The Reaction of Cyanide with Peroxidatic Forms of Cytochrome Oxidase[†]

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ABSTRACT: The interaction of peroxidatic derivatives of cytochrome *c* oxidase with cyanide has been investigated by optical spectroscopy and the stopped-flow method. Two reactions were found in the conversion of peroxy cytochrome oxidase to its cyanide complex. The first reaction is characterized by the loss of the 607 nm band, an increase in absorbance at 655 nm, and a decrease in absorbance at 432 nm resulting from a blue-shift of the Soret band; this reaction occurred with a bimolecular rate constant of about 90 M^{-1} s^{-1}. The second reaction is observed as an absorbance increase at 585 and 432 nm; the latter was due to a red-shift of the Soret band. This second process proceeded with a rate constant of about 22 M^{-1} s^{-1}. Both reaction rates are linearly dependent on the concentration of cyanide between 5 and 100 mM. The reappearance of the 655 nm band at the completion of the first reaction suggests that cytochrome a_3 becomes transiently high-spin, a finding which implies that cyanide is not initially bound to this heme center. It appears that preparations of oxidized CcO contain small but variable amounts of the peroxy form. The variable content of this form is probably responsible for the different response of oxidized oxidase to low concentrations of cyanide [Berka, V., Vygodina, T., Musatov, A., Nicholls, P., & Konstantinov, A. A. (1993) *FEBS Lett. 315*, 237−241] and may explain the biphasic reduction of the binuclear center with dithionite [Cooper, C. E., Junemann, S., Ioannidis, N., & Wrigglesworth, J. M. (1993) *Biochim. Biophys. Acta 1144*, 149−160].

Mitochondrial cytochrome *c* oxidase is a terminal enzyme in the respiratory chain and catalyzes the oxidation of ferrocytochrome *c* by molecular oxygen. This electron transport reaction is coupled to proton pumping from the matrix side of the mitochondrial inner membrane to the cytosolic side (Wikström, 1977).

The enzyme contains four catalytically active metal centers—cytochrome *a* and Cu_A which comprise the electron delivery sites, and cytochrome a_3−Cu_B designated as the site of oxygen reduction and of ligand binding. Electrons from ferrocytochrome *c* are transferred through Cu_A and cytochrome *a* to the cytochrome a_3−Cu_B site, where oxygen is reduced to water. The reduction of oxygen to water apparently proceeds with the intermediate formation of peroxy (CcO-P)[1] and ferryl (CcO-F) derivatives of cytochrome a_3 (Blair et al., 1985; Chance et al., 1975; Clore et al., 1980; Han et al., 1990; Ogura et al., 1990; Babcock et al., 1992; Babcock & Wikström, 1992).

Most ligand binding studies of cytochrome oxidase have been performed with the enzyme in the oxidized, as isolated, state; this state we will call CcO-O. Recently the reactions of cyanide with CcO-P and CcO-F have been described (Mitchell et al., 1992). CcO-P reacts with cyanide apparently to yield the classical CcO-cyanide complex (CcO-CN) as the final product. It was noticed, however, that during this reaction the amount of CcO-P decomposed by cyanide did not correspond to the amount of cytochrome a_3 present as the cyanide complex (Mitchell et al., 1992).

A further peculiarity in the interaction of oxidized CcO with cyanide is the variable response that is observed. At least two kinds of spectral changes have been demonstrated by optical spectroscopy. With concentrations of cyanide above 1 mM, one consistently observes the standard absorbance changes associated with a red-shift of the Soret maximum (Antonini et al., 1971; Berka et al., 1993; Nicholls et al., 1976; Van Buuren et al., 1972). However, at concentrations of cyanide less than 1 mM, the reaction of oxidized CcO is variable and dependent on the preparation (Berka et al., 1993).

These interesting facts prompted us to the study further the interaction of CcO-P, a mixture of CcO-P and CcO-F (CcO-PF), and CcO-O with cyanide. We find that the decomposition of CcO-P and the formation of the final cyanide complex proceed in two stages with the intermediate formation of cytochrome a_3 in a high-spin state similar to that present in the original untreated enzyme. In the second phase, cytochrome a_3 is returned to the low-spin state. The rate of both phases depends linearly on the cyanide concentration.

Similar kinetic rates and spectral changes are observed in the reaction of cyanide with CcO-P, CcO-PF, and CcO-O; consequently, we conclude that oxidized CcO contains some small and variable amounts of the CcO-P intermediate and that the variable response of oxidized CcO to low concentrations of cyanide is a result of reaction of cyanide with CcO-P present to varying degrees in existing enzyme preparations.

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[1] Abbreviations: CcO-O, CcO-CN, oxidized (as isolated) rapid form of cytochrome oxidase and its cyanide derivative; CcO-P, CcO-PCN, peroxide adduct of cytochrome oxidase and its cyanide derivative; CcO-F, ferryl derivative of cytochrome oxidase; CcO-PF, mixture of CcO-P and CcO-F; DM, dodecyl maltoside; Tris, tris(hydroxymethyl)aminomethane.

