Cytochrome *c* Oxidase Binding of Hydrogen Peroxide†

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ABSTRACT: Oxidized cytochrome *c* oxidase can bind hydrogen peroxide, as evidenced by changes in its spectrum and its ability to use hydrogen peroxide as an electron acceptor in cytochrome *c* oxidation. The affinity of the oxidized enzyme for hydrogen peroxide is high, with a K_d of less than 10 μM, and the binding is inhibited by ligands of cytochrome a_3. Oxidized cytochrome *c* oxidase, in submitochondrial particles or solubilized in several ionic and nonionic detergents, binds peroxide with comparable affinities. The size of the spectral shift observed upon peroxide binding depends on the pH of the solution and differs in extinction coefficient between preparations, but all preparations tested appeared to bind peroxide. The differences in the magnitude of the spectral shift upon peroxide binding to different preparations suggest that oxidized cytochrome *c* oxidase as prepared may be made up of more than one species and that the proportion of the species which binds peroxide varies with the preparation. These studies of the binding of peroxide clarify the mechanism by which cytochrome *c* oxidase catalyzes the reduction of oxygen to water without the formation of free-radical intermediates.

Cytochrome *c* oxidase catalyzes the reduction of molecular oxygen by electrons from cytochrome *c*. The last enzyme in the electron transport path, it is a membrane-bound protein with several subunits and prosthetic groups, and although it has been intensively studied for several years, its composition and functional characteristics remain elusive [for reviews, see Malmström (1974) and Wikstrom & Krab (1979)]. Overall, the reaction it catalyzes is simple:

$$O_2 + 4\text{cytochrome } c(\text{II}) + 4H^+ \rightarrow 2H_2O + 4\text{cytochrome } c(\text{III}) \quad (1)$$

This is a thermodynamically favorable reaction, with a $\Delta G^{\circ\prime} = -26$ kcal mol^{-1}.[1] The large negative free energy is generally believed to be coupled to an energy-requiring reaction, the membrane translocation of protons (Wikström & Krab, 1979), and eventually, via the proton gradient, to the production of ATP. But while the four-electron reduction of molecular oxygen to water is energetically favorable, each cytochrome *c* molecule can transfer only one electron at a time. The single electron reduction of molecular oxygen, however, is decidedly unfavorable.

$$O_2 + \text{cytochrome } c(\text{II}) \rightarrow O_2^- + \text{cytochrome } c(\text{III}) \quad (2)$$

The $\Delta G^{\circ\prime}$ for the one-electron reduction of molecular oxygen by cytochrome *c* as described in eq 2 is +20 kcal mol^{-1}. Any process using this reaction as a component step would necessarily be slow. Assuming the back reaction to be diffusion limited, the second-order rate constant for the single electron transfer would be about 10^{-5} M^{-1} s^{-1}. The thermodynamic situation might be improved somewhat if the superoxide formed was stabilized by binding to cytochrome *c* oxidase; but the binding affinity would have to be very high, with a K_d of about $<10^{-14}$ M^{-1} needed to account for the observed kinetics for oxygen reduction via cytochrome *c* oxidase (Gibson et al., 1965a). Alternatively, if cytochrome *c* oxidase had a large, negative redox potential at the heme of cytochrome a_3 or at Cu_{a_3},[2] this would favor the one-electron reduction of oxygen. However, the redox potentials for the heme of cytochrome a_3 and Cu_{a_3} are positive (Leigh et al., 1974; Lindsay et al., 1975).

Not only is the single electron reaction thermodynamically unfavored, the reaction product, the superoxide radical or its protonated form, the hydronium ion, is capable of generating various highly reactive compounds which are potentially damaging to biological systems [for a review, see Fridovich (1976)]. This would also be true for the other odd-number electron transfer: $H_2O_2 + e^- \rightarrow OH^- + \cdot OH$.

Studies of cytochrome *c* oxidase suggest that biologically hazardous and energetically unfavored states have been avoided by having multiple electron-accepting sites (Blumberg & Peisach, 1979), allowing the sequestering of more than one electron by cytochrome *c* oxidase and the rapid or simultaneous transfer of two or more electrons to molecular oxygen (Lindsay et al., 1975). The kinetics of electron transfer in cytochrome *c* oxidase indicate that at most only two electrons can be transferred to molecular oxygen before the oxygen binding sites are electron depleted and that these sites must be rereduced by other metal centers in the molecule (Gibson et al., 1965a; Chance & Leigh, 1977). A two-electron reduction of molecular oxygen would produce a peroxy intermediate. The reaction can be represented by

$$O_2 + 2\text{cytochrome } c(\text{II}) + 2H^+ \rightarrow H_2O_2 + 2\text{cytochrome } c(\text{III}) \quad (3)$$

