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Tryptophan 334 oxidation in bovine cytochrome *c* oxidase subunit I involves free radical migration

Patrizia Lemma-Gray, Susan T. Weintraub, Christopher A. Carroll, Andrej Musatov, Neal C. Robinson*

Department of Biochemistry, The University of Texas Health Science Center, MC 7760, 7703 Floyd Curl Drive, San Antonio, TX 78229-3900, USA

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Abstract **A single tryptophan ($W_{334(I)}$) within the mitochondrial-encoded core subunits of cytochrome *c* oxidase (CcO) is selectively oxidized when hydrogen peroxide reacts with the binuclear center. $W_{334(I)}$ is converted to hydroxytryptophan as identified by reversed-phase HPLC-electrospray ionization tandem mass spectrometry analysis of peptides derived from the three SDS–PAGE purified subunits. Total sequence coverage of subunits I, II and III was limited to 84%, 66% and 54%, respectively. $W_{334(I)}$ is located on the surface of CcO at the membrane interface. Two other surface tryptophans within nuclear-encoded subunits, $W_{48(IV)}$ and $W_{19(VIIc)}$, are also oxidized when hydrogen peroxide reacts with the binuclear center (Musatov et al. (2004) Biochemistry 43, 1003–1009). Two aromatic-rich networks of amino acids were identified that link the binuclear center to the three oxidized tryptophans. We propose the following mechanism to explain these results. Electron transfer through the aromatic networks moves the free radicals generated at the binuclear center to the surface-exposed tryptophans, where they produce hydroxytryptophan.**

Keywords: Cytochrome oxidase; Tryptophan oxidation; Hydrogen peroxide; Mass spectrometry; Proteolytic digestion; Aromatic pathway

1. Introduction

Cytochrome *c* oxidase (CcO) is susceptible to damage by reactive oxygen species (ROS) that are byproducts of cellular metabolism [1,2]. A major source of ROS is electron leakage from the mitochondrial electron transport chain, specifically from Complex I (NADH dehydrogenase) and Complex III (cytochrome bc_1), both of which generate superoxide anions [3–5]. The superoxide anion is subsequently converted to hydrogen peroxide by superoxide dismutase. Under normal physiological conditions, the average cellular hydrogen peroxide concentration is relatively low (1–100 nM), but the local concentration near the mitochondrial inner membrane would be much higher. Hydrogen peroxide itself is not highly reactive, but reacts with transition metals to produce extremely reactive hydroxyl radicals [6].

Cytochrome *c* oxidase can be oxidatively damaged by hydrogen peroxide without involvement of other reactive oxygen species. Hydrogen peroxide binds to the heme of cytochrome a_3 to form two catalytic intermediates, peroxy- and ferryl-CcO, which are potential sources of free radicals. In fact, radicals are formed near the binuclear center during CcO turnover [7,8]. Generation of such free radicals is likely to be responsible for the slow, but progressive loss of enzymatic activity upon exposure of CcO to hydrogen peroxide [9]. Coincident with this loss in activity is the oxidation of two peripheral tryptophans, $W_{48(IV)}$ and $W_{19(VIIc)}$, and dissociation of subunits VIa and VIIa. Selective oxidation of these tryptophans, each located within a nuclear-encoded subunit, is somewhat surprising since they are on the surface of CcO, quite far (44–60 Å) from the binuclear center. The mechanism by which $W_{19(VIIc)}$ or $W_{48(IV)}$ are selectively oxidized has not been identified, but neither oxidation product is formed if CcO is first inhibited by cyanide. This suggests that the first step must involve a direct reaction of hydrogen peroxide with the binuclear center [9]. We, therefore, postulated that radical migration from the binuclear center through Subunit I must be responsible for oxidation of $W_{48(IV)}$ and $W_{19(VIIc)}$ [9]. Such a mechanism suggests that other amino acid residues may be oxidized in the core of CcO. We have now developed methodology to detect oxidized sites within the three, very hydrophobic, core mitochondrial-encoded subunits by HPLC-ESI/MS/MS analysis of peptides derived from SDS–PAGE purified subunits. Using this approach, we were able to identify an additional site within the core of CcO that is oxidized during exposure to H_2O_2.

2. Materials and methods

2.1. Materials

Hydrogen peroxide (30 wt% solution in water), cytochrome *c*, bovine liver catalase, insulin B (oxidized form), elastase and cyanogen bromide were purchased from Sigma–Aldrich Chemical Co. Dodecylmaltoside was purchased from Anatrace, Inc. Sequencing grade bovine pancreatic trypsin and chymotrypsin were purchased from Roche Diagnostic Corporation. Microcon YM-3 membranes were purchased from Millipore Co. All other chemicals were of analytical grade.