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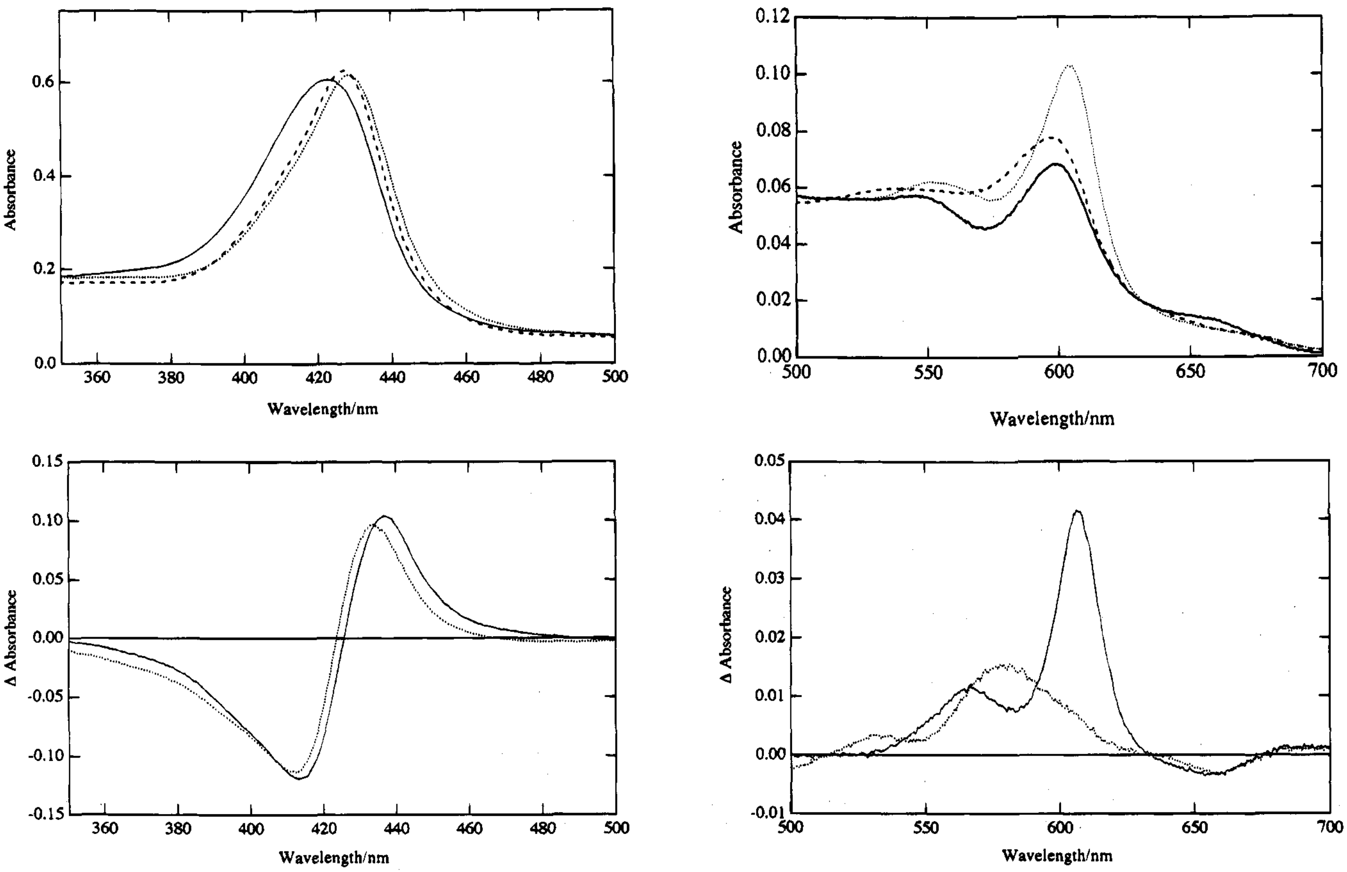

FIGURE 1: Absolute and difference spectra of CcO-P and CcO-F. Top panels: The absolute spectra of CcO-O (—), CcO-P (···), and CcO-F (- - -). Bottom panels: The difference spectra of CcO-P minus CcO-O (—) and CcO-F minus CcO-O (···). The spectrum of CcO-F was measured immediately after addition of 1 mM H_2O_2 to CcO-O. The spectrum of CcO-P was recorded when the absorbance at 607 nm was fully developed. The enzyme concentration was 3.8 μM. The buffer was 100 mM Tris buffer$-SO_4$/pH 8.0 containing 0.1% DM. Temperature = 22 °C.

MATERIALS AND METHODS

The fast form of cytochrome oxidase (Palmer et al., 1988) was prepared by the modification of the method of Hartzell and Beinert (1974) developed in this laboratory (Baker et al., 1987). To remove the traces of ammonium sulfate from the preparation, the enzyme was diluted several times into 100 mM Tris−sulfate buffer, pH 8.0, containing 0.1% DM (hereafter called buffer) followed by concentration using Amicon Centriflo ultrafiltration cones. This procedure yields enzyme with an absorbance maximum in the Soret band at 423 nm at pH 8.

