Hydrogen peroxide is not released following reaction of cyanide with several catalytically important derivatives of cytochrome c oxidase

Marian Fabian[1], Graham Palmer*

Department of Biochemistry and Cell Biology MS 140, Rice University, 6100 Main, Houston, TX 77005-1892, USA

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Abstract We have looked for the production of hydrogen peroxide following reaction of oxidized cytochrome c oxidase and two oxy derivatives (compounds P and F) with cyanide. In each case the final product was the cyanide adduct of cytochrome c oxidase. In no case release of hydrogen peroxide was detected, as gauged by the scopoletin plus horse radish peroxidase assay. The simplest conclusion is that none of these forms of the enzyme contains intact hydrogen peroxide.

Key words: Cytochrome c oxidase; Peroxy derivative; Oxyferryl derivative; Hydrogen peroxide; Cyanide

1. Introduction

Cytochrome c oxidase (CcO) catalyzes the reduction of dioxygen to water using reducing equivalents provided by ferrocytochrome c; this reaction occurs at a binuclear center composed of cytochrome a_3 and Cu_B. Various approaches, notably transient kinetic studies on the reaction of reduced enzyme with O_2 [1–6] and reversal of electron transfer induced using conditions of high electron and proton affinity plus a high membrane potential [7,8], have led to the identification of a variety of intermediates in the reoxidation pathway.

Two of these intermediates are of particular interest because they appear to be intimately involved in proton translocation [9]. These are compounds P (CcO-607) and F (CcO-580). These species were originally identified as optical intermediates arising during reverse electron transfer. It was proposed [7,8] that P is a species in which the catalytically important binuclear center contains the elements of hydrogen peroxide and that F is a species in which the heme component of the binuclear center has been oxidized to the ferryl (Fe(IV)) state.

While the existence of the entities P and F is generally accepted their specific chemical composition is controversial. Thus it has been suggested that (i) CcO-607 and CcO-580 are indeed the peroxy and ferryl intermediates [7–19]; (ii) CcO-607 and CcO-580 forms represent two different binding sites for hydrogen peroxide present in oxidized CcO [20]; (iii) CcO-607 is a ferryl form while CcO-580 is species of oxidized oxidase with cytochrome a_3 low-spin [21]; and (iv) CcO-607 and CcO-580 species are both ferryl forms but with a free radical center in the CcO-607 species [22]. Indeed Kitagawa's laboratory has provided rather compelling Raman data that an Fe = O bond is present at the heme iron of cytochrome a_3 in both compounds [23–25] while Fabian and Palmer, on the basis of chemical experiments, suggested that compound F itself may be at the peroxide level [26].

In an attempt to throw some light on this controversy we reasoned that treatment of derivatives of the enzyme in which peroxide is believed to be bound at the binuclear center with cyanide, a ligand with exceptionally high affinity for the binuclear center, should lead to the release of peroxide, and this released peroxide should be detectable using simple analytic procedures. In this communication we report that binding of cyanide to (i) native CcO; (ii) compound P produced using the CO method [26]; and (iii) compound F produced using H_2O_2 [26] does not result in the release of detectable amounts of hydrogen peroxide as gauged by oxidation of the fluorescent probe scopoletin in the presence of horse radish peroxidase.

2. Materials and methods

Tris base, 7-hydroxy-6-methoxy-2H-1-benzopyran-2-one (Scopoletin) and horse radish peroxidase (HRP) were purchased from Sigma, NaCN from Mallinckrodt, hydrogen peroxide and 4-morpholine ethane-sulfonic acid (MES) from Aldrich, *N*-dodecyl-β-D-maltoside (DM) from Anatrace and Triton X-100 (peroxide free; TX-100) from Boehringer.

Cytochrome c oxidase (CcO) was isolated by the method of Soulimane and Buse [27] with small modifications. During the isolation K_2SO_4 was used in place of chloride salts and for the extraction of bc_1 and CcO by TX-100 the mitochondrial protein concentration was 20 and 10 mg/ml respectively. The enzyme concentration was determined at pH 8.0 from its absorbance at 424 nm by using $A_{mM} = 158$ mM^{-1} cm^{-1}. Unless otherwise specified the buffer was 100 mM Tris pH 8.1 containing 0.1% DM and 100 mM K_2SO_4.