The $\Delta G^{\circ\prime}$ for the reaction above is +3.0 kcal mol^{-1}, and the reaction is not therefore favorable under standard conditions. If the reaction were catalyzed by cytochrome *c* oxidase, and the oxidized cytochrome *c* oxidase bound the peroxy intermediate (a not unlikely occurrence), the binding would stabilize the intermediate and increase the free energy of this reaction. A dissociation constant of 10 μM for peroxide would give a $\Delta G^{\circ\prime} = -3.8$ kcal mol^{-1} for the reaction:

$$O_2 + \begin{matrix} a(\text{III}) & a_3(\text{II}) \\ Cu_a(\text{II}) & Cu_{a_3}(\text{I}) \end{matrix} \rightarrow \begin{matrix} a(\text{III}) & a_3(\text{III}) \\ Cu_a(\text{II}) & Cu_{a_3}(\text{II}) \end{matrix} \cdot O_2^{2-} \quad (4)$$

A likely mechanism for the reduction of molecular oxygen by cytochrome *c* oxidase would therefore involve a peroxy in-

† From the Marine Biomedical Center, Duke University Marine Laboratory, Beaufort, North Carolina 28516. *Received August 24, 1981*; *revised manuscript received January 13, 1982*. This work was supported in part by National Institutes of Health Grants HL 15460 and ESO-1908 and National Science Foundation Grant PCM 7906462.

[1] Estimates of $E^{\circ\prime}$ are as follows: cyt a_3 heme(III) Cu_{a_3}(II)/cyt a_3 heme(II) Cu_{a_3}(I), $E^{\circ\prime} = 360$ mV; $O_2/H_2O_2^-$, $E^{\circ\prime} = 295$ mV (Lindsay et al., 1975); O_2/O_2^-, $E^{\circ\prime} = -600$ mV (Malmström, 1974).

[2] Abbreviations: the heme of cytochrome a_3 is the CO-binding heme and Cu_{a_3} is its associated copper. Cytochrome *a* contains the remaining heme and Cu_a is its associated copper (Wharton & Gibson, 1976).

0006-2960/82/0421-2661$01.25/0

termediate (Brunori et al., 1979; Chance et al., 1975; Malmström, 1974). A direct test of this mechanism would be to see if cytochrome *c* oxidase can form an adduct with hydrogen peroxide. A number of equilibrium and kinetic spectrophotometric measurements of hydrogen peroxide binding to oxidized cytochrome *c* oxidase are reported in this paper. A preliminary report describing some of this work has appeared (Bickar et al., 1980). The results demonstrated that cytochrome *c* oxidase can reversibly bind hydrogen peroxide. This binding is shown to be inhibited by other ligands of the heme of cytochrome a_3 and compatible with theories of cytochrome *c* oxidase catalyzed oxygen reduction which postulate a peroxide intermediate.

Experimental Procedures

Submitochondrial particles were prepared from beef heart following the procedure of Yonetani (1961), except the initial grinding and washing stages to remove myoglobin were eliminated; the heart muscle was instead cut into small cubes. The cubes were homogenized in a blender with buffer. The rest of the procedure was followed as reported. No spectrally detectable myoglobin contamination was found when this method was used.

Cytochrome *c* oxidase (ferrocytochrome *c*:oxygen oxidoreductase, EC 1.9.3.1) was purified from the isolated submitochondrial particles. The procedure used was essentially that of Yonetani (1961), but the overnight precipitation step was limited to 6 h, and 1 mM EDTA was added to the buffers throughout. Care was taken that the entire procedure, from fresh heart to frozen, purified cytochrome *c* oxidase, take less than 24 h. The quality of each enzyme preparation was determined by polyacrylamide gradient $NaDodSO_4$ gel electrophoresis (Wilson et al., 1980) and spectral criteria (Gibson et al., 1965b; Kuboyama et al., 1972). Only enzyme preparations with 10 sharp bands (identified as subunits 1–7 and "contaminants" a–c) and 444–424 nm absorbance ratios of greater than 2.25 for the dithionite-reduced enzyme were used in binding experiments (Gibson et al., 1965b). Cytochrome *c* oxidase concentrations were expressed in terms of total heme (heme *a*) and estimated by using a millimolar extinction coefficient of 21 for dithionite-reduced cytochrome *c* oxidase at 605 nm (Brunori et al., 1979). Enzyme preparations were tested in 0.1 M sodium phosphate and 1 mM EDTA with 2% sodium cholate or 1% Tween 80 (Wilson et al., 1980) and in 0.05 M Tris-HCl with 0.1 mM EDTA, 0.1% lauryl maltoside (Calbiochem), and 0.1 M NaCl (Rosevear et al., 1980). Stock hydrogen peroxide solutions were in the same buffer as the sample to which they were added and were prepared from 30% hydrogen peroxide (Fisher Scientific) stored at 4 °C. Peroxide concentrations were determined by using an extinction coefficient of 40 M^{-1} cm^{-1} at 240 nm (Bergmeyer, 1974). *tert*-Butyl hydroperoxide was prepared according to the procedure of Milas & Surgenor (1946). Static titrations of cytochrome *c* oxidase with hydrogen peroxide were performed in matched cuvettes at 25 °C as difference spectra on an Aminco DW2-A (American Instrument Co.) spectrophotometer. The kinetics of cytochrome *c* oxidation were determined by stopped-flow spectrophotometry in a Gibson–Durrum stopped-flow spectrophotometer with a 1.46-cm observation chamber. Sodium ascorbate, cytochrome *c* (type III), and glucose oxidase (type II) were purchased from Sigma and used without further purification. Cytochrome *c* concentrations were determined spectrophotometrically in the presence of dithionite by using an E_{mM} = 27.6 at 550 nm (Antonini et al., 1977). All other reagents were reagent grade or better. Data collection and analysis were facilitated by use of a DASAR (American Instrument Co.) analogue/digital converter and a PDP 11/34 minicomputer (Digital Equipment Corp.).

