggesting that it was formed in the transmembrane region, a region that is not

accessible to proteases. Its signal was greatly enhanced when CcO cysteine residues were chemically modified by *N*-ethylmaleimide, when the tryptophan residues in CcO were oxidized by *N*-bromosuccinimide, and when tyrosine residues on the surface of CcO were iodinated, showing that a radical equilibrium was established among the cysteine, tryptophan and tyrosine residues of the protein-centred radical. Pre-treatment of CcO with cyanide prevented detectable MNP adduct formation, confirming that the haem *a*₃-Cu_B binuclear centre was the initial reaction site. When the CcO was pre-treated with 10 mM (100 equivalents) of CumOOH, the enzyme activity decreased by more than 20%. This inhibition was persistent after dialysis, suggesting that the detected protein-centred radical was, in part, involved in the irreversible inactivation by CumOOH. Visible spectroscopic analysis revealed that the haem *a* of CcO was not affected during the reaction. However, the addition of pyridine to the reaction mixture under alkaline conditions resulted in the destruction of the haem centre of CcO, suggesting that its protein matrix rather than its haem *a* is the target of oxidative damage by the organic hydroperoxide.

Key words: compound F, compound P, free radical, oxidative damage.

INTRODUCTION

Oxidative stress in mitochondria has been widely recognized to be a causative factor in a variety of diseases, including aging, neurodegeneration and cancer [1–4]. During mitochondrial respiration, there is a continuous release of electrons from their normal pathway to molecular oxygen to form superoxide anion [5]. Increased superoxide production from the mitochondrial electron-transport chain (ETC) is associated with a low level of phosphorylation. When the concentration of ADP is lower, the flow of electrons is slower, and there is an accumulation of electron-transfer components in their reduced states (i.e. reduced flavin mononucleotide, ubiquinone and reduced cytochrome *b*₅₆₆) which can reduce oxygen upstream of the mitochondrial cytochrome *c* oxidase (CcO) to form superoxide [6–9]. It has been estimated that approx. 2% of the oxygen uptake in state 4 respiration (respiration slows due to all ADP being converted into ATP) by isolated mitochondria results in production of H₂O₂ under physiological conditions [7,10]. Since the major source of reactive oxygen species (ROS) is associated with oxidative phosphorylation, respiratory-chain dysfunction has

been recognized and identified in several neurological diseases [2,4], such as Alzheimer's disease [11], Huntington's disease [12] and Parkinson's disease [12]. Generally, respiratory-chain defects are defined as those detected in the mitochondrial complexes I–IV. Several reports have demonstrated that ROS can cause oxidative inactivation of the components of the mitochondrial ETC during exposure of submitochondrial particles ('SMPs') to fluxes of O₂^{•−}, [•]OH or H₂O₂ [13,14].

CcO is one of the essential energy-transducing electron-transfer complexes. It is a critical segment of the mitochondrial respiratory chain. The enzyme catalyses the electron transfer from ferrocytochrome *c* to dioxygen, and concomitantly translocates protons across the membrane, providing the driving force for ATP synthesis. The enzyme contains five metalloprotein redox centres: two *a*-type haems (haem *a* and haem *a*₃) and one copper (Cu_B) are located on subunit I, while two coppers (Cu_A) are located on subunit II.

It has been demonstrated that CcO can undergo a 'peroxidase-like' reaction at its haem *a*₃-Cu_B binuclear centre, a reaction that has a high *K*_m for H₂O₂ with participation by compound P and compound F intermediates [15,16]. We have employed the ESR

Abbreviations used: CcO, cytochrome *c* oxidase; *t*-BuOOH, *t*-butyl hydroperoxide; CumOOH, cumene hydroperoxide; DMPO, 5,5-dimethyl-1-pyrroline *N*-oxide; DTPA, diethylenetriaminepenta-acetic acid; ETC, electron-transport chain; 13-HpODE, 13-hydroperoxy-9,11-octadecadienoic acid; MNP, 2-methyl-2-nitrosopropane; NEM, *N*-ethylmaleimide; ROS, reactive oxygen species; UV/VIS, UV-visible.

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spin-trapping technique to investigate the oxidative damage induced in CcO by H_2O_2 [17]. It is quite clear that, following peroxidation with H_2O_2 , CcO forms protein-centred radicals.

However, the ROS induced in the mitochondrial milieu are not restricted to H_2O_2 . In an earlier report, spin-trapping studies with DMPO on CcO interaction with H_2O_2 also revealed cyanide-sensitive superoxide radical adduct formation [18]. The mechanism of the reaction implies slow one-electron oxidation of excess H_2O_2 by compounds P and F to generate superoxide. When the influx of ROS is greater than the ability of the biological system to cope with them, eventually lipid hydroperoxide can be produced from the lipid-peroxidation chain reaction [19]. Lipid peroxidation can lead to oxidative damage of biological molecules other than lipids.