Enzyme concentration (aa_3) was determined by using an absorbance coefficient of 158 mM^{-1} cm^{-1} at 423 nm for the untreated enzyme. The concentration of peroxy form (CcO-P) was determined from the difference spectra of peroxy-CcO minus oxidized CcO using $\Delta A_{607-630} = 11$ mM^{-1} cm^{-1} (Wikström & Morgan, 1992). The concentration of H_2O_2 was determined from the absorbance at 240 nm using a coefficient of 40 M^{-1} cm^{-1} (Bergmayer et al., 1970).

Optical spectra were recorded in an IBM 9430 spectrophotometer interfaced to a microcomputer for data storage, spectral analysis, and presentation. Stopped-flow experiments were performed using a Kinetic Instruments apparatus with a 2 cm path length observation cell. The starting solutions were diluted 1:1 after mixing. The kinetic data were collected and analyzed using the OLIS stopped-flow operating system.

The peroxy (CcO-P), the ferryl (CcO-F), and the mixture of the peroxy and ferryl forms (CcO-PF) were prepared by the following procedures: (1) CcO-P (Bickar et al., 1984; Mitchell et al., 1992): CcO-O was dissolved in buffer and bubbled with CO for about 2 min with no precautions to exclude air. The conversion of the enzyme to CcO-P was followed using the absorbance increase at 607 nm which was complete within 5 min. From the data of Wikström and Morgan (1992), we estimate that about 80−90% of the enzyme is converted to CcO-P. The half-life of spontaneous decomposition of CcO-P measured as an absorbance decrease at 607 nm is about 20 min at 22 °C. (2) CcO-F: Addition 1 mM H_2O_2 to CcO-O immediately converts the enzyme to the ferryl form as judged from the optical changes at 575−580 and at 535 nm; only a trace of the peak at 607 nm persists in this material (see Figure 1). (3) CcO-PF was prepared by the addition of 0.3 mM H_2O_2 to the oxidized oxidase; the conversion to the mixture takes 3−5 min.

RESULTS

Reaction of the Peroxy Form with Cyanide. When CO is bubbled through an aerobic solution of oxidized cytochrome oxidase for about 2 min, there are significant changes in the optical spectrum which develop over a 5 min period. These consist of a red-shift of the Soret, from 423 to 428−429 nm (Figure 1; top panels), an intensification of the visible spectrum between 530 and 620 nm, and a loss of the 655 nm band (Figure 1; top panels). The difference spectrum between this CO-treated enzyme and untreated enzyme shows a relatively intense and narrow band at 607 nm with a small shoulder at 565 nm, a broad trough centered at 650