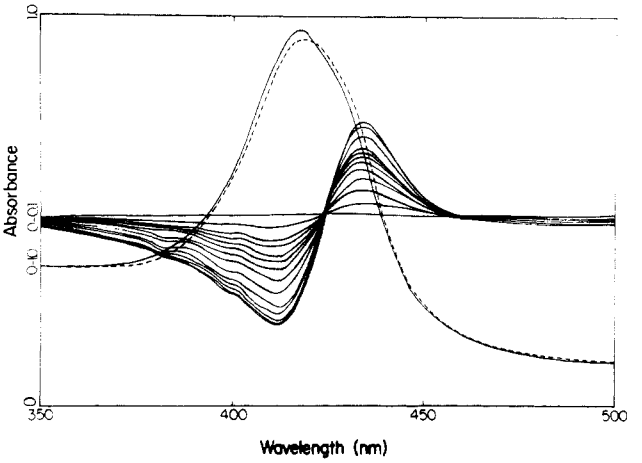

FIGURE 1: Soret peak of cytochrome *c* oxidase in the unliganded (—) and 100 M peroxide-bound form (---) and difference spectra. The difference spectra were obtained by titrating with additions of 0.005-mL aliquots of 1.7 mM H_2O_2 in buffer to 3 mL of 12.2 μM cytochrome *c* oxidase in the sample chamber and an equivalent volume of buffer to 3 mL of cytochrome *c* oxidase in the reference cell. Spectra were recorded immediately at 5 nm/s, and new peroxide and buffer additions proceeded at 1-min intervals. Cytochrome *c* oxidase and H_2O_2 were buffered with 0.1 M sodium phosphate, 1 mM EDTA, and 1% Tween 80, pH 7.5, at 20 °C.

Results

The binding of hydrogen peroxide by oxidized cytochrome *c* oxidase causes a shift in the wavelength maximum of the Soret absorption peak from 417 to 422 nm and a slight decrease in the total integrated absorbance. The visible absorbance peak is also changed, with a shift in the absorption wavelength maximum to 601 from 599 nm. In this region there is a slight increase in total integrated absorbance. The Soret results for the enzyme as isolated are shown in Figure 1. The peroxide-bound form has a slightly smaller extinction coefficient and a somewhat broader peak, compared to the unliganded enzyme. *tert*-Butyl hydroperoxide caused similar spectral changes, but the affinity of cytochrome *c* oxidase for the larger molecule appeared to be lower.

In Figure 2 the change in absorbance with increasing peroxide concentration is shown. The Scatchard plot insert of Figure 2 indicates that the titration curve can be described by a single, noncooperative binding site with a K_d of about 10 μM. A slight catalase activity, found in most cytochrome *c* oxidase preparations, would decrease the actual concentration of peroxide added. This suggests that the actual K_d may be somewhat less than 10 μM.