One class of compounds has proved to be greatly useful in studying oxidative damage resulting from lipid hydroperoxide: organic hydroperoxides. Of this class, two in particular, cumene hydroperoxide (CumOOH) and *t*-butyl hydroperoxide (*t*-BuOOH), have been widely used to investigate the mechanism of haem protein-catalysed free radical production [20–22]. Early studies indicated that *t*-BuOOH and other organic peroxides could induce changes in the UV–visible (UV/VIS) absorption spectrum of CcO (formation of compounds P and F) [23–25]. To date, there is little evidence that describes how organic hydroperoxides interact with membrane-bound CcO, and lead to toxicological consequences. Elucidating the above reaction mechanism is of fundamental importance in understanding the oxidative damage of the ETC.

In the present study, we have used ESR spin-trapping to investigate the mechanism of radical production from the reaction of CcO with the above organic peroxides. In addition to the oxygen- and carbon-centred radicals derived from the organic peroxide, we have found that the protein-centred radical of CcO is involved in the oxidative damage induced by the organic peroxides. The implications of this finding with regard to the protein damage of CcO are discussed.

MATERIALS AND METHODS

Reagents

Diethylenetriaminepenta-acetic acid (DTPA), *t*-BuOOH (70 %), 5,5'-dithiobis-(2-nitrobenzoic acid), horse-heart cytochrome *c* and manganese superoxide dismutase were purchased from Sigma Chemical Co. (St Louis, MO, U.S.A.), and used as received. CumOOH (80 %), 2-methyl-2-nitrosopropane (MNP), *N*-bromosuccinimide, *N*-ethylmaleimide (NEM), potassium cyanide, pyridine and sodium iodoacetate were purchased from Aldrich Chemical Co. (Milwaukee, WI, U.S.A.). Catalase (bovine liver) was purchased from Boehringer Mannheim (Indianapolis, IN, U.S.A.). The spin trap 5,5-dimethyl-1-pyrroline *N*-oxide (DMPO) was purchased from Fluka Chemical Co. (St Louis, MO, U.S.A.), vacuum-sublimed twice and stored under nitrogen at -70°C until needed. All reactions were performed in a 50 mM phosphate buffer, pH 7.4, treated with Chelex overnight. DTPA was added to a final concentration of 1 mM after treatment with Chelex.

Mitochondrial CcO preparation and assay

Highly purified CcO was isolated from submitochondrial particles using the method developed by Yu et al. [26]. Optical spectra were measured using an SLM AMINCO DW-2000 UV/VIS spectrophotometer (SLM Instruments, Inc, Rochester, NY, U.S.A.). The enzyme concentration (per a_{430}) of CcO was calculated from the spectrum after dithionite reduction using a

molar absorption coefficient of $24\text{ mM}^{-1}\cdot\text{cm}^{-1}$ at 605 nm. The enzyme activity of CcO was assayed by measuring cytochrome *c* oxidation. An appropriate amount of CcO was added to an assay mixture (1 ml) containing 50 mM phosphate buffer, pH 7.4, and 60 μM reduced cytochrome *c*. CcO activity was determined by measuring the decrease in absorbance at 550 nm.

ESR experiments

ESR experiments were performed with a Bruker EMX spectrometer operating at 9.8 GHz with 100 kHz modulation frequency at $23\text{--}24^\circ\text{C}$. The reaction mixture was transferred to a 10-mm quartz ESR flat cell, which was then positioned into the SHQ cavity (Bruker Instrument, Billerica, MA, U.S.A.). The sample was scanned using the following parameters: centre field, 3490 G ($1\text{ G} = 10^{-4}\text{ T}$); microwave power, 20 mW; modulation amplitude, 1 G; time constant, 655.36 ms; gain, 1×10^5 . The spectral simulations were performed using the WinSim program of the NIEHS public ESR software package, available on the Internet (<http://epr.niehs.nih.gov/pest.html>; [27]).

Isolation and qualitative analysis of haem *a* from CcO

The haem *a* from the purified CcO was extracted and analysed by the procedure published previously [28].

Analysis of haem *a* from CcO by analysis of pyridine haemochromagen spectra

An appropriate amount of purified haem *a* or purified CcO was dissolved in 50 mM NaOH containing 15 % (v/v) pyridine, and the spectra were recorded using an SLM AMINCO DW-2000 UV/VIS spectrophotometer.

Iodination of tyrosine residues on the surface of the CcO

Aliquots (1 ml) of CcO (200 μM) in 50 mM sodium phosphate buffer, pH 7.4, containing 1 % (w/v) sodium cholate were mixed with NaI (final concentration of 100 mM). Iodination of tyrosine residues of CcO was initiated by the addition of two *N*-chlorobenzenesulphonamide-immobilized beads (Iodo-beads; Pierce, Rockford, IL, U.S.A.) [29]. The reaction was allowed to proceed at $23\text{--}24^\circ\text{C}$ for 10 min. To stop the reaction, the solution was removed from the vessel and subjected to dialysis against the same buffer.