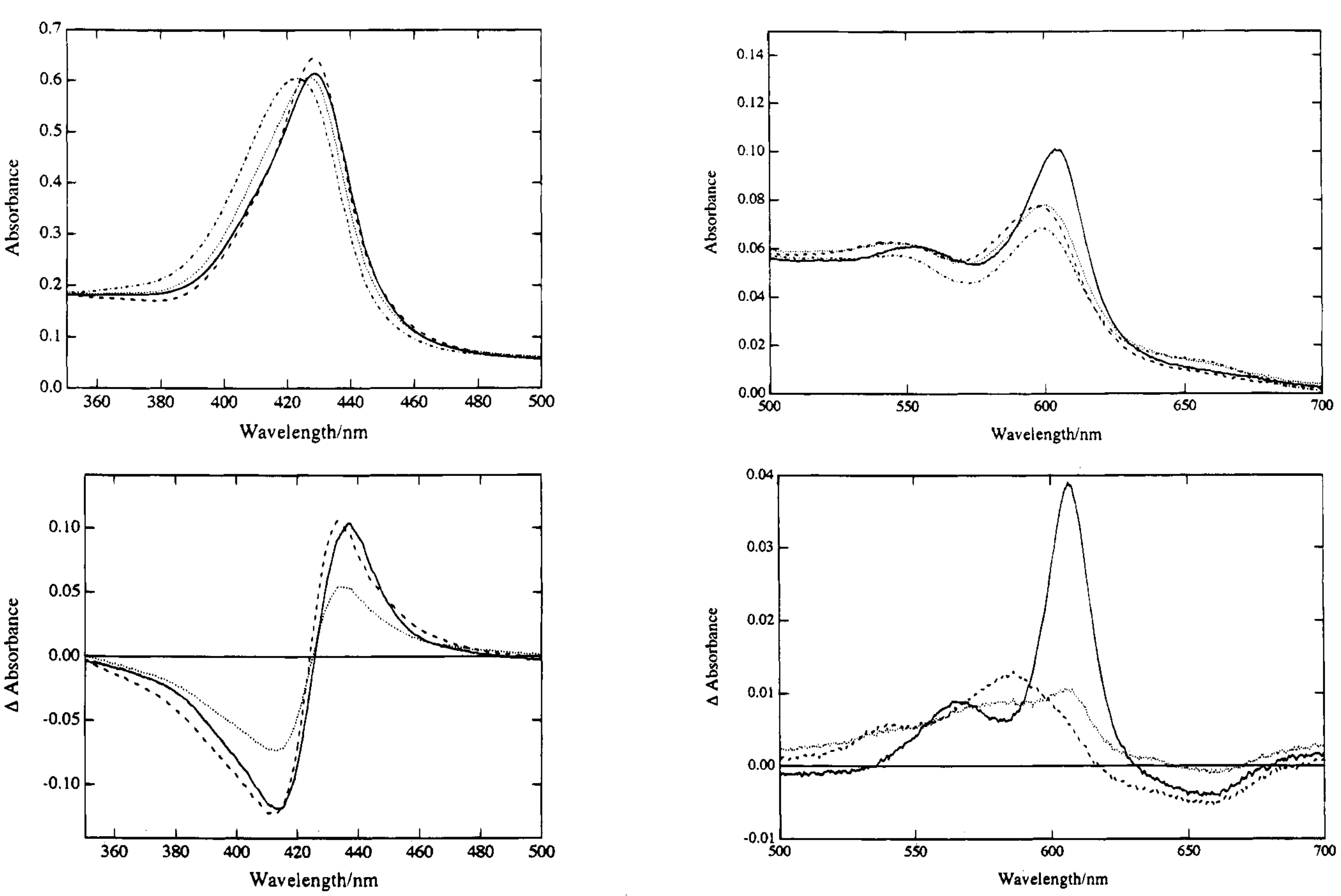

FIGURE 2: Spectral changes following addition of cyanide to CcO-P. Top panels: Absolute spectra of 3.8 μM CcO-O (— • —) and CcO-P (—). Ten seconds after addition of 0.5 mM cyanide, an absorbance spectrum was recorded at 100 nm s^{-1} (···). A second spectrum was recorded 10 min later (- - -). Bottom panels: the difference spectra CcO-P minus CcO-O (—), 10 s after cyanide addition minus CcO-O (···), and 10 min after cyanide addition minus CcO-O (- - -). Conditions as in Figure 1.

nm (Figure 1; bottom panels) and a red-shift of the Soret band with an isosbestic point at 426 nm (Figure 1; bottom panel). Similar spectral changes have been observed earlier (Bickar et al., 1984; Nicholls & Chanady, 1981; Vygodina & Konstantinov, 1989; Wrigglesworth, 1984; Weng & Baker, 1991) and have been assigned to CcO-P (Wikström & Morgan, 1992), a derivative in which dioxygen is reduced to the formal oxidation state of peroxide and bound to cytochrome a_3. Using a value of 11 mM^{-1} cm^{-1} for the absorbance difference between 607 and 630 nm (Wikström & Morgan, 1992), we routinely find that 80–90% of cytochrome a_3 has been converted to this derivative.

For comparison, the absolute spectrum and the difference spectrum of CcO-F minus CcO-O are also presented in Figure 1; the ferryl form was prepared by the addition of 1 mM H_2O_2 to 3.8 μM oxidized oxidase. Again there is a red-shift of the Soret, this time to 428 nm, together with a decrease in the 655 nm band, but the increase in the 530–620 nm region is smaller than that observed with CcO-P (Figure 1; top panels). The difference spectra show an isosbestic point at 424 nm, a weak broad band at 575–580 nm, and a trough at 655 nm (Figure 1; bottom panels). Similar difference spectra have been reported earlier for CcO-F prepared either by (i) reoxidation of partially reduced oxidase (Witt & Chan, 1987), (ii) reverse electron transfer in mitochondria (Wikström & Morgan, 1992), or (iii) addition of an excess of hydrogen peroxide to CcO-O (Weng & Baker, 1991; Mitchell et al., 1992; Vygodina et al., 1993).

Addition of cyanide to CcO-P leads to complex spectrophotometric changes (Figure 2). Within 1 min after reaction of 0.5 mM cyanide with 3.8 μM CcO, the intense peak at 607 nm is almost completely lost, and there is an increase in absorbance at 655 nm, though the original value is not fully restored (Figure 2; top right); at the same time, the Soret band is blue-shifted (Figure 2; top left). Over the next 10 min there is a slow decrease in absorbance at 655 nm with the final value being below that observed with compound P. At the same time, there is an increase in absorbance at 585 nm, and the Soret band is again shifted, but now in the opposite direction (Figure 2; top panels). These opposing shifts of the Soret band, first to the blue and then to the red, are clearly visible in the difference spectra (Figure 2; bottom left). At the end of 10 min, the difference spectrum has maxima at 432 and 585 nm, a minimum at 413 nm, and a clear trough at 650 nm (Figure 2; bottom panels). This final difference spectrum is essentially identical to that obtained when cyanide is added to untreated oxidized enzyme (Van Buuren et al., 1972). Furthermore, both the circular dichroism spectra and the magnetic circular dichroism (MCD) spectra of CcO-OCN and CcO-PCN are identical in the Soret region (data not shown).

To more accurately characterize the early stages of the reaction of cyanide with CcO-P, the reaction was studied using a stopped-flow instrument. Upon mixing 3.8 μM CcO-P with 20 mM cyanide, three kinetic phases are observed at 432 nm (Figure 3; top panel). The first of these occurs with a decrease in absorbance with an observed rate of 1.04 s^{-1} (see inset). It is subsequently followed by a biphasic absorbance increase which is described by two exponentials with rate constants of 0.22 ± 0.02 and 0.02 ± 0.002 s^{-1}, respectively. This pattern is qualitatively that expected from the time evolution of the difference spectra (Figure 2; bottom left). The first two processes contribute most of the absorbance change; the slowest reaction has a rate constant similar to that for the reaction of the parent, untreated enzyme with this concentration of cyanide and presumably represents a contribution from a subpopulation of CcO-O which had not reacted with carbon monoxide.