Figure 3 shows an extended wavelength region for the peroxide-induced difference spectrum of a preparation of cytochrome *c* oxidase with 100 μM H_2O_2 at pH 7.45 in 0.1 M $NaPO_4$ and 1% Tween 80. In this case the enzyme was "pulsed" (reduced and reoxidized) immediately prior to peroxide addition. The maximum difference extinction coefficients were −5.4 mM^{-1} at 413 nm, 6.0 mM^{-1} at 436 nm, 0.6 mM^{-1} at 570 nm, and 1.6 mM^{-1} at 607 nm. Preparations in 0.1 M potassium phosphate and 1 mM EDTA, pH 7.5, with 1% Tween 80 or 2% sodium cholate or in 0.1 M NaCl, 0.1 mM EDTA, 0.05 M Tris, pH 7.5, and 0.1% lauryl maltoside had difference extinction coefficients with similar wavelength dependences. Figure 3 also demonstrates that the binding of H_2O_2 is readily, albeit slowly, reversed by adding catalase. Addition of catalase in an insolubilized, polymer-bound form made it possible to demonstrate the repetitive binding and

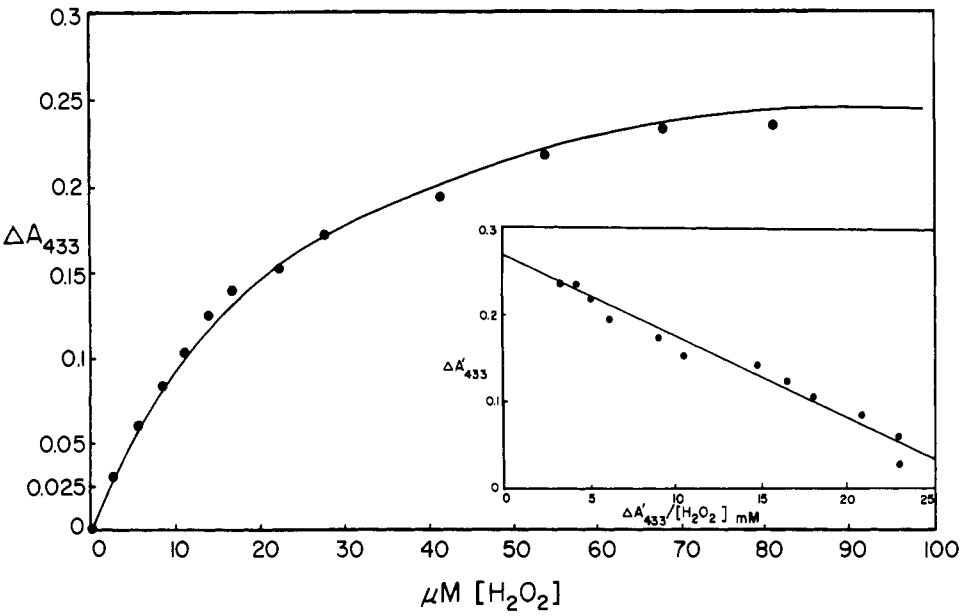

FIGURE 2: Titration of cytochrome *c* oxidase with hydrogen peroxide. Conditions are as described in Figure 1. In the Scatchard plot (the inset), ΔA_{433} is the absorbance change after correction for the enzyme dilution from peroxide and buffer addition.

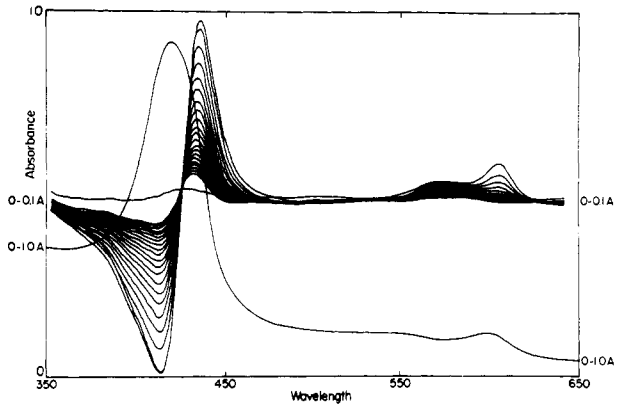

FIGURE 3: Spectrum and difference spectra of 13 M cytochrome *c* oxidase treated with 100 μM H_2O_2 in the sample cell. Conditions were as in Figure 1 except the enzyme was pulsed (reduced and then reoxidized) immediately prior to H_2O_2 addition. The time course of H_2O_2 dissociation was recorded by adding 60 nM catalase to both sample and reference cells and scanning at 5 nm/s at 2-min intervals.