Two oxy intermediates of CcO designated as CcO-607 (i.e. compound P) and CcO-580 (i.e. compound F) were prepared from the oxidized CcO. CcO-607 was formed by bubbling CO gently through oxidized enzyme (CcO-O) in an open cuvette for about 30 s at room temperature. The cuvette was then transferred to the spectrophotometer and the production of CcO-607 was assessed spectrophotometrically. The maximum conversion CcO-O to CcO-607 (92%) was reached in about 8 min. Then the sample was degassed several times on ice to remove the residual CO and concentration of CcO-607 was determined from the difference spectrum CcO-607 minus CcO-O using $\Delta A_{607-630} = 11.0$ mM^{-1} cm^{-1} [8].

CcO-580 was prepared at pH 5.9 (100 mM MES in place of Tris) by the addition of 20 μM H_2O_2 to 6.7 μM CcO-O. The difference optical spectrum of CcO-580 minus CcO-O is pH dependent and at acidic pH the maximum of the α band is at 575 nm compared to 578–580 nm at pH 8.0 [26]. The conversion of CcO-O to CcO-580 was followed in

*Corresponding author. Fax: +1 (713) 285 5154.
E-mail: graham@bioc.rice.edu

[1]Permanent address: Institute of Experimental Physics, Slovak Academy of Sciences, 043 53 Kosice, Slovak Republic.

Abbreviations: CcO, cytochrome c oxidase; CcO-O, oxidized cytochrome c oxidase; CcO·CN, cytochrome c oxidase-cyanide derivative; P (CcO-607), peroxy form of cytochrome c oxidase; F (CcO-580), oxyferryl form of cytochrome c oxidase; HRP, horse radish peroxidase; MES, 4-morpholine ethane-sulfonic acid; DM, *N*-dodecyl-β-D-maltoside; TX-100, Triton X-100; Tris, Tris hydroxymethylaminomethane

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spectrophotometer using the absorption changes at 575–630 nm. The maximum yield of CcO-580 was observed in about 6 min. At this point scopoletin (24 μM) and HRP (0.94 μM) were added and the residual hydrogen peroxide was reduced to water by HRP oxidation of scopoletin. The concentration of CcO-580 was determined from the difference optical spectrum of peroxide treated CcO minus CcO-O using $\Delta A_{575-630} = 5.3$ mM^{-1} cm^{-1} [8].

The concentration of stock solution of hydrogen peroxide was obtained from absorption at 240 nm by using $A_m = 40$ M^{-1} cm^{-1} [28]. For the detection of hydrogen peroxide during the reaction of oxidase with cyanide fluorescent method with scopoletin and HRP was employed [29]. The basis of the method is that fluorescent scopoletin is oxidized to the nonfluorescent derivative by HRP at the presence of H_2O_2. A typical sample for the detection of H_2O_2 during the reaction of oxidase with 1 mM cyanide contains 0.94 μM HRP, 13.6 μM scopoletin and 6.7 μM CcO in 100 mM buffer (Tris/MES), 100 mM K_2SO_4 and 0.1% DM.

Optical spectra were measured in Hewlett Packard Diode Array 8452 A spectrophotometer at room temperature (24°C). The fluorescence measurements were performed on an SLM Aminco Luminescence Spectrometer using an excitation wavelength of 365 nm, an emission wavelength of 460 nm and spectral bandwidths of 4 nm.

3. Results

We have attempted to detect the release of H_2O_2 following the binding of cyanide to three forms of cytochrome c oxidase: oxidized enzyme (CcO-O), CcO-607 and CcO-580.

At pH 8.1 CcO-O interacts with 1 mM cyanide in a single exponential phase (Fig. 1A) which is complete in approximately 25 min as gauged from the optical changes in the Soret region. The fluorescence emission of a parallel sample shows a small increment immediately following addition of cyanide and a subsequent slow decrease. The initial increment appears to be due to a reaction of cyanide with HRP for it can be observed in a sample containing only HRP and scopoletin (Fig. 2, upper trace). The subsequent slow decrease is probably associated with spontaneous oxidation of scopoletin and is observed in a sample without cyanide (Fig. 2, lower trace).

To verify that the peroxide detection system was functioning correctly addition of a quantity of hydrogen peroxide comparable to the amount of enzyme was found to give a relatively rapid and substantial decrease in scopoletin emission (Fig. 1A).

The reaction of CcO-607 with cyanide is a two step process [30]. In the first step cyanide induces a fast conversion of CcO-607 to a form very similar to CcO-O accompanied with a blue shift of the Soret absorption. In the second step the complex of oxidized CcO with cyanide is formed (Fig. 2). There are no changes in scopoletin emission associated with either of these two phases (Fig. 1B).

