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Formation and reduction of a 'peroxy' intermediate of cytochrome *c* oxidase by hydrogen peroxide

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1. In the presence of micromolar concentrations of H_2O_2, ferric cytochrome *c* oxidase forms a stable complex characterized by an increased absorption intensity at 606–607 nm with a weaker absorption band in the 560–580 nm region. Higher (millimolar) concentrations of H_2O_2 result in an enzyme exhibiting a Soret band at 427 nm and an α-band of increased intensity in the 589–610 nm region. 2. Addition of H_2O_2 to ferric cytochrome *c* oxidase in the presence of cyanide results in absorbance increases at 444 nm and 605 nm. These changes are not seen if H_2O_2 is added to the cyanide complex of the ferric enzyme. 3. The results support the idea that direct reaction of H_2O_2 with ferric cytochrome a_3 produces a 'peroxy' intermediate that is susceptible to further reduction by H_2O_2 at higher peroxide concentrations. Electron flow through cytochrome *a* is not involved, and the final product of the reaction is the so-called 'pulsed' or 'oxygenated' ferric form of the enzyme.

Cytochrome *c* oxidase normally catalyses the reduction of molecular O_2 to water:

$$4\text{Cyt. } c^{2+} + 4H^+ + O_2 \rightarrow 4\text{Cyt. } c^{3+} + 2H_2O \quad (1)$$

However, experiments performed by Bickar *et al.* (1982) and Orii (1982) on the peroxidase activity of the enzyme indicate that H_2O_2 can also act as an electron acceptor for cytochrome *c* (Cyt. *c*) oxidation:

$$2\text{Cyt. } c^{2+} + 2H^+ + H_2O_2 \rightarrow 2\text{Cyt. } c^{3+} + 2H_2O \quad (2)$$

A peroxidatic-type reaction such as this might be expected to occur from the various postulated partial reactions of the enzyme (Gibson *et al.*, 1965; Greenwood *et al.*, 1974; Chance *et al.*, 1975; Erecinska & Wilson, 1978; Reed & Landrum, 1979; Wikström *et al.*, 1981) in which an initial two-electron reduction of molecular O_2 may produce a bound peroxy intermediate:

$$2\text{Cyt. } c^{2+} + (2H^+) + O_2 \rightarrow 2\text{Cyt. } c^{3+} + (H_2^{2+})O_2^{2-} \quad (3)$$

which can then be fully reduced to water by further two-electron transfer:

$$2\text{Cyt. } c^{2+} + 2H^+ + (H_2^{2+})O_2^{2-} \rightarrow 2\text{Cyt. } c^{3+} + 2H_2O \quad (4)$$

Information on the initial two-electron reaction (eqn. 3) has been obtained mainly from the reaction of the mixed-valence enzyme ($a^{3+}Cu_A^{2+}a_3^{2+}Cu_B^{+}$) with O_2. At temperatures above −100°C a compound is rapidly formed, exhibiting an increased absorption intensity in the 605–610 nm region, that has been variously identified as being due to a peroxy intermediate containing ferryl (Fe^{IV}) iron (Chance *et al.*, 1975), a superoxide complex with ferrous iron and blue 'Type 1' copper (Chance *et al.*, 1979), a μ-peroxo species of ferric iron and Cu_B^{II} (Clore *et al.*, 1980; Wikström *et al.*, 1981), and even a mixed-valence CO complex formed by re-binding of photodissociated CO to a modified a_3 centre (Nicholls, 1978). If the compound is indeed a 'peroxy' intermediate (or mixture of intermediates), it might be thought to form also in the reaction of the ferric enzyme with H_2O_2. The affinity of ferric cytochrome *c* oxidase for H_2O_2 appears to be high (Bickar *et al.*, 1982; Orii, 1982), and indeed binding of peroxidase to the oxidized enzyme is reported to induce a spectral shift of the Soret and α-absorption maxima from 417 nm to 422 nm and from 599 nm to 601 nm respectively (Bickar *et al.*, 1982).

The present paper reports the results of experiments showing that a stable intermediate of the reaction of H_2O_2 with ferric cytochrome *c* oxidase has spectral properties similar to those of 'Com-

pound C' described by Chance *et al.* (1975) and that full reduction of the intermediate can be achieved by H_2O_2 in a catalase-like reaction.