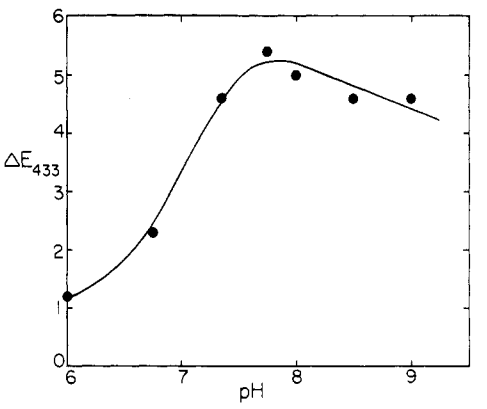

FIGURE 4: Effect of pH on the absorbance changes associated with peroxide binding to cytochrome *c* oxidase. Difference extinction coefficients at 433 nm were measured by addition of equal volumes of hydrogen peroxide (final concentration 1 mM) or buffer to equal volumes of cytochrome *c* oxidase in sample or reference cuvettes. The enzyme was prepared by diluting 2 mM cytochrome *c* oxidase to 10 μM in 0.05 M Tris, 0.1 mM EDTA, 0.1% lauryl maltoside, and 0.1 M NaCl.

release of low concentrations of peroxide by cytochrome *c* oxidase.

Peroxide binding by cytochrome *c* oxidase may also be reversed by adding sodium azide or sodium cyanide, ligands believed to bind at the heme of cytochrome a_3. In experiments in which peroxide binding is shown as difference spectra, as in Figure 3, the addition of cyanide or azide to both the sample and reference cells decreases the difference in signal and can eventually eliminate it. However, when the peroxide concentration is above 10 mM, a slow apparently irreversible spectral shift ensues, causing an increase in absorbance around 440 nm. The inhibition of the peroxide-induced signal by cyanide or azide suggests that these ligands bind at the heme of cytochrome a_3.

The extinction coefficients for the peroxide-induced spectral shifts of cytochrome *c* oxidase absorbance are pH dependent. In Figure 4 the difference extinction coefficient at 433 nm is given for a single preparation of cytochrome *c* oxidase at pHs from 6.0 to 9.0. The change in absorbance associated with peroxide binding reaches a maximum at around pH 7.75 that is roughly 5 times as large as that at pH 6.0. Cytochrome *c* oxidase was fully saturated with hydrogen peroxide at all pHs, as indicated by the lack of spectral change with additional hydrogen peroxide. At pH 9.8 the shape of the difference spectrum is radically changed, as is the spectrum of untreated cytochrome *c* oxidase. The changes in the absorbance spectrum probably reflect substantial unfolding of the protein around the heme (Takemori & King, 1965). The change in the difference spectrum of peroxide binding at this high pH suggests that the way in which peroxide is bound at high pH differs from peroxide binding at neutrality.

Different preparations of cytochrome *c* oxidase varied in their interactions with peroxide binding in another way that was not due to pH. While all preparations of the enzyme as isolated appeared to bind peroxide, some had much smaller extinction coefficients than those reported for the "pulsed" oxidase of Figure 3. Differences between preparations were apparent even in the presence of a large excess of hydrogen peroxide. The preparation studied which showed the smallest spectral change upon peroxide addition had about one-tenth the absorbance change shown in Figure 2, while other preparations had a 4-fold larger change. Although different preparations varied in their difference extinction coefficients, the position of the extinction coefficient maxima was not

detectably different between preparations.

Cytochrome *c* oxidase in preparations of mitochondrial particles will also bind peroxide, as evidenced by peroxide-induced spectral changes at 607 nm in difference spectrum experiments (not shown). The changes in absorbance were consistent with that observed for the isolated enzyme. The effect of hydrogen peroxide on the spectrum of cytochrome *c* oxidase is, in this case, however, much more transient, presumably because endogenous enzymes can catalyze the breakdown of peroxide and thereby remove it from the solution.

Cytochrome *c* oxidase can utilize hydrogen peroxide as a substrate for the oxidation of reduced cytochrome *c*. This can be shown by comparing the cytochrome *c* oxidase catalyzed oxidation of cytochrome *c* with either molecular oxygen or hydrogen peroxide as a cosubstrate. The following results address this point.

Under conditions where cytochrome *c* oxidase activity was measured polarographically in the presence of 5 mM ascorbate, 0.5 mM *N,N,N',N'*-tetramethylphenylenediamine, and 20 μM cytochrome *c* (data not shown), hydrogen peroxide acted as an inhibitor of oxygen consumption. The inhibition of oxygen consumption was reversible by addition of catalase. The rate of oxygen consumption after the peroxide has been removed was identical with the rate prior to peroxide addition. A more detailed treatment of the kinetics of the interactions of peroxide and cytochrome *c* oxidase will be reported in another publication.