A similar result was obtained upon reaction of CcO-580 with cyanide. Because the optical spectra of the CcO·CN complex and CcO-580 are very similar we used the wavelength couple 548 nm minus 574 nm to measure the conversion of CcO-580 to CcO·CN (Fig. 1C). In this case also the independence of fluorescence emission with time implies that the conversion of CcO-580 to CcO·CN is not accompanied by release of H_2O_2 (Fig. 1C).

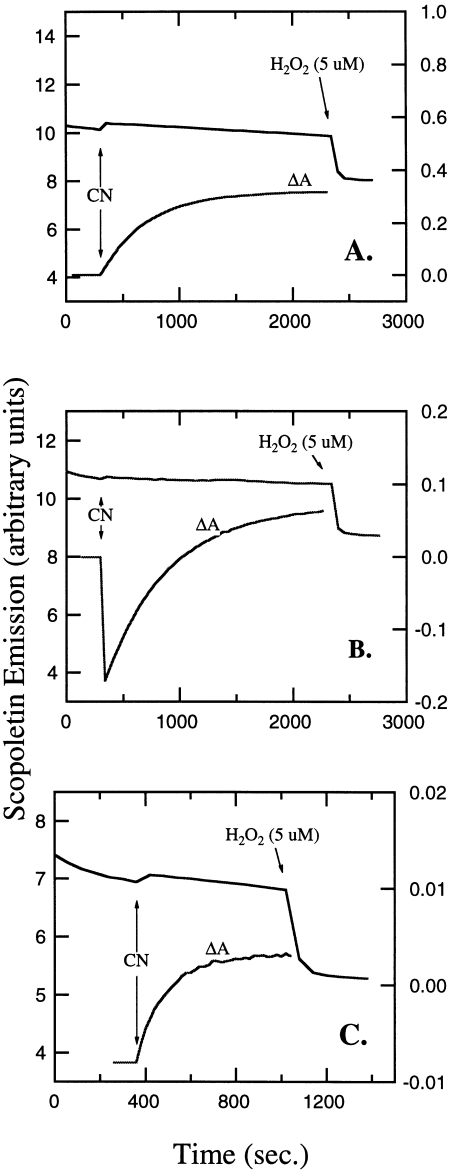

Fig. 1. Fluorescence and absorbance changes following the addition of cyanide to three forms of cytochrome oxidase. A: Oxidized CcO. The reaction of 1 mM cyanide with 6.7 μM oxidized CcO was followed using changes in the absorbance difference 432 nm minus 412 nm (right axis) at 24°C. Hydrogen peroxide production was monitored (left axis) under the same conditions with 6.7 μM CcO, 0.94 uM HRP and 13.6 μM scopoletin present. At the time when the absorbance changes were complete 5 μM H_2O_2 was added. The buffer for both experiments was 100 mM Tris pH 8.1 plus 100 mM K_2SO_4 and 0.1% DM. B: CcO-607. The binding of 1 mM cyanide to 5.6 μM CcO-607 (total enzyme was 6.7 μM) was measured as changes in the absorbance difference 434 nm minus 412 nm (right axis). Hydrogen peroxide production was monitored (left axis) under the same conditions. All other conditions as in A. C: CcO-580. The binding of 1 mM cyanide to 3.6 μM CcO-580 (total enzyme was 6.7 μM) was measured as changes in the absorbance difference 548 nm minus 574 nm (right axis). Hydrogen peroxide production was monitored (left axis) under the same conditions. The buffer was 100 mM MES pH 5.9 containing 100 mM K_2SO_4 and 0.1% DM. All other conditions as in A.

4. Discussion

The simplest explanation of the observation that no peroxide is released following addition of cyanide to CcO-607 (compound P) is that the components of peroxide are no longer intact in this derivative. This conclusion is of course consistent with recent Raman data [23–25] that appear to show that P, like F, contains a Raman mode typical of the Fe = O stretching vibration which requires that the oxygen-oxygen bond is broken.