RESULTS

Reaction of CcO with *t*-BuOOH in the presence of DMPO

When CcO was allowed to react with *t*-BuOOH in the presence of 100 mM DMPO, an ESR spectrum was produced (Figure 1). The spectrum could be simulated with a mixture of three DMPO adducts: methyl alkoxyl (36 %; $a^{\text{N}} = 14.45\text{ G}$, $a^{\text{H}}\beta = 10.75\text{ G}$ and $a^{\text{H}}\gamma = 1.35\text{ G}$) [30], *t*-butoxyl (47 %; $a^{\text{N}} = 14.9\text{ G}$, and $a^{\text{H}}\beta = 16.15\text{ G}$), and methyl radical (17 %; $a^{\text{N}} = 16.25\text{ G}$, and $a^{\text{H}}\beta = 23.35\text{ G}$) [30]. These adducts were produced only if both CcO and peroxide were present (results not shown). To demonstrate that the primary product was the *t*-butoxyl radical (*t*-BuO $\cdot$), we repeated the experiment with an increased concentration of DMPO (400 mM). As indicated in Figure 2(A), the relative amounts of DMPO/ $\cdot\text{OCH}_3$ and DMPO/ $\cdot\text{CH}_3$ were reduced, and the contribution of DMPO/ $\cdot\text{Ot-Bu}$ was increased.

The detected methyl radical adduct (DMPO/ $\cdot\text{CH}_3$) was attributed to the unimolecular β -scission of *t*-BuO $\cdot$. The source of the methyl radical was confirmed by substituting

Figure 1 Computer simulation and deconvolution of the ESR spectrum obtained from the reaction mixture containing CcO, t-BuOOH, and DMPO

(A) The computer simulation (dashed line) is superimposed on the experimental spectrum obtained using CcO (50 μ M), t-BuOOH (10 mM) and DMPO (100 mM). The purified CcO in 50 mM sodium phosphate buffer, pH 7.4, containing 1% (w/v) sodium cholate was mixed with DMPO in the presence of 1 mM DTPA. The reaction was initiated by adding t-BuOOH to the mixture at 23–24 °C. The experimental spectrum was immediately recorded at 23–24 °C. (B–D) The individual simulations of each species in the composite spectrum. The hyperfine values used for each species are described in the Results section. (B) The simulated spectrum for DMPO/•Ot-Bu: line width, 0.82 G; line shape, 19% Lorentzian, 81% Gaussian; and mole ratio, 0.47. (C) The simulated spectrum for DMPO/•OCH₃: line width, 0.64 G; line shape, 3% Lorentzian, 97% Gaussian; and mole ratio, 0.36. (D) The simulated spectrum for DMPO/•CH₃: line width, 0.87 G; line shape, 65% Lorentzian, 35% Gaussian; and mole ratio, 0.17. The instrumental parameters are described in the Materials and methods section, and the scan time was 327 s for the 100 G scan.

t-[¹²C]BuOOH with t-[¹³C]BuOOH so that the former was replaced by the heavier isotope with a different spin number ($I = 1/2$) (results not shown).

The result also suggested that the enzymic reaction could proceed via the mechanism of reductive scission, involving the haem a_3 -Cu_B binuclear centre of CcO. To test this involvement, we added potassium cyanide (100 nmol of KCN/nmol of aa_3) to block the binuclear centre of CcO before adding the t-BuOOH. As shown in Figure 2(C), the addition of KCN to the reaction system significantly inhibited alkoxyl radical adduct formation, indicating a haem-catalysed reaction was involved.

The KCN-inhibited ESR spectrum (Figure 3) also revealed an additional species with hyperfine coupling constants ($a^N = 15.4$ G and $a^H\beta = 18.90$ G), which are those of the DMPO/•CN adduct [31] (simulation shown in Figure 3A, the dashed line). To confirm further the detection of the cyanyl radical adduct, we substituted potassium cyanide isotopically labelled with ¹³C ($I = 1/2$) (K¹³CN), which converted the 6-line spectrum

Figure 2 Effect of increasing DMPO concentration and KCN on the ESR spectrum obtained from the reaction mixture containing CcO, t-BuOOH, and DMPO

(A) The computer simulation (dashed line) is superimposed on the experimental spectrum obtained using CcO (50 μ M), t-BuOOH (10 mM) and DMPO (400 mM). The reaction conditions in the system were identical with those described in the legend for Figure 1, except that the DMPO concentration was elevated to 400 mM. (B) Same as in (A), except the enzyme was omitted from the system; (C), same as in (A), except that CcO was incubated with 5 mM KCN at 23–24 °C for 5 min, and then reacted with t-BuOOH. The instrumental parameters are described in the Materials and methods section, and the scan time was 327 s for the 100 G scan.