In rapid-mixing experiments with oxygen as a cosubstrate, a solution of cytochrome *c* in 0.1 M phosphate buffer with 0.5 M glucose, 1 mM EDTA, and 1% Tween, pH 7.5, was sealed in a septum bottle and deoxygenated by repeated evacuation and saturation with nitrogen. The cytochrome *c* was then reduced with an equal concentration (2-fold excess in electron equivalents) of sodium ascorbate from a freshly prepared 1 M stock solution. After the cytochrome *c* was reduced, cytochrome *c* oxidase, also deoxygenated by evacuation and saturation with nitrogen, was added. Some of this solution was withdrawn from the septum bottle under positive nitrogen pressure into a 20-mL holding syringe previously flushed with nitrogen. The holding syringe was connected to one drive syringe of a stopped-flow spectrophotometer. The solution was pushed back and forth between the drive syringe and the holding syringe several times to ensure complete equilibration, and after a few minutes the solution in the drive syringe returned to the characteristic color of reduced cytochrome *c*. The other drive syringe was filled with solution from a holding syringe containing the same buffer but equilibrated with room air and with no reducing agents or proteins. The rate of cytochrome *c* oxidation upon rapid mixing with the oxygen-containing buffer was observed by the change in light transmission at 563 nm, an isosbestic point for the reduced and oxidized forms of cytochrome *c* oxidase (Brunori et al., 1979). The time course of the reaction is shown in Figure 5 (curve A). The rate observed was 0.43 s^{-1}.

Rapid-mixing experiments were then conducted with hydrogen peroxide as cosubstrate. For removal of the oxygen and addition of peroxide, the syringe containing air-equilibrated buffer was removed, and glucose oxidase was added to it. The holding syringe was again attached to the drive syringe and the solution pushed back and forth between syringes. The reduction of oxygen to hydrogen peroxide at these concentrations of glucose and glucose oxidase requires 6 min to reach levels of molecular oxygen below detectability by an oxygen electrode. After 15 min the rapid-mixing experiments were repeated. Under these conditions the rate of cytochrome *c* oxidation observed is that characteristic of cytochrome *c* oxidase with peroxide as cosubstrate. The time course shown in Figure 5B has a rate of 0.038 s^{-1}. The holding syringe was then again removed and sufficient hydrogen peroxide added to yield a concentration of 635 μM. The rapid-mixing experiments were then repeated at the higher H_2O_2 concentration. The results of these experiments with peroxide as an oxidant are shown in Figure 5 and have a rate of 0.046 s^{-1}. Thus, with peroxide as a cosubstrate, the reaction is about 10 times slower than with O_2 as a cosubstrate under these measuring conditions.

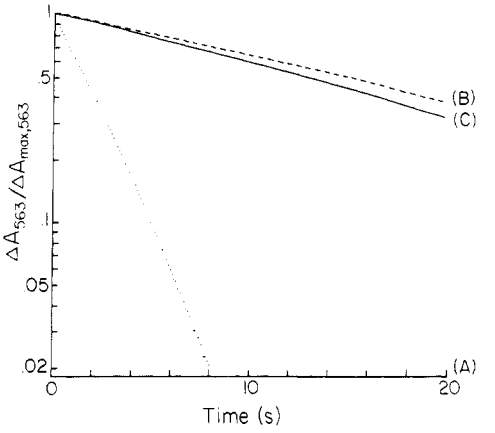

FIGURE 5: Time course of cytochrome *c* oxidation after rapid mixing of reduced cytochrome *c* and cytochrome *c* oxidase with oxygen (A) or peroxide (B and C) as measured at 563 nm. Reactants were at 20 °C in 0.1 M sodium phosphate, 1 mM EDTA, 0.5 M glucose, and 1% Tween at pH 7.5. Concentrations of reactants after mixing were as follows: (A) 58 μM cytochrome *c*, 1 μM cytochrome *c* oxidase, 135 μM oxygen; (B) same as above, but without oxygen and with 135 μM H_2O_2 and 1.3 mg/mL glucose oxidase; (C) same as above, but with 635 μM H_2O_2. See text for details.

Discussion

The foregoing results show that hydrogen peroxide binds to cytochrome *c* oxidase at the heme of cytochrome a_3. The peroxide forms a stable peroxide adduct, with a $K_d \leq 10$ μM. The peroxide can be displaced by other ligands of the heme of cytochrome a_3 or removed by removing peroxide from the solution. These aspects of peroxide binding are compatible with current descriptions of cytochrome *c* oxidase activity (Chance et al., 1975; Wilson & Erecinska, 1979) and models of the metal centers (Blumberg & Peisach, 1979).