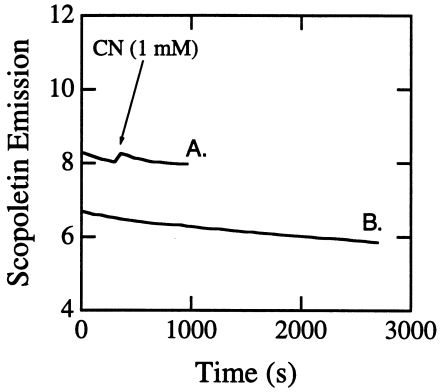

Fig. 2. A: Fluorescence changes following addition of 1 mM cyanide to 13.6 μM scopoletin plus 0.94 μM horse radish peroxidase. B: Slow decrease in the fluorescence emission of scopoletin in the absence of cyanide. The reaction mixture contained 5.5 μM cytochrome c oxidase, 13.6 μM scopoletin plus 0.94 μM horse radish peroxidase. The buffer is the same as that used in Fig. 1A.

Anomalies in the reaction of CcO-607 with cyanide were initially observed by Mitchell et al. [31]; subsequently Berka et al. [32] suggested that the small blue shift initially observed in the reaction of oxidized enzyme with cyanide could be the result of a reaction of cyanide with a small population of P present in the resting enzyme. We subsequently showed that the reaction of P is itself biphasic with both phases more rapid than the reaction of oxidized enzyme with cyanide [30].

It is important to note that the product of this reaction (and indeed of CcO-580) is indistinguishable from the authentic oxidase-cyanide adduct as judged by optical and mcd spectra; in particular the mcd is most revealing as the cyanide adduct exhibits the characteristic doubling of mcd intensity in the Soret (data not shown). It thus appears that the original heme ligand present in CcO-607 has been displaced and the iron is present as Fe(III).

Given that the product does indeed appear to be the ferric enzyme one explanation for the biphasic kinetics of CcO-607 supposes that the first phase is the reduction of Fe(IV) to Fe(III) by an initial reaction with cyanide followed by reaction of the heme iron with a second equivalent of cyanide to yield the Fe(III)-cyanide adduct.

A less likely possibility which was proposed earlier [30], and one for which there is no precedent, also requires the reaction of two equivalents of cyanide with the enzyme. The first reacts directly with the bound peroxide to cleave the O-O bond and produce a novel species according to the scheme:

$$Fe^{3+}-O-O-H + CN^- \Rightarrow Fe^{3+}-OH + CNO^-.$$

The heme iron subsequently reacts with the second cyanide to produce the final product.

There are several difficulties with assigning P as an oxy iron species. First there is good evidence that P is 1-electron equivalent more oxidized than F [10] but the location of this oxidizing equivalent is not apparent. It is certainly not the porphyrin macrocycle for the conversion of P to F that proceeds with almost no change in the Soret absorption while attempts to demonstrate a protein free radical in P have failed [26]. Furthermore were this oxidizing equivalent located away from the heme (either on the protein or perhaps in the form of CuB(III)), there would be no good reason why the heme spectrum would change so dramatically when P is converted to F. Second the optical spectrum of P in the alpha band region is unusually narrow for a high oxidation state of iron [33]; indeed the difference spectrum of P between 500 and 700 nm is remarkably similar in shape and intensity to that of the a_3^{2+}-CO difference spectrum though the latter is shifted some 18 nm to the blue.

The absence of any detectable peroxide following addition of cyanide to the native enzyme is to be expected for there has never been any reason to believe that this species contains hydrogen peroxide; however our suggestion [26] that F might be a peroxide species would appear to be ruled out by the present data.

If CcO-580 is also a ferryl species then one would anticipate biphasic kinetics similar to those observed when CcO-607 reacts with cyanide. However the reaction is monophasic and about 5 times faster than the fastest phase observed with resting CcO [30,31]. Mono-exponential kinetics could arise if the first step was reduction of Fe(IV) and was rate-limiting; this could explain the higher rate of reaction of both CcO-580 and CcO-607 compared to CcO itself for the former two species have been assigned very high redox potentials [8]. But the faster rate of binding of cyanide to CcO-580 does not by itself indicate that the reaction is a redox event. For example the rate of binding of cyanide to the azide adduct of CcO is about 10 times the rate of binding to unliganded enzyme [34]; similarly the rate of binding of cyanide to chloride enzyme is faster than that to chloride-free enzyme [35].

It should be noted that the activity of the scopoletin-HRP assay is reduced by the presence of cyanide. Thus the complete reaction of the hydrogen peroxide used as calibrant (Fig. 1) required about 3 min as compared with reaction time of the order of seconds obtained when cyanide is omitted. Nevertheless the clear and readily measured decrease in fluorescence observed following addition of hydrogen peroxide validates the use of this assay.

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