Materials and methods

Cytochrome *c* oxidase was prepared from ox heart by the method of Kuboyama *et al.* (1972), with Tween 80 substituting for Emasol, and was stored under liquid N_2 until use. The final preparation of the enzyme had a haem *a*/protein ratio of 10.1 μmol/g and maximal turnover (electrons/cytochrome aa_3) at 30°C of 70 s^{-1} measured by oxygen-electrode assay in the presence of 0.5% Tween 80 in 100mM-potassium phosphate buffer, with ascorbate, *NNN'N'*-tetramethyl-*p*-phenylenediamine dihydrochloride and horse heart cytochrome *c* (Sigma type VI) as substrate. Spectrophotometry was carried out with a Cary 210 instrument and all reactions were done at 30°C. Ferric cytochrome *c* oxidase–cyanide complex was formed by incubating 39 μM enzyme with 3mM-KCN for 6 days in 100mM-potassium phosphate buffer, pH7.5, containing 0.05% Tween 80 at 4°C.

Results

A closer examination of the difference spectrum of the absorbance region under similar conditions to those used by Bickar *et al.* (1982) (with untreated oxidized cytochrome *c* oxidase as reference) shows that the complex formed by the addition of micromolar concentrations of H_2O_2 to a solution of oxidized cytochrome *c* oxidase has a spectrum with an α-band at 606nm and a pronounced shoulder at 570–580nm (Fig. 1). At these low peroxide concentrations, the complex is stable (for at least 30min), but if higher (millimolar) concentrations of peroxide are added to the sample the absorbance peak in the difference spectrum shifts to 583nm (Fig. 1). The absolute spectrum of this new species (Fig. 2) now has a Soret band at 427nm and an α-band of increased intensity in the 589–610nm region, both features characteristic of the so-called 'oxy' (low-spin) species, usually produced by aerating the fully reduced enzyme (Sekuzu *et al.*, 1959) or by illuminating the mixed-valence enzyme–CO complex in the presence of O_2 (Nicholls, 1978).

By analogy with the more usual methods for producing the 'oxy' enzyme, the results with relatively high concentrations of H_2O_2 suggest that under these conditions H_2O_2 can also act as an electron donor to the ferric enzyme. This appears to be the case from the results shown in Fig. 3, where the addition of H_2O_2 to the oxidized enzyme in the presence of cyanide results in absorbance increases at 444nm and 605nm. The reaction of cyanide with oxidized cytochrome *c* oxidase is relatively slow, having a rate constant for cyanide

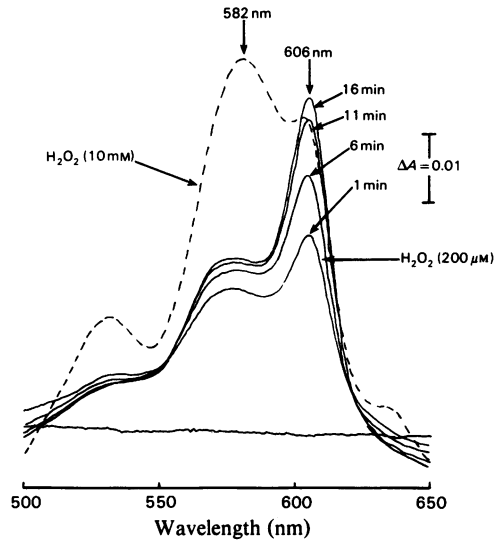

Fig. 1. *Difference spectra of cytochrome c oxidase–H_2O_2 complexes*
Cytochrome *c* oxidase (40 μM) was dissolved in 90mM-potassium phosphate buffer, pH7.4, containing 1% Tween-80 at 30°C in both sample and reference cuvettes (each of 1ml final volume). Then 2 μl of 100mM-H_2O_2 was added to the sample cuvette, and, after 1min, spectra were scanned at 5min intervals (continuous lines). After 30min H_2O_2 was added to a final concentration of 4mM to the sample cuvette and the spectrum was scanned after a further 1min (broken line).

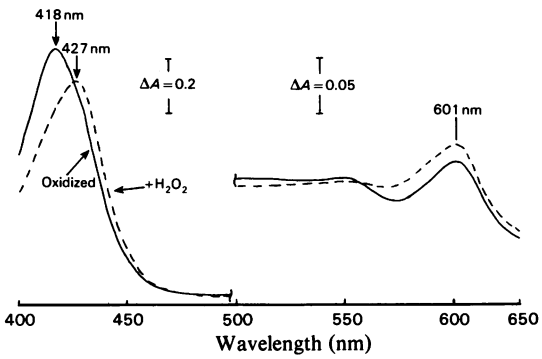

Fig. 2. *Effect of millimolar concentrations of H_2O_2 on the absolute spectrum of ferric cytochrome c oxidase*
Cytochrome *c* oxidase (6 μM) was dissolved in 90mM-potassium phosphate buffer, pH7.4, containing 0.5% Tween-80 at 30°C and the spectrum was measured (continuous line). H_2O_2 was added to a final concentration of 4.3mM, and the spectrum was scanned after 15min (broken line).