*Corresponding author. Fax: +1 210 567 6595.
E-mail address: robinson@uthscsa.edu (N.C. Robinson).

Abbreviations: CcO, cytochrome *c* oxidase; HPLC, high-performance liquid chromatography; HPLC-ESI/MS/MS, reversed-phase HPLC with electrospray ionization tandem mass spectrometry detection and analysis; MALDI-TOF/MS, matrix-assisted laser desorption ionization time-of-flight mass spectrometry; ROS, reactive oxygen species

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2.2. Enzyme isolation

CcO was isolated from Keilin–Hartree beef heart particles as described by Mahapatro and Robinson [10]. The isolated enzyme was solubilized at ~100 μM in 100 mM NaH_2PO_4 buffer, pH 7.4, containing 25 mM sodium cholate and 1.0 mM EDTA, quickly frozen by dripping the CcO solution into liquid nitrogen and the individual pellets (~25 μL) stored at −80 °C. Before each experiment, a frozen pellet of CcO (~2.5 nmol) was thawed, 2 μmole dodecylmaltoside was added and sodium cholate was subsequently removed by extensive dialysis. The enzyme activity was 370–390 s^{-1} as calculated from the first order rate of ferrocytochrome *c* oxidation measured spectrophotometrically in 25 mM phosphate buffer, pH 7.0, containing 2 mM dodecylmaltoside [11]. Reduced and oxidized visible spectra, subunit composition, phospholipid content and aggregation state of the purified enzyme were analyzed as previously described [12,13]. Cyanide reactivity of the dodecylmaltoside-solubilized enzyme was biphasic with a second order rate constant of 2.3 M^{-1} s^{-1} with 1 mM KCN at pH 7.8 [14]. Cyanide-inhibited CcO was prepared by reacting 20 μM CcO with 5 mM KCN for 18 h at 4 °C. Formation of the CN–CcO complex was confirmed by spectroscopic analysis.

2.3. Reaction of CcO with hydrogen peroxide

CcO (20 μM, solubilized in 20 mM Tris–SO_4, pH 7.4, containing 2 mM dodecylmaltoside) was reacted with 1 mM H_2O_2 (ε_{240} = 40 M^{-1} cm^{-1} [15]) for 30 min at RT to produce a mixture of peroxy- and ferryl-CcO as is evident from the absolute and difference spectra (Fig. 1). The two difference spectrum maxima at ~580 nm and ~607 nm and the $\Delta A_{434-412}$ ($\Delta\varepsilon_{434-412}$ = 65 mM^{-1} cm^{-1} [16]) indicate that 45–60% of heme a_3 is converted to a mixture of the two oxy-intermediates. A small amount of heme bleaching occurs during this reaction since the Soret absorbance intensity decreases 5–10% (Fig. 1). After 30 min, 0.25–0.27 mM H_2O_2 remained in solution as determined using the ferrous ion oxidation method in the presence of xylenol orange, butylated hydroxytoluene and sulfuric acid (FOX2 method) [17]. For this analysis, unreacted H_2O_2 was separated from the H_2O_2–CcO mixture using a Microcon YM-3 Centrifugal Filter Unit (molecular weight cut-off 3000); 10 μL of filtrate was reacted with 900 μL FOX2 reagent for 30 min at RT after dilution with 90 μL methanol. The H_2O_2 concentration was determined spectrally at 560 nm.

Possible destruction of heme a_3 and/or heme *a* during reaction of CcO with H_2O_2 was tested by quantifying the dithionite reducibility of heme *a* and the cyanide binding to the binuclear center. The reaction of 4.8 μM CcO with 1 mM H_2O_2 at RT was stopped after 30 min by addition of 50 μM catalase. A shift in the absorbance maximum from 426 nm to 423–422 nm over the next 45 min suggested a slow decay of oxy-intermediates. Subsequent addition of 5 mM KCN shifted the maximum absorbance to 427 nm, consistent with the formation of a CcO-cyanide complex. Addition of dithionite produced a spectrum typical of the mixed-valence CcO–cyanide complex. The concentration of CcO, i.e., (heme $a + a_3$)/2, in the oxidized and reduced CN complexes was calculated using ε_{427} = 168 mM^{-1} cm^{-1} and ε_{605} = 38.9 mM^{-1} cm^{-1}, respectively [18]. The concentration of CcO was found to be 4.27 μM, i.e., ~88% of the hemes remained reactive towards dithionite or cyanide, which is consistent with the ~10% heme bleaching that occurred during exposure of CcO to H_2O_2 (Fig. 1).