(DMPO/•¹²CN) into a 12-line DMPO/•¹³CN spectrum ($a^N = 15.4$ G, $a^H\beta = 18.90$ G and $a^{13C}\beta = 12.95$ G; Figures 3C and 3D). DMPO/•CN was generated only if both the enzyme and organic hydroperoxide were present (results not shown). The formation of the cyanyl radical implies that this reaction is peroxidase-like and involves the ferryl-oxo intermediates of CcO, compound P and compound F.

It has been of concern that organic peroxides are often contaminated with H₂O₂, and the latter can be generated in the reaction mixture during the course of the experiment. H₂O₂ will react with oxidized CcO much faster than the bulky organic peroxides, and will give rise to highly reactive compounds P and F and superoxide radicals, which can subsequently interact with organic peroxides in secondary reactions [23]. This possibility was eliminated by control experiments in which catalase (200 μ g/ml) and manganese superoxide dismutase (1000 units/ml) were added to the reaction mixture, which did not affect radical formation (results not shown). This result supports the facile reaction of t-BuOOH or CumOOH (see below) with the binuclear site in CcO [24].

Figure 3 Computer simulation of the ESR spectra obtained from the reaction mixture containing CcO, KCN, t-BuOOH and DMPO

(A) The computer simulation (dashed line) is superimposed on the experimental spectrum obtained using CcO (50 μ M), KCN (5 mM), t-BuOOH (10 mM) and DMPO (400 mM). The reaction conditions in the system were the same as those described in the legend for Figure 2(C). (B) The simulated spectrum for DMPO/ ^{12}CN : line width, 0.53 G; line shape, 27% Lorentzian, 73% Gaussian; and mole ratio, 0.20. (C) Same as in (A), except that the K^{12}CN was replaced with K^{13}CN . (D) The simulated spectrum for DMPO/ ^{13}CN : line width, 0.53 G; line shape, 29% Lorentzian, 71% Gaussian; and mole ratio 0.13. The instrumental parameters are described in the Materials and methods section, and the scan time was 327 s for the 100 G scan.

MNP spin-trapping of the protein-derived and other small carbon-centred radicals derived from CumOOH

We were able to characterize further the carbon-centred radical(s) formed during the reaction of CcO with organic peroxide CumOOH by using the nitroso spin-trap, MNP. MNP adducts yield more information about the trapped radical, because the radicals are added directly to the nitrogen that becomes the nitroxide.

Figure 4(A) shows the ESR spectrum when 50 μ M CcO was used. In the initial scan, the ESR spectrum was dominated by broad lines characteristic of the immobilized MNP radical adducts derived from proteins (Figure 4A). In later scans, MNP radical adducts characterized by sharp lines were also present and readily detected (Figure 4D). The small, sharp-lined adducts were assigned on the basis of computer simulation (Figures 4E–4G) as follows: methyl, $a^{\text{N}} = 17.36$ G and $a^{\text{H}}\beta = 14.35$ G (3H); hydroxymethyl, $a^{\text{N}} = 15.34$ G and $a^{\text{H}}\beta = 6.2$ G (2H); and 'CHRR', $a^{\text{N}} = 15.72$ G and $a^{\text{H}}\beta = 1.68$ G (1H) (where R and R' denote two different substituent alkyl groups), indicating a carbon-centred radical with one β proton coupling. The hyperfine

Figure 4 ESR spectrum obtained from the reaction of CcO with CumOOH in the presence of MNP

(A) The reaction mixture containing 50 μ M CcO, 20 mM CumOOH, catalase (200 μ g/ml) and 11 mM MNP was subjected to ESR measurement. The spectrum was collected at the initial scan. (B) Same as in (A), except that CcO was replaced with buffer. (C) Same as in (A), except that CumOOH was eliminated from the system. (D) Same as in (A), except that the reaction mixture was incubated for 15 min at 23–24 $^{\circ}\text{C}$ before ESR measurement. The individual simulations for MNP/ CH_3 (E), MNP/ CH_2OH (F) and MNP/'CHRR' (G) are shown respectively. The hyperfine values used for each species are described in the Results section; instrumental parameters are described in the Materials and methods section, and the scan time was 656 s for the 100 G scan.

coupling constants were similar to those reported by Chamulitrat et al. [21]. The fact that these adducts were detected later, after longer incubation times, indicates that the protein-centred radical is the primary radical formed upon reaction of CcO with CumOOH, whereas the small radical adducts are secondary radicals derived from CumOOH.

The concentration of CcO affected the ESR spectrum of the MNP radical adducts. As the concentration of protein was increased, the immobilized nitroxide became stronger. Figure 5(A) represents the ESR spectrum when the concentration of CcO was raised to 100 μ M. When the protein-centred radicals (Figure 5A) were separated from the small, sharp-lined radical adducts by dialysis (Figure 5B), the immobilized radical adducts were quite stable and persistent. The immobilized nitroxide has a splitting of approx. 64.5 G, as measured between the low-field maximum and the high-field minimum (i.e. $2a_{\text{N}}^{\text{H}}$).