We have also studied the reaction at 610 nm, a wavelength isosbestic for the conversion of untreated enzyme to the enzyme–cyanide derivative. At this wavelength, we only observe an absorbance decrease. This could be well described as a simple first-order reaction with a rate constant of 0.9 ± 0.08 s^{-1} (Figure 3; bottom panel) and would appear to be the same process as the most rapid phase detected at 432 nm; the small discrepancy in rates presumably reflects the difficulties in accurately fitting multiexponential processes involving similar rate constants (Figure 3; inset).

A rate constant of ca. 1 s^{-1} is about 50 times larger than that observed in the reaction of untreated enzyme with this concentration of cyanide. It is also about 10 times larger than the value deduced from the second-order rate constant reported by Mitchell et al. (1992) in their study of the interaction of cyanide with P. However, using the data shown in Figure 6A of that paper, we calculate a half-time of reaction some 10 times larger than that quoted by Mitchell et al. (1992).

The kinetic phase with a rate constant of 0.22 s^{-1} observed at 432 nm would appear to represent a process in which the initial product from the reaction of CcO-P with cyanide undergoes a secondary transformation.

Because the rate of reaction of cyanide with CcO-O reaches a limiting value at high cyanide concentrations, it has been proposed that this reaction proceeds with the formation of an intermediate (Van Buuren et al., 1972). However, plots of both the fastest rate of reaction of CcO-P with cyanide (for simplicity monitored at 610 nm) and the intermediate rate (determined at 432 nm) as a function of cyanide concentration are accurately linear up to the highest concentration tested (0.1 M cyanide after mixing). It would appear therefore that these two reactions represent separate and distinct bimolecular processes and not an initial bimolecular reaction of CcO-P with cyanide with a subsequent unimolecular rearrangement.

For completeness, we have tested the reaction of a PF mixture with cyanide under the same conditions as above. CcO-PF was prepared by the addition of 0.3 mM H_2O_2 to 10 μM oxidized oxidase; the difference spectrum (CcO-PF minus CcO-O) is shown in Figure 4 (left panel). In contrast to our observations with CcO-P, the absorbance decrease at 610 nm following the mixing of 10 μM CcO-PF with 20 mM cyanide cannot be fitted as a single phase but requires a minimum of two exponentials (Figure 4; right panel) with rate constants of 0.8 s^{-1} (75%) and 0.05 s^{-1} (25%), respectively [to minimize complications arising from the partial reduction of oxidase induced by addition of cyanide to mixtures of CcO and H_2O_2 (Wrigglesworth, 1984) we performed these reactions in the presence of 1 mM ferricyanide]. The value of 0.05 s^{-1} is similar to that of 0.1 s^{-1} calculated from the second-order rate constant reported by Mitchell et al. (1992) for the reaction of CcO-F with cyanide.

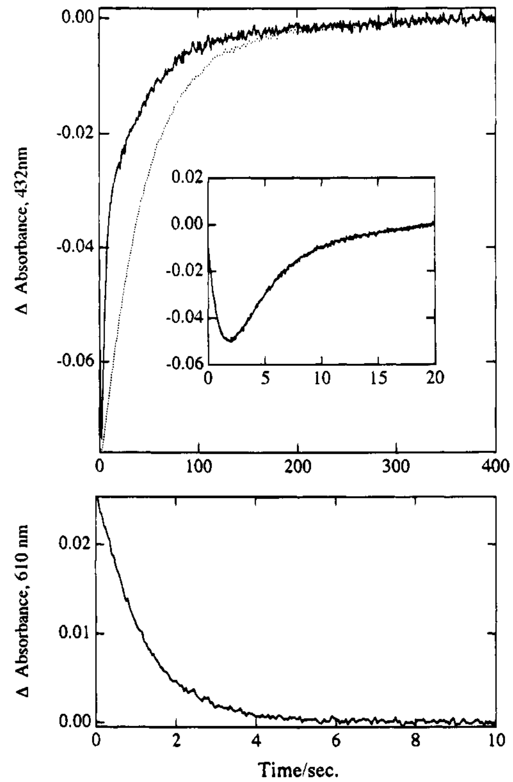

FIGURE 3: Stopped-flow kinetics of absorbance changes of CcO-O and CcO-P induced by cyanide. 3.8 μM of either CcO-O or CcO-P was mixed with 20 mM cyanide, and absorbance changes were recorded. Top panel: (--) CcO-O at 432 nm; (—) CcO-P at 432 nm. Inset: The initial changes of CcO-P at 432 nm. Bottom panel: CcO-P at 610 nm. The measurement conditions were as in Figure 1.