The pH dependence of the extinction coefficients of the difference spectrum associated with peroxide binding may be due to (a) change in the formal charge of the heme iron, (b) variable degrees of protonation of peroxide, or (c) some allosteric effect of protons on the cytochrome *c* oxidase structure. We note in regard to the first possibility that at pH 8, where the difference extinction coefficient for peroxide binding has a maximum, a number of heme-containing proteins are known to react with hydrogen peroxide to form complexes in which the iron atom is formally in a low spin, Fe(IV) state (Aviram et al., 1978; Campbell et al., 1980). However, since hydrogen peroxide binding to the heme of cytochrome a_3 of cytochrome *c* oxidase is reversible, either an electron transfer to peroxide from the ferric cytochrome a_3 does not commonly occur with cytochrome *c* oxidase, so that the formal charge is unchanged, or the cytochrome a_3 to peroxide electron transfer is reversible. In proteins with only a single heme redox site, when the heme is originally in the ferric state, hydrogen peroxide, a two-electron acceptor, can remove only one electron from the metal. The free radical reactions which can follow are generally

destructive to the protein (Tomoda et al., 1978; King et al., 1967). Cytochrome *c* oxidase, with four coupled redox sites, might be able to avoid free radical formation during a reaction of peroxide and ferric heme. This suggests that electron transfer from peroxide and formation of ferryl iron might be possible. Conversely, the electron-transfer hypothesis requires that the Fe(IV) complex formed be stable and be able to return to Fe(III) upon displacement of peroxide by cyanide ligation or by removing hydrogen peroxide from the solution. We favor the simple peroxide ligation model, without charge transfer, because the optical changes observed at any pH have little in common with the optical changes seen upon peroxide oxidation of hemes from ferric to ferryl in other heme proteins (Wittenberg, 1978; Dolphin et al., 1971).

If the changes in difference extinction coefficients are not due to a change in the formal charge of the iron of cytochrome a_3, they may be due to deprotonation of bound hydrogen peroxide in the high pH region. The form of peroxide bound to cytochrome *c* oxidase at pH 6 (as illustrated in Figure 4) is probably the neutral molecule. We note that catalase is believed to be able to bind molecular hydrogen peroxide at physiological pH, unlike simpler iron complexes which can only bind the conjugate base (Brown et al., 1970). If cytochrome *c* oxidase, like catalase, can bind the protonated form, it might be expected that binding of the conjugate base of peroxide would give a different spectral shift. Alternatively, we cannot discount the possibility that the changes in difference spectra with pH may be the result of changes far removed from the peroxide binding site. Methemoglobin provides an example of both direct and indirect influences. Changing the Fe(III) ligand from water in "acid" metHbA to hydroxide in "alkaline" metHbA causes changes in optical spectra. But additionally, the binding of inositol hexaphosphate at a site removed from the heme can also alter the methemoglobin spectrum (Perutz et al., 1974). Either proton abstraction from hydrogen peroxide or protein-induced effects or both effects could underlie the observed pH dependence of the extinction coefficients in peroxide binding to cytochrome *c* oxidase. Resonance Raman or Mossbauer spectroscopy should be able to distingish changes in peroxide ligation or electron transfer from cytochrome a_3 to peroxide. We are currently exploring this question.

The low temperature work of Chance and others (Chance et al., 1975) suggests that peroxide is bound to ferric cytochrome a_3 rather than causing it to oxidize to Fe(IV). Indeed, the peroxide adduct of ferric cytochrome *c* oxidase appears to be a stable room temperature analogue of "compound C", an intermediate in oxygen reduction observed between −100 and −60 °C. The transient "607-nm complex" of Nicholls & Chanady (1981) and a form described by Wikström (1981) also appear to be equivalent to the peroxide complex described in this paper.

Differences in extinction coefficients upon peroxide binding were observed between preparations of cytochrome *c* oxidase. Although there is a possibility that the preparations differ in their constituent subunits or in contaminating materials, we hypothesize that the phenomenon is linked instead to the intriguing fact that fully oxidized cytochrome *c* oxidase can exist in more than one form (Brunori et al., 1979; Brittain & Greenwood, 1976). If at least one form does not bind hydrogen peroxide to any significant degree, changes in the size of the difference extinction coefficient upon addition of peroxide would be expected to accompany changes in the proportions of the forms. This would require that transition rates between forms be very slow, because the presence of peroxide would otherwise shift the equilibrium to favor the peroxide binding form and thus eliminate the differences between extinction coefficients. Two forms that have been defined are "resting", which is a less active form of cytochrome *c* oxidase, and "pulsed", a more active form.[3] Pulsed cytochrome *c* oxidase is cytochrome *c* oxidase which has been reduced and quickly reoxidized. Resting cytochrome *c* oxidase, in contrast, is simply the name given the enzyme-as-isolated. We believe, however, that the enzyme-as-isolated is composed of cytochrome *c* oxidase molecules in different states.