binding of only 1.8 $M^{-1}\cdot s^{-1}$ at pH7.4, whereas cyanide binding to the ferrous enzyme is relatively fast (150 $M^{-1}\cdot s^{-1}$) (Van Buuren *et al.*, 1972). Thus cyanide can be used to 'trap' any ferrous enzyme

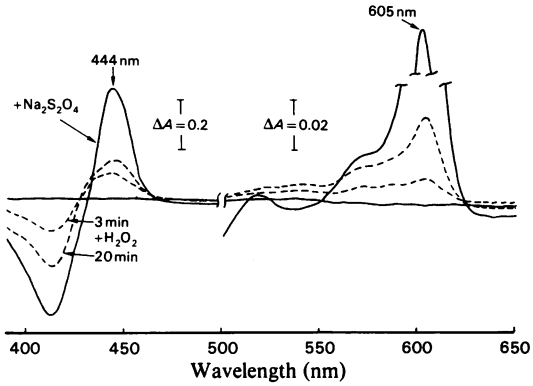

Fig. 3. *Effect of H_2O_2 on the ferric cytochrome c oxidase difference spectrum in the presence of cyanide*
Cytochrome *c* oxidase (7.7 μM) was dissolved in 100mM-potassium phosphate buffer, pH7.4, containing 0.013% Tween-80 and 3mM-KCN at 30°C in both sample and reference cuvettes (each of 1ml final volume). H_2O_2 was added to a final concentration of 4mM to the sample cuvette, and the spectrum was scanned after 3min and 20min (broken lines). A few crystals of solid $Na_2S_2O_4$ were added to the sample cuvette, and the spectrum was re-scanned after a further 10min (continuous line).

formed by reaction with H_2O_2. In the presence of cyanide, it can be seen that the absorbances at 444nm and 605nm increase, over a period of 20min, to 37% and 24% respectively compared with the spectrum of the dithionite-reduced enzyme (Fig. 3), indicating haem reduction by H_2O_2 followed by reaction with cyanide.

Formation of the ferrous a_3–cyanide complex by H_2O_2 appears to require 'free' ferricytochrome a_3 to be available. It is not formed from the cyanide complex of the ferric enzyme (Fig. 4), where reduction of cytochrome a_3 is blocked (Nicholls & Hildebrandt, 1978). Addition of H_2O_2 to the stable ferric enzyme–cyanide complex results in only slight absorbance changes at 444nm and 605nm (Fig. 4). Ferric cytochrome *a* in this complex still remains available for reduction by other reductants, as can be evinced by the addition of dithionite to the reaction mixture.

Discussion

The present results confirm the observations by Bickar *et al.* (1982) that, at low H_2O_2 concentrations, H_2O_2 can form a room-temperature-stable complex with ferric cytochrome *c* oxidase. A close examination of the α-absorbance-band region as presented here shows it to have many spectral similarities with several of the intermediates detected in the reaction of the fully reduced and partially reduced enzyme with O_2 (Chance *et al.*, 1975; Chance & Leigh, 1977; Clore