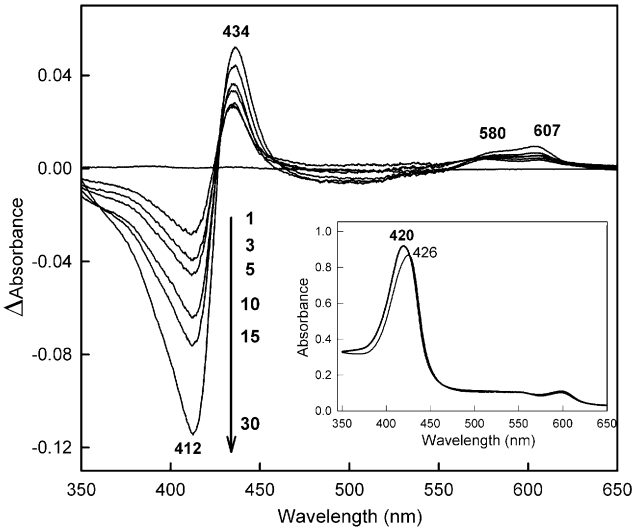

Fig. 1. H_2O_2-induced changes in the absolute and difference spectrum of cytochrome *c* oxidase. Main panel: time-dependent difference spectra of H_2O_2-reacted CcO minus oxidized CcO. *Inset*: the absolute spectra of oxidized CcO (thick line) and CcO after reaction with 1 mM H_2O_2 for 30 min (thin line). Experimental conditions were 6 μM CcO in 20 mM Tris–SO_4 buffer at pH 7.4 containing 2 mM dodecylmaltoside.

2.4. In-gel proteolytic digestion of core CcO subunits

The subunits of CcO (~40 pmole) were separated by SDS–PAGE using a 15% acrylamide separating gel. The protein bands corresponding to subunits I, II and III were excised, destained and incubated separately with elastase, chymotrypsin, or trypsin (25 μL, 0.02 μg/μL) for 18 h, at 37 °C.

2.5. Mass spectrometry

The resulting proteolytic peptides were concentrated to 10 μL using C18MB OMIX pipette tips (Varian) and analyzed by mass spectrometry using either matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF/MS) or HPLC-ESI/MS/MS. MALDI-TOF mass spectra were acquired on an Applied Biosystems Voyager-DE STR in reflectron mode using either α-cyano-4-hydroxycinnamic acid or dihydroxybenzoic acid as matrix. HPLC-ESI/MS/MS was performed on a Thermo Finnigan LCQ that had been adapted for microspray ionization. On-line HPLC separation of the protein digests was accomplished with a Michrom BioResources Paradigm MS4 micro HPLC as follows: PicoFrit™ column (New Objective; 75 μm, i.d.) packed to 10 cm with C18 adsorbent (Vydac; 218MS 5 μm, 300 Å); mobile phase A, 0.5% acetic acid/0.005% trifluoroacetic acid; mobile phase B, 90% acetonitrile/0.5% acetic acid/0.005% trifluoroacetic acid; gradient, 2–72% B in 30 min; flow rate, 0.4 μL/min. The MS/MS spectra of the protein fragments were searched against the Swiss-Prot database using the Mascot (Matrix Science) search engine. Assignments of MS/MS fragments were verified by comparison with fragmentation predicted in silico by GPMAW (Lighthouse Data).

3. Results

Hydrogen peroxide reacts with heme a_3 of CcO to produce a mixture of peroxy- and ferryl-CcO. During the reaction, tyrosine and/or tryptophan radicals are known to be generated near the binuclear center [19], $W_{19(VIIc)}$ and $W_{48(IV)}$ become oxidized [9], and the enzymatic activity decreases ~60% [9]. A small amount of heme bleaching occurs during this reaction since the Soret absorbance intensity decreases 5–10% (Fig. 1) and the cyanide reactivity and dithionite reducibility decrease ~12% (Section 2.3). However, the magnitude of the heme destruction is certainly not enough to account for the substantial activity loss.

To test the hypothesis that exposure of CcO to H_2O_2 may also oxidize tryptophans within the CcO core, peptides generated from purified subunits I, II and III were analyzed by MALDI-TOF/MS and HPLC-ESI/MS/MS. The best procedure for generating sets of peptides with high sequence coverage of the core subunits was identified by MALDI-TOF/MS screening of proteolytic digests (Table 1). Tryptic digestion of either the entire CcO complex, or SDS–PAGE purified subunits I, II and III resulted in less than 5% MALDI-TOF/MS coverage of the three core subunit sequences. Cyanogen bromide cleavage gave much better sequence coverage, but the resulting large, multi-charged peptides are not suitable for HPLC-ESI/MS/MS analysis, which must be used to identify