Experiments with another cytochrome a_3 ligand, cyanide, have demonstrated that cytochrome *c* oxidase-as-isolated is in fact composed of molecules of two distinct states with significantly different reactivities (Van Buuren et al., 1972). Cyanide binds to pulsed cytochrome *c* oxidase in a monophasic fashion with a second-order rate constant of 22 M^{-1} s^{-1} (Brittain & Greenwood, 1976). Cyanide binds to cytochrome *c* oxidase-as-isolated in a biphasic fashion whose slower phase is about 1.8 M^{-1} s^{-1} (Van Buuren et al., 1972). These results suggest that pulsed oxidase is composed of one form of cytochrome *c* oxidase, with monophasic binding kinetics, while the enzyme-as-isolated has at least two different forms.

The major component of preparations of isolated cytochrome *c* oxidase appears to be a form with low activity toward peroxide. The proportion of the low activity form, however, varies from preparation to preparation. (We speculate that the ratio of each form may depend on the treatment of enzyme during isolation or on the state of the organism before slaughter. Contented cows may give more than good milk.) Preparations of cytochrome *c* oxidase which had small difference extinction coefficients for peroxide binding showed larger peroxide-induced extinction coefficients after they were pulsed. The pulsed form of cytochrome *c* oxidase was used to generate the data of Figure 3. It is clear that hydrogen peroxide can bind to the pulsed form, perhaps exclusively or at least with a much higher rate or affinity than to the major component of the enzyme-as-isolated.

A major conclusion of the results presented here is that hydrogen peroxide can be used as an electron acceptor in the oxidation of reduced cytochrome *c* by cytochrome *c* oxidase and as an inhibitor of oxygen consumption. This is evidence that the peroxide can be bound to the cytochrome *c* oxidase in a functionally active way. There is a slower rate of cytochrome *c* oxidation when peroxide is the coupled oxidant than when molecular oxygen is the coupled oxidant. The slower rate may reflect a ligand-dependent difference in the rate of the internal electron transfer from cytochrome *a* to cytochrome a_3. Alternatively, it may be due to the difference in the interactions of peroxide and oxygen with cytochrome a_3. Hydrogen peroxide is not considered a physiological substrate of cytochrome *c* oxidase, and the rate of association of peroxide with the enzyme may be slower than that of molecular oxygen. The cytochrome *c* oxidation experiments done tell us very little about whether or not the enzyme uses precisely the same steps when peroxide is used as a substrate as in the case when molecular oxygen is reduced. Peroxide, unlike oxygen, can bind to the fully oxidized form of the enzyme. Mechanisms may therefore be possible with peroxide as the substrate which would not occur with oxygen. Details of the kinetics of hydrogen peroxide binding are of relevance, but the kinetic

[3] Other forms of the enzyme have also been described. At least three fairly stable species of oxidized cytochrome *c* oxidase can be observed by EPR, which correspond to resting, pulsed, and a third species which gradually forms from pulsed (Brudvig et al., 1981). Additionally, spectral and kinetic differences have been shown between the form which prevails during turnover and either resting or pulsed cytochrome *c* oxidase (Brunori et al., 1979).

analysis is beyond the scope of the present paper and will be presented in another publication.

The reduction of molecular oxygen to water is the reaction which provides the driving energy for several cytochrome *c* oxidase catalyzed reactions. Our study supports models in which the oxygen reduction is carried out in two, sequential, two-electron reductions. The interval between reduction to peroxide and reduction to water under physiological conditions is unknown. However, in vivo spectrophotometric measurements of the oxidation state of cytochrome *c* oxidase show that in some cases the enzyme is partially reduced even in the presence of molecular oxygen (Rosenthal & LaManna, 1977). This suggests that the internal electron transfer, rate limiting in vitro, may be slowed even further, and the sequential two-electron transfers may be temporally very separate indeed.

Hydrogen peroxide is the two-electron reduction product of molecular oxygen. Reversible binding of peroxide by cytochrome *c* oxidase carries implications about the function of the enzyme. The experiments reported here provide compelling evidence that hydrogen peroxide is a true intermediate in the catalytic mechanism of the enzyme, not merely a chemical probe of the active site. It has previously been postulated that peroxide, bound to various forms of cytochrome *c* oxidase, is the cause of changes in spectra or enzyme activity (Lemberg & Mansley, 1966; Chance et al., 1975; Brunori et al., 1979; Nicholls & Chanady, 1981; Harmon & Wikström, 1978). The formation and isolation of a stable, room temperature peroxide adduct of cytochrome *c* oxidase allows for exploration of these hypotheses and for further investigations of the role of intermediate states of cytochrome *c* oxidase in the enzymatic oxidation of cytochrome *c*.

Acknowledgments

Helpful discussions with Dr. Mike Wilson of the University of Essex and Dr. Maurizio Brunori of the University of Rome are gratefully acknowledged.

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