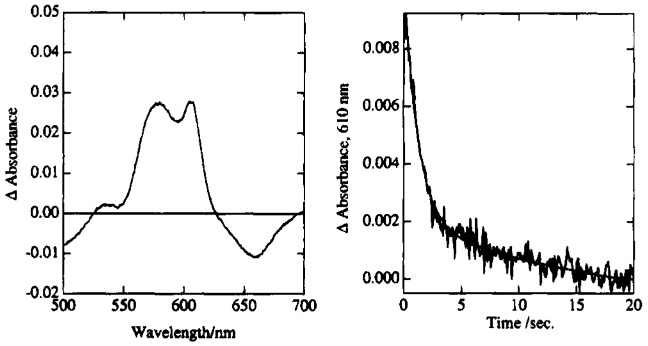

FIGURE 4: Difference spectrum and reaction kinetics of CcO-PF with cyanide. Left panel: 10 μM CcO-O was converted to CcO-PF by reaction with 0.3 mM hydrogen peroxide and the difference spectrum of CcO-PF minus CcO-O recorded directly. Right panel: The reaction of CcO-PF with cyanide followed at 610 nm. Concentrations after mixing: 5 μM CcO and 10 mM cyanide. The other conditions were as in Figure 1 except that the buffer contained 1 mM ferricyanide.

To confirm this similarity, we prepared CcO-F by reacting 15 μM CcO plus 1 mM ferricyanide with 3 mM H_2O_2 followed by 50 nM catalase (to destroy excess peroxide). The product was mixed with 20 mM potassium cyanide in the stopped-flow apparatus, and the absorbance changes at 562 and 546 nm were recorded. In four experiments, the observed rate of reaction of the difference absorbance (562–546 nm) ranged from 0.05 to 0.073 s^{-1}. The relative amplitudes of the kinetic phases are consistent with the amounts of P and F present in the mixture as deduced from the optical spectrum; these amounts were 80% and 20%, respectively. It is thus reasonable to conclude that the slower

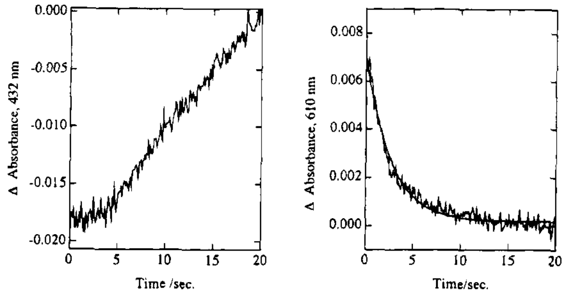

FIGURE 5: Kinetics of absorbance changes of CcO-O induced by cyanide. Left panel: The reaction of 2 μM oxidized CcO with 10 mM cyanide recorded at 432 nm. Right panel: The reaction of 5 μM CcO with 10 mM cyanide recorded at 610 nm. The measurement conditions were as in Figure 1.

rate obtained with the PF mixture is indeed the reaction of CcO-F.

Reaction of Oxidized Oxidase with Cyanide. The reaction of CcO-O with cyanide is routinely measured by following the changes in absorbance in either the Soret or the α-band regions of the visible spectrum. When followed in a manual spectrophotometer, the reaction of this preparation of enzyme appears to be a single exponential with a measured first-order rate constant of 0.02 ± 0.002 s^{-1} using our standard conditions of assay (Baker et al., 1987). The monoexponential reaction of oxidase with cyanide is typical of the fast form of oxidized CcO as was demonstrated earlier (Baker et al., 1987; Moody et al., 1991).

The same behavior is normally observed when the reaction is studied at 432 nm in the stopped-flow instrument. However, with some preparations of enzyme, we find that a small lag precedes the normal absorbance increase. In one instance, the reaction of 4 μM CcO-O with 20 mM cyanide, this lag was complete within 5 s (Figure 5; left panel) and is thus not detected in manual experiments. An absorbance decrease occurring in the same time interval is also observable at 610 nm, which, as noted above, is isosbestic for the normal reaction (Figure 5; right panel). The change at 610 nm can be described by a single exponential with an observed rate which varied from 0.6 to 1.1 s^{-1} in different preparations (Figure 5). These data are unchanged when 1 mM ferricyanide is included with either reactant.

This faster rate seen at 610 nm is similar to that observed in the direct reaction of cyanide with CcO-P described above and suggests that CcO-O, as we isolate it, contains some of this species. In one enzyme preparation, almost 20% of the enzyme appeared to be present as CcO-P; more typically, the CcO-P content is in the range 0–8%.

DISCUSSION

It should be stated at the outset that CcO-P and CcO-F as originally described by Wikström (1981) are anticipated intermediates in the reaction of reduced CcO with dioxygen and that there are convincing resonance Raman data (Babcock et al., 1992; Ogura et al., 1990; Han et al., 1990) in support of the existence of these species. However, the identity of CcO-P and CcO-F with the 607 and 580 nm absorbing species prepared by the chemical treatments used in this study is not well established, and definitive spectral data on this correspondence remain to be produced.