To test whether the generation of the protein radical is initiated in the haem a_3 -Cu_B binuclear centre, we pre-treated CcO with KCN (100 nmol of KCN/nmol of a_{a_3}), which inhibited protein

Figure 5 Effect of KCN on the ESR spectrum obtained from the reaction mixture containing CcO, and CumOOH in the presence of MNP

(A) The reaction mixture containing 100 μ M CcO, 10 mM CumOOH, catalase (100 μ g/ml) and 11 mM MNP was subjected to ESR analysis. The spectrum was recorded at the initial scan. (B) The sample used in (A) was recovered from the flat cell and dialysed against 100 ml of 50 mM sodium phosphate buffer, containing 1.0% (w/v) sodium cholate, pH 7.4, for 2 h, before the ESR spectrum was re-recorded. (C) Same as in (A), except that the CcO was pre-treated with 10 mM KCN for 5 min at 23–24 °C. The instrumental parameters are described in the Materials and methods section, and the scan time was 656 s for the 100 G scan.

radical adduct formation by 90% (Figure 5C and Table 1). A four-line ESR spectrum with hyperfine coupling constants $a^N = a^H\beta = 14.38$ G from MNP/ $\cdot$ H, a product of MNP reduction [32], was obtained (Figure 5C). The addition of cyanide to the pre-formed radical adducts of MNP did not affect the spectrum of these adducts in any way (results not shown).

When the sample from Figure 5(B) was subjected to Pronase digestion, the immobilized MNP adduct was resistant to proteolytic digestion, suggesting that protein radical formation may occur at the transmembrane region, an area that is not accessible to protease. However, it was observed that partial fragmentation of the immobilized nitroxide with Pronase revealed a weak three-line spectrum (results not shown). The hyperfine coupling constant with $a^N = 15.5$ G is similar to the MNP/tyrosyl adduct, as seen in metmyoglobin [33] and cytochrome *c* [34].

When CumOOH was substituted with *t*-BuOOH or the lipid hydroperoxide 13-hydroperoxy-9,11-octadecadienoic acid (13-HpODE), a similar protein radical adduct of MNP was observed with the same $2a^N$ value, which was found to be sensitive to cyanide (results not shown).

The spectrum of the immobilized adduct depended on the concentrations of CcO and hydroperoxide. As the concentration of CcO was increased, the signal from the immobilized nitroxide

Table 1 Effect of various chemical modifications of CcO on the ESR signal of protein-centred radicals induced by CumOOH

CcO (100 μ M, as modified by the indicated compounds) in 50 mM sodium phosphate buffer, pH 7.4, containing 1% sodium cholate was mixed with MNP (11 mM) in the presence of 1 mM DTPA and catalase (100 μ g/ml). The reaction was initiated by adding CumOOH (10 mM) and allowed to incubate at room temperature for 10 min. The reaction mixture was immediately dialysed against the same buffer for 2 h. Aliquots of the dialysate were withdrawn for recording the ESR spectra and determining the enzyme concentration (on the basis of a_{a_3}). The generated radical concentration (protein radical adduct/ a_{a_3}) was calculated by dividing the double integration of the obtained ESR spectrum with the a_{a_3} concentration of CcO.

Reagents used	Chemical reactions	Protein radical adduct/ a_{a_3} (%)†
None	Native CcO*	100
NEM	Alkylation of cysteine residues	220
Sodium iodoacetate	Carboxymethylation of cysteine residues	219
5,5'-Dithiobis-(2-nitrobenzoic acid)	Thiylation of cysteine residues	198
<i>N</i> -Bromosuccinimide	Oxidation of tryptophan residues	210
KI + <i>N</i> -chlorobenzenesulphonamide	Iodination of tyrosine residues	220
Diethyl pyrocarbonate	Ethoxycarbonation of histidine residues	100
KCN	Binding to the haem a_3 -Cu _B centre	10

* The radical concentration obtained from native CcO in which no modifying compound was added is scaled as 100%.

† Data are the average values of two batches of CcO. The deviation among the different batches was < 5%.