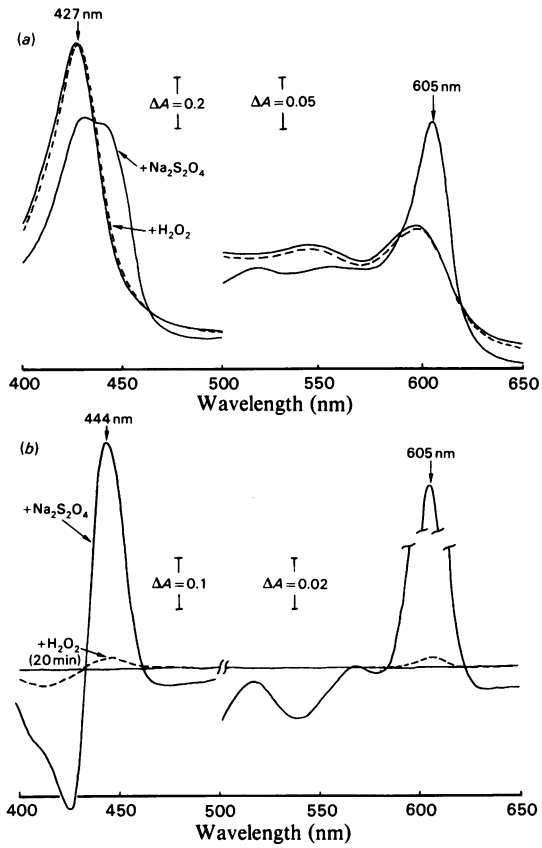

Fig. 4. *Effect of H_2O_2 on the spectrum of the ferric cytochrome c oxidase–cyanide complex*
Ferric cytochrome *c* oxidase–cyanide complex was mixed to a final concentration of approx. 10 μM enzyme in 100mM-potassium phosphate buffer, pH7.4, containing 0.013% Tween-80. H_2O_2 was added to a final concentration of 4mM, and the spectrum was scanned after 20min (broken line). A few crystals of solid $Na_2S_2O_4$ were added to the sample cuvette, and the spectrum was re-scanned after 10min. (*a*) Absolute spectra; (*b*) difference spectra with ferric cytochrome *c* oxidase–cyanide complex in the reference cuvette.

& Chance, 1978; Nicholls, 1978, 1979; Chance *et al.*, 1979; Clore *et al.*, 1980; Denis, 1981), and the 'peroxy' intermediate produced by partial reversal of the oxidase reaction (Wikström, 1981). The general features of the spectra of these complexes include an increased absorption intensity in the 605–610nm region compared with the fully oxidized enzyme, a distinct peak at 606–607nm and a weaker absorption band in the 560–580nm region. (The latter is often obscured when the difference spectrum is measured relative to the CO complex of the half-reduced enzyme.) These features can be seen in the present complex, which in addition

shows a decrease in absorption around 650–660nm, indicating an absence of the 655nm absorption band usually present in the unliganded ferric enzyme (Denis, 1977, 1981; Chance & Leigh, 1977; Clore *et al.*, 1980).

A possibility that is consistent with these findings is that the direct reaction of H_2O_2 with the oxidized enzyme generates a 'peroxy' complex in accordance with eqn. (3). The method of generation precludes the necessity of electron donation from a_3 and its associated copper, but, in the absence of any e.p.r. data, the present evidence is insufficient to assign detailed electronic configurations to the intermediate. However, the absence of CO from the reaction mixture confirms the conclusions of Wikström *et al.* (1981) and Nicholls & Chanady (1981) that CO is not a necessary component or participant in the production of the complex.

The stability of the present complex at room temperature is likely to be a consequence of the highly oxidized conditions of its production. Only in the presence of appropriate reductants could further two-electron transfer take place (eqn. 4). According to the present results, H_2O_2 can act as a suitable reductant if sufficiently high peroxide concentrations are used. The reaction appears to occur directly at the a_3Cu_B site, and not to involve cytochrome *a*. Any electron flow from peroxide through cytochrome *a* to cytochrome a_3 would be expected to cause reduction of cytochrome *a* in the cyanide complex of the ferric enzyme. This is not found to be the case to any significant extent. The reducing ability of H_2O_2 on the complex may be mediated, in part, by its content of superoxide (Bernofsky & Wanda, 1983), but a catalase-like reaction is possible.

A weak catalase-like activity of the enzyme has been reported by Orii & Okunuki (1963) and Krasna (1965), and more recently this finding has been re-examined by Baum *et al.* (1983). Unfortunately, it is not yet known to what extent if any the catalase-like activity of cytochrome *c* oxidase preparations is due to contamination by catalase or to effects due to denatured oxidase. However, the fact that H_2O_2 is capable of direct interaction with the present highly purified preparation, and that further reduction of the intermediate by H_2O_2 can occur, indicates that cytochrome *c* oxidase indeed possesses catalase-like activity, although the kinetic consequences of this activity remain to be determined.

The final product of the reaction of the enzyme with high concentrations of H_2O_2 is characterized by a red-shifted Soret band (to 427nm) relative to the ferric 'resting' enzyme. The spectrum is similar to that shown by the reduced enzyme after aeration to the so-called 'oxygenated' (Sekuzu *et al.*, 1959) or 'pulsed' (Antonini *et al.*, 1977) form. Ferric cytochrome *c* oxidase is known to exist in (at least) two functionally distinct forms, which have been distinguished on the basis of catalytic activity (Wilson *et al.*, 1981), conformation (Kornblatt *et al.*, 1975) and spin state (Nicholls & Hildebrandt, 1978). The transition from the 'resting' to the 'pulsed' or 'oxygenated' form is sensitive to the flow of electrons through the complex (Antonini *et al.*, 1977). The present results with cyanide indicate that electron flow through cytochrome *a* may not be a necessary event in this transition. Formation and reduction of a peroxy intermediate at the a_3Cu_B site appears to be sufficient to convert the enzyme from the ferric 418nm-absorbing species into the ferric 427nm-absorbing form.

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