The ultimate product of the interaction of CcO-P with cyanide appears to be the oxidized oxidase–cyanide complex because we find the final optical spectrum (Figure 2) and the CD and MCD spectra to be essentially the same as those observed when untreated oxidase is reacted with cyanide (Babcock et al., 1976; Van Buuren et al., 1972). This conversion of CcO-P to the oxidase–cyanide complex clearly proceeds in two stages. In the first stage, CcO-P is decomposed, as indicated by the blue-shift of the Soret band, the loss of 607 nm absorbance, and the partial recovery of absorbance at 655 nm (Figures 2 and 3). The bimolecular rate constant for this process is about 90 M^{-1} s^{-1} (measured at 610 nm). On the basis of these optical changes, we propose that the decomposition of CcO-P leads initially to the formation of a high-spin state of cytochrome a_3, while the reaction of the second cyanide restores cytochrome a_3 to the low-spin state. The binding of cyanide to cytochrome a_3, whether bridging, as is believed to be the case in oxidized enzyme, or terminal, as is believed to occur in reduced enzyme, always makes a_3 low-spin. It would thus appear that in the product of the reaction of the first cyanide with CcO-P the cyanide is not bound to the heme iron of cytochrome a_3 during this first reaction, for, if it were, the high-spin electronic state would be highly improbable and the absorbance increase at 655 nm would not be observed (Figure 2). It seems reasonable to conclude that cyanide is first bound to some other site of the binuclear center, conceivably Cu_B.

Scheme 1[a]

$$\text{Fe-OOH Cu}_B \xrightleftharpoons{+\text{CN}^- + \text{H}^+} \text{Fe-OCN Cu}_B + \text{H}_2\text{O}$$

$$\text{Fe-OCN Cu}_B \xrightleftharpoons{+\text{CN}^-} \text{Fe-CN Cu}_B + \text{OCN}^-$$

$$\text{Fe-CN Cu}_B \rightleftharpoons \text{Fe-CN-Cu}_B$$

[a] The first equivalent of cyanide attacks the bound peroxide with cleavage of the O–O bond. The products of the reaction are cyanate and water, one of these molecules remains bound to the heme iron. As both water and cyanate are relatively weak field ligands, the heme iron reverts to the high-spin state. The second equivalent of cyanate serves to displace the bound cyanate and return a_3 to the low-spin state.

The second reaction is observed as a subsequent red-shift of the Soret band accompanied by an absorbance decrease at 655 nm and an absorbance increase at 585 nm (Figure 2). The reaction is characterized by the bimolecular rate constant of 22 M^{-1} s^{-1} (measured at 432 nm). These spectral changes are similar to the normal changes observed during the reaction of CcO-O with cyanide (Van Buuren, 1972). In this second reaction, cytochrome a_3 is probably converted back from the high-spin to the low-spin state.

There appear to be at least three possible explanations for these data (Schemes 1, 2, and 3). In Scheme 1, the first equivalent of cyanide attacks the bound peroxide with cleavage of the O–O bond. The products of the reaction would be cyanate and water, one of which would remain bound to the heme iron. In this scheme, the cyanate is shown as remaining, but this is an arbitrary choice. As both water and cyanate are relatively weak field ligands, the heme iron would revert to the high-spin state. The second equivalent of cyanide serves to displace the bound cyanate and return a_3 to the low-spin state. In principle, this hypothesis could be tested by demonstrating the formation of cyanate; unfortunately, this species readily hydrolyzes to regenerate cyanide.

The second scheme (suggested by a referee) draws on the known ligand affinity of Cu_B (Brudwig et al., 1981; Li &

Scheme 2[a]

$$\mathrm{Fe\text{-}OOH\ Cu_B} \overset{+\,\mathrm{CN^-}}{\rightleftharpoons} \mathrm{Fe\text{-}OOH\ Cu_B\text{-}CN}$$

$$\mathrm{Fe\text{-}OOH\ Cu_B\text{-}CN} \overset{+\,\mathrm{CN^-}\,+\mathrm{H^+}}{\rightleftharpoons} \mathrm{Fe\text{-}CN\quad Cu_B\text{-}CN} + \mathrm{H_2O_2}$$

$$\mathrm{Fe\text{-}CN\quad Cu_B\text{-}CN} \rightleftharpoons \mathrm{Fe\text{-}CN\text{-}Cu_B} + \mathrm{CN^-}$$

[a] The first equivalent of cyanide binds to $\mathrm{Cu_B}$, thereby inducing a change in spin-state of cytochrome a_3. The second cyanide then inserts inself as a bridge between cytochrome a_3 and $\mathrm{Cu_B}$, returning cytochrome a_3 to the low-spin state and promoting the release of the first cyanide from $\mathrm{Cu_B}$ (possibly by some kind of trans effect).

Scheme 3[a]

$$\mathrm{Fe\text{-}OOH\ Cu_B} \overset{+\,\mathrm{H^+}}{\rightleftharpoons} \mathrm{Fe\qquad Cu_B} + \mathrm{H_2O_2}$$

$$\mathrm{Fe\quad Cu_B} \overset{+\,\mathrm{CN^-}}{\rightleftharpoons} \mathrm{Fe\quad Cu_B\text{-}CN}$$

$$\mathrm{Fe\quad Cu_B\text{-}CN} \overset{+\,\mathrm{CN^-}}{\rightleftharpoons} \mathrm{Fe\text{-}CN\ \ Cu_B\text{-}CN}$$

[a] CcO-P is proposed to be in rapid equilibrium with a small fraction of the enzyme in the unligated form. The first equivalent of cyanide reacts with this unligated form to drive the equilibrium to the right. Cyanide must be attached to $\mathrm{Cu_B}$ to account for the anticipated high-spin character of the unligated heme iron of cytochrome a_3. The second equivalent of cyanide then reacts directly with the heme iron, restoring the low-spin electronic state.