Figure 6 Titration of peroxide-induced protein radical derived from CcO with various concentrations of CumOOH

The purified CcO in 50 mM sodium phosphate buffer, pH 7.4, containing 1% (w/v) sodium cholate was mixed with MNP in the presence of 1 mM DTPA and catalase. The reaction was initiated by adding the indicated concentrations of CumOOH and allowed to incubate at 23–24 °C for 10 min. The reaction mixture was immediately dialysed against the same buffer for 2 h. Aliquots of the dialysate were withdrawn for recording the ESR spectra and determining the enzyme concentration (on the basis of the a_{a_3} amount). The generated radical concentration (spin number/ a_{a_3}) was calculated by double integration of the ESR spectrum.

became stronger (results not shown). When purified CcO was titrated with various concentrations of CumOOH, the radical adduct (represented as protein radical adduct/ a_{a_3}) increased as the concentration of peroxide increased (Figure 6). Maximum production was reached at a ratio of 100 mol of CumOOH/mol

Figure 7 Effect of CumOOH and MNP on the enzymic activity of purified CcO

Aliquots (200 μ l) containing CcO (100 μ M) and CumOOH in 50 mM sodium phosphate buffer, pH 7.4, containing 1% (w/v) sodium cholate and 1 mM DTPA were allowed to incubate at 23–24 $^{\circ}$ C for 10 min. The reaction mixture was then dialysed against the same buffer for 2 h to remove excess amounts of CumOOH. Cytochrome *c* oxidation activity was determined for aliquots of the dialysate. The activity was calculated on the basis of the aa_3 concentration determined by the difference spectrum of dithionite reduction and ferric cyanide oxidation. (A) intact CcO. (B) Reaction mixture contained CcO and 5 mM CumOOH. (C) Same mixture as in (B), except that CumOOH was increased to 10 mM. (D) Same mixture as in (B), but the thiol groups of CcO were carboxylmethylated with sodium iodoacetate.

of CcO; further increases in hydroperoxide concentration failed to increase protein radical generation, although the protein gradually became aggregated.

Involvement of cysteine, tryptophan and tyrosine residues on the surface of CcO in the electron transfer with detected protein-centred radical

The resistance of the CcO radical to proteolytic digestion implies that the detected globin radical(s) of CcO is located within the transmembrane region. Therefore it would be of interest to know whether blocking specific amino acid residues of CcO by chemical modification would affect the protein radical. As shown in Table 1, when the cysteine residues on the surface of CcO were chemically modified by NEM (alkylation of thiol), sodium iodoacetate (carboxylmethylation of thiol), or 5,5'-dithiobis-(2-nitrobenzoic acid), the ESR signal of the protein-centred radical was enhanced by almost 100%. A similar enhancement was observed when tryptophan residues in CcO were oxidized by *N*-bromosuccinimide [35]. The signal intensity increased by nearly 120% when the tyrosine residues on the surface of the CcO were iodinated by sodium iodide in the presence of *N*-chlorobenzenesulphonamide [29]. These results established radical transfer between the detected protein radical and the cysteine, tryptophan and tyrosine residues on the surface of CcO when the oxidative damage was initiated by either organic hydroperoxide or lipid hydroperoxide. In contrast, the intensity of the protein-centred radical was not affected by ethoxycarbonylation of histidine residues of CcO by diethyl pyrocarbonate, suggesting

Figure 8 Visible absorption spectra of CcO and metmyoglobin following the addition of CumOOH

The reaction mixture contained CcO (100 μ M) and CumOOH (10 mM) in 50 mM sodium phosphate, pH 7.4, also containing 1% (w/v) sodium cholate and 1 mM DTPA. Aliquots (10 μ l) were withdrawn and mixed with 980 μ l of the above buffer, before spectroscopic measurements. Absorption spectra were recorded every 3 min. The spectra show intact CcO (continuous line), a mixture of CcO and CumOOH [dashed lines, labelled 'initial scan' and '15 min' (the sixth scan) as shown], and a mixture of metmyoglobin (1.6 μ M) and CumOOH (40 μ M; inset). The spectra were collected under the same conditions as those for CcO.

that the histidyl imidazole groups on the surface of the CcO were not involved.

Involvement of the detected protein-centred radical in the oxidative damage of CcO by the CumOOH

Incubation of 100 μ M CcO with 5 mM CumOOH, pH 7.4 [in the presence of 1% (w/v) sodium cholate] at 23–24 $^{\circ}$ C for 10 min caused enzymic activity (assayed with cytochrome *c* oxidation) to decrease up to 16% after removal of excess peroxide by dialysis (Figure 7, bar B). The above results imply that the formation of the protein-centred radical(s) is involved in the irreversible loss of enzyme activity by CumOOH. Further increasing the CumOOH (up to 10 mM) enhanced the inhibitory effect (up to 23–25% inhibition; Figure 7, bar C), presumably due to increasing the amount of the protein-centred radical formation (Figure 6). Either alkylation of CcO with NEM or carboxylmethylation with sodium iodoacetate did not affect the enzymic activity of CcO (results not shown). However, blocking the thiol groups on the surface of CcO with NEM (or sodium iodoacetate) made enzyme activity more vulnerable to the CumOOH treatment (up to 32–35% inhibition at a ratio of NEM-CcO:CumOOH of 1:100; Figure 7D), demonstrating the protective role of cysteine residues in CcO.

Protein matrix of CcO, but not the haem *a* of CcO, is the target of oxidative damage by organic hydroperoxides

To understand further the mechanism of organic hydroperoxide-induced oxidative damage to CcO, it is necessary to know whether the prosthetic groups of CcO are affected. It has been

Figure 9 Alkaline pyridine haemochromagen spectral change of purified haem *a* following the addition of CumOOH

The conditions for isolating haem *a* from CcO and its pyridine haemochromagen spectrum are described in the Materials and methods section. The spectra were recorded every 3 min (up to four scans). The spectra show purified haem *a* (3.6 μ M; continuous line), and the reaction of purified haem *a* and 2 mM CumOOH (the four dashed lines).

Figure 10 Alkaline pyridine haemochromagen spectra of CcO following the addition of CumOOH

CcO (100 μ M), 20 μ l in 50 mM sodium phosphate, pH 7.4, containing 1% (w/v) sodium cholate and 1 mM DTPA was mixed with 1 ml of 50 mM NaOH containing 15% (v/v) pyridine. The mixture was subjected to visible spectroscopic measurement (continuous line). CumOOH was then added to the mixture to a final concentration of 1 mM, and the spectrum (dashed line) was recorded every 3 min for up to four scans.

reported previously that organic and lipid hydroperoxides can degrade protoporphyrin IX and bleach the Soret band of some haem proteins, such as cytochrome *c* [22], chloroperoxidase [21], metmyoglobin (inset of Figure 8), and cytochrome P450 [36].

However, when we reacted CcO with CumOOH, the Soret band of haem *a* was not bleached (Figure 8, solid line). Similar results were also observed in the reaction of CcO with *t*-BuOOH and 13-HpODE (results not shown). This is consistent with the ESR signals of CcO detected at low temperature (77° K), in which the intensities of low-spin haem ($g = 2.99$) and rhombic iron ($g = 4.30$) were not significantly affected by CumOOH treatment (results not shown). The haem *a* in CcO contains iron protoporphyrin IX with a 1-formyl substituent and a 7-hydroxyl farnesylethyl group. To examine whether the difference in structure from the bleachable haem proteins above contributed to the resistance of peroxide treatment, we isolated the haem *a* from CcO by using a procedure published previously [28]. The pyridine haemochromagen spectrum of purified haem *a* exhibited a characteristic absorption of the α band (λ_{max} shifted to 588 nm by dithionite reduction) (Figure 9, solid line). When the purified haem *a* was incubated with either CumOOH (Figure 9) or *t*-BuOOH (results not shown), the Soret bands were bleached, as seen in the UV/VIS spectra (Figure 9, dashed line), showing that purified haem *a* is degraded by organic hydroperoxide.

The above result can be demonstrated further by the pyridine haemochromagen spectrum of intact CcO in the presence of CumOOH. A shift in the Soret bands can be elicited by pyridine, which is known to be capable of dissociating haem from the haem protein under alkaline conditions [37]. The pyridine haemochromagen spectrum of CcO (Figure 8, dashed line) revealed a significant shift of α - and γ -bands. When CumOOH was included in the CcO/pyridine system, the Soret bands were gradually bleached (Figure 10, dashed line), whereas, in the presence of pyridine alone, no haem *a* was destroyed (Figure 10, solid line). This result suggests that it is the protein matrix of the CcO that protects its haem *a*, and prevents it from being degraded by the CumOOH.

DISCUSSION

In the present study, the main radicals observed in our ESR spin-trapping experiments with *t*-BuOOH were *t*-butoxyl, methoxyl, methyl and a protein-derived immobilized radical. With DMPO as the spin trap, our results demonstrate that *t*-BuO $\cdot$ is the primary radical generated from the reaction of the CcO with *t*-BuOOH (Figure 2A). Therefore we propose a mechanism involving the reductive cleavage of *t*-BuOOH by the CcO haem a_3 -Cu $_B$ binuclear centre, as illustrated in reaction 1, where compound F is a ferryl-oxo intermediate of CcO.

This hypothesis of haem-initiated reductive scission is consistent with the decrease in DMPO/ $\cdot$ Ot-Bu when the CcO is pre-treated with cyanide (Figure 2C).

In addition, after cyanide pre-treatment the cyanyl radical adduct of DMPO was detected (Figure 3A) and verified with K ^{13}CN (Figure 3B). Presumably, cyanyl radical can be produced, because the reaction of CcO with organic hydroperoxide also proceeds by a peroxidase-like mechanism involving a porphyrin cation radical intermediate (compound P). That is, if the *t*-BuOOH undergoes a heterolytic 2-electron reduction, as shown in reaction (2), then one-electron oxidation of cyanide to cyanyl radical could be catalysed by the intermediates of compound P and compound F [38] (reactions 3 and 4), as was previously seen in the peroxidation of cyanide by horseradish peroxidase in the presence of H $_2$ O $_2$ [31].