Palmer, 1993; Schoonover & Palmer, 1991), the recent demonstration that the reaction of reduced CcO with CO proceeds with this ligand binding initially at $\mathrm{Cu_B}$ (Einarsdottir et al., 1993; Lemon et al., 1993), and the observation that cyanide appears to bind to $\mathrm{Cu_B}$ in partially reduced enzyme (Yoshikawa & Caughey, 1990). In this scheme (Scheme 2), it is proposed that the first cyanide binds to $\mathrm{Cu_B}$; the second cyanide subsequently binds to cytochrome a_3, displacing the peroxide and forming a bridge between a_3 and $\mathrm{Cu_B}$. The formation of this bridge would weaken the affinity of $\mathrm{Cu_B}$ for the first cyanide which then dissociates.

Both Schemes 1 and 2 are consistent with recent infrared data which show that only one cyanide is bound to oxidized CcO (Li & Palmer, 1993; Yoshikawa & Caughey, 1990); however, in Scheme 2, it is not clear why there would be conversion of cytochrome a_3 to the high-spin state in the intermediate produced following reaction with the first equivalent of cyanide.

A third possibility (Scheme 3) requires that CcO-P is in rapid equilibrium with a small fraction of the enzyme in the unligated form and that the first equivalent of cyanide reacts with this unligated form to drive the equilibrium to the right. In this case, this cyanide must also be coordinated to $\mathrm{Cu_B}$ to account for the anticipated high-spin character of the unligated heme iron of cytochrome a_3. The second equivalent of cyanide then reacts directly with the heme iron, restoring the low-spin electronic state. In this third scheme, the end product has 2 equiv of cyanide bound at the binuclear center, a situation that is inconsistent with the infrared data described above. However, the possibility exists that upon conversion of the parent oxidase to CcO-P there is a change in the structure of the ligand binding site such that more than one cyanide molecule can be bound. This change to the more open state could be induced by a redox reaction at the cytochrome a_3–$\mathrm{Cu_B}$ site (Yoshikawa & Caughey, 1990). Infrared measurements on the reaction product will be of value in testing this.

Recently Berka et al. (1993) have reported spectral changes of oxidized oxidase induced by low concentrations of cyanide (less than 1 mM) which are dependent on the enzyme preparation. These spectral changes proceed in two steps. The first, represented mainly by a blue-shift of the Soret band and an absorbance decrease at 607 nm, was interpreted as evidence for the presence of an intermediate in the reaction of CcO-O with cyanide. The second step represents those changes which are normally observed in the reaction of oxidized oxidase with cyanide. For the following two reasons, we propose that the changes in the first step are in fact due to the reaction of a small fraction of the enzyme which is present in the peroxy form: (i) the kinetics and spectral changes observed by Berka et al. (1993) are similar to those observed with authentic CcO-P; and (ii) the presence of these spectral changes varies with preparation, consistent with the notation that the amount of CcO-P will vary with preparation.

Similarly, Cooper et al. (1993) have recently shown that, in their preparation of slow cytochrome oxidase, the reduction of cytochrome a_3 by dithionite is biphasic. The more rapid of the two phases is associated with a minority form of the binuclear center that does not exhibit the $g' = 12$ epr signal and which has a Soret maximum somewhat red-shifted than the bulk of the enzyme. They propose that, in this more rapidly reacting species, cytochrome a_3 is in the Fe(IV) state while $\mathrm{Cu_B}$ is cuprous. Given the close location of these metals, we find this suggestion inherently implausible because electron transfer between the two metal centers in these oxidation states must be quite facile. If, on the other hand, the more rapidly reacting form of their enzyme represents a small proportion of CcO-P in their preparation, then both the absence of the $g' = 12$ signal and the red-shift of the Soret are easily understood.

The reaction of CcO-F with cyanide also appears to proceed with a characteristic rate of 0.05–0.06 $\mathrm{s^{-1}}$ (Figure 4). We have been unable to detect this second process in the reaction of CcO-O with cyanide for single-exponential and biexponential fits to these latter data do not appear to be significantly different. However, as we are attempting to observe a minor component of rate 0.06 $\mathrm{s^{-1}}$ in the presence of a major component of rate 0.02 $\mathrm{s^{-1}}$, we cannot unequivocally state that there is not any CcO-F present in the parent enzyme.

It is not known whether these small amounts of peroxidized enzyme are already present in the enzyme when it is isolated from the original heart muscle or are produced by reaction of the enzyme with variable amounts of hydrogen peroxide which may be present in trace amounts in one-or-more of the reagents employed during purification. The fast form of cytochrome oxidase reacts quite rapidly and with high affinity with hydrogen peroxide (Palmer et al., 1988; Weng & Baker, 1991), and it is quite conceivable that the enzyme scavenges any available hydrogen peroxide during its purification. As the decomposition of detergents is a plausible source of hydrogen peroxide, we have adopted the practice of not using detergent solutions that are more than 1 week old.

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