The methyl radical detected with both DMPO (Figures 1A and 1D) and MNP (Figures 4D and 4E) can be attributed to the unimolecular β -scission of $t\text{-BuO}^\bullet$ [18,19] (reaction 5). These results were confirmed by substituting $t\text{-}^{13}\text{C}\text{BuOOH}$ ($I = 1/2$) for $t\text{-}^{12}\text{C}\text{BuOOH}$ ($I = 0$).

The protein-derived radical was detected as the immobilized MNP adduct. The organic hydroperoxides $t\text{-BuOOH}$, CumOOH and 13-HpODE all induce the same adduct, as shown by the identical $2a_N$ (64.5 G) for each. The elimination of this protein-centred radical by cyanide indicates that the haem $a_3\text{-Cu}_B$ binuclear centre of CcO is involved in its formation. Presumably, the initial site for the reaction was not the amino acid residues of CcO, but the haem, generating compound P (a ferryl porphyrin cation radical; reaction 2 in the case of $t\text{-BuOOH}$), which then oxidized the vicinal amino acid residues. A similar result was also observed in a study of the protein oxidation of metmyoglobin [33].

Although the CcO radical adduct(s) of MNP were quite stable and resisted proteolysis with Pronase, such resistance is typically not observed in metmyoglobin [33] or cytochrome c [34]. We conclude that CcO's unique protein radical must be located within the region of transmembrane helices that is not accessible to the protease. This location is supported further by the failure of chemical modifications of the four amino acid residues to abolish the ESR signal of the protein radical adduct (Table 1). These amino acid residues, which could be oxidized to form free radicals on the surface of CcO, include cysteine (alkylation and carboxymethylation), tyrosine (iodination), tryptophan (oxidation) and histidine (ethoxycarbonylation).

The protein-derived radical appears to be connected with damage to the enzymic function of CcO. As our analysis of enzyme activity clearly shows, when the protein radical is formed, CcO irreversibly loses part of its activity (Figure 7). In particular, we observed that blocking the thiol groups on the surface of CcO did make the enzyme more vulnerable to redox attack (Figure 7D). This result supports a protective role for cysteine residues in CcO, as has been seen for oxidative damage to BSA [39].

UV/VIS spectral analysis of the Soret absorption band provides strong evidence that oxidative damage of CcO by organic hydroperoxides does not involve the destruction of haem a (Figure 8). In contrast, the haem is destroyed in the reaction of organic hydroperoxide with several other haem proteins, i.e. metmyoglobin, cytochrome P450, cytochrome c and chloroperoxidase. Although the structure of haem a in CcO differs from the structure of iron protoporphyrin IX in the latter haem proteins, it is unlikely that this structural difference can account for the haem a protection in CcO, because the protoporphyrin IX of haem a was bleached by organic hydroperoxide in the alkaline pyridine haemochromagen spectra of both purified haem a and intact CcO (Figures 9 and 10). Thus the protection of haem a must be attributed to the environment of CcO's active site.

The high-resolution three-dimensional X-ray structure of CcO revealed that the haem a and haem a_3 of CcO are located 13 Å (1 Å = 0.1 nm) into the inner membrane of 48 Å thickness [40]. Each protoporphyrin IX is held by its four transmembrane helices of subunit I [41]. Furthermore, 28 transmembrane helices per CcO molecule are observed from the X-ray crystallographic data [41]. Therefore it is logical to propose that the protoporphyrin ring is protected by the protein matrix of CcO, and is thus inaccessible to redox attack by organic hydroperoxide. It is noteworthy that the iron of haem a in CcO is still accessible to the organic hydroperoxide in order to form the reactive ferryl-oxo ($\text{Fe}^{\text{IV}}=\text{O}$) intermediate (reactions 1 and 2).

If it is not the haem that is damaged, then the damage site must be the site of the protein-derived radical. It is notable that, even after extensive dialysis against the phosphate buffer (for up to 6–8 h), the ESR signal of this protein radical adduct is quite stable. Other protein-derived MNP adducts with similar stability are the tyrosyl radical adducts in metmyoglobin (induced by H_2O_2) [33] and BSA (induced by hydroxyl radical attack) [39]. Partial fragmentation of the protein radical adduct in CcO with Pronase revealed a weak three-line spectrum with $a^N = 15.5$ G, a parameter possibly indicative of the tyrosyl radical adduct of MNP. The spectrum we observed had a broader line width than authentic MNP/Tyr adduct [33] because Pronase degrades only partially the hydrophobic region containing transmembrane helices of CcO, leading to slow motional effects, and making the assignment of tyrosyl radical with perdeuterated MNP (MNP- d_9) unfeasible [33,34]. However, direct evidence for detecting protein-derived tyrosyl radical from the peroxidation of the CcO and compound P formation has been reported [42,43]. From our results here, we conclude that, in addition to oxygen and carbon-centred radical(s), protein radicals need to be considered as one important toxicological consequence when the oxidative damage of CcO is induced by organic or lipid hydroperoxide.

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