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INTERACTIONS OF CYTOCHROME aa_3 WITH OXYGEN AND CARBON MONOXIDE

THE ROLE OF THE 607 nm COMPLEX

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Summary

The 607 nm complex of cytochrome *c* oxidase, formed aerobically in the presence of CO, appears as an intermediate during the oxidation of CO to CO_2 by the enzyme. Maximal steady-state formation of this complex requires oxygen, high levels of carbon monoxide, and the presence of an endogenous hydrogen donor system or the addition of small amounts of reductant (both with isolated enzyme and mitochondrial preparations). The 607 nm complex can be formed after removing CO from the mixed-valence CO complex (cytochrome $a^{3+}a_3^{2+}CO$) by aerating the presumably CO-free product. The elements of CO are, therefore, probably not part of the 607 nm complex nor of the related 'compound C' produced at low temperatures.

The relationship between cytochromes *a* and a_3 within the cytochrome aa_3 complex remains problematic, as does the pathway by which oxygen is reduced by this enzyme. Two oxygen-sensitive species are known, the fully reduced enzyme ($a^{2+}a_3^{2+}$) and the partially reduced enzyme, or mixed-valence species, obtained in the presence of CO($a^{3+}a_3^{2+}$). The oxygen reaction with the fully reduced enzyme has been studied at room temperature by Greenwood and Gibson [1] and at low temperature by Chance et al. [2], while the reaction with partially reduced enzyme has been examined at room temperature by Greenwood et al. [3] and at low temperature by Chance and his colleagues [2,4].

The intermediates seen in the reaction with fully reduced enzyme are not

Abbreviations: Cu_U, EPR-undetectable copper of cytochrome aa_3; Cu_D = EPR-detectable copper of cytochrome aa_3.

stable at room temperature, although several species are seen at low temperatures [2] and under flow-flash conditions [1,5]. On the other hand the product of the reaction with partially reduced enzyme has an unusual α-peak (at 606–607 nm) and is not identical with ferric enzyme; it remains stable over periods of minutes at low temperature [4,6] and tens of seconds at room temperature [3].

This 607 nm product has been variously identified as:

(a) a blue (Type I) cupric complex involving the EPR invisible copper [4,6];

(b) a form of oxy(ferri)cytochrome *c* oxidase [3]; and

(c) a product formed by rebinding of photodissociated carbon monoxide to a 'modified' ferrous cytochrome a_3 centre [7,8].

The last suggestion was based largely on the observation that the spectrum of the 607 nm complex (with α, β and Soret bands) is that of a liganded ferrous cytochrome a_3 species and analogous to that of the carbon monoxide complex, although the absorption bands are shifted some 10 nm to the red in the Soret region and 16 nm to the red in the visible region. Moreover a similar intermediate can be generated in solution at room temperature by bubbling CO gas into an aerobic sample of cytochrome *c* oxidase [7,8].

Two other kinds of investigation have been directed recently at these intermediates. Clore et al. [9,10] and Reinhammar et al. [11] have reported on the EPR spectra characteristics of the intermediates. The 607 nm intermediate itself appears not to be characterized by any new Cu signal [10] although rapid freezing identifies a possibly related intermediate that does show a new Cu signal [11], tentatively attributed to the Cu atom associated with cytochrome a_3. Secondly, Young et al. [12] and Young and Caughey [13] have reexamined the oxidation of carbon monoxide by the enzyme, a process first reported by Fenn and Cobb [14] for intact systems and proven to be oxidase-dependent by Tzagoloff and Wharton [15]. They identified an aerobic steady-state intermediate closely related to the ferric state during CO oxidation [12].

We have therefore carried out the experiments briefly described here in an attempt to clarify the relationship between the oxygen and carbon monoxide reactions of the oxidase and the role of the 607 nm complex in mediating the interaction of the two gas species.

Materials and Methods

Cytochrome *c* oxidase (bovine heart) was prepared and stored as described by Nicholls [8], either by the cholate method of Kuboyama et al. [16] or by the Triton X-114 extraction method of Hartzell and Beinert [17], and used as indicated in the individual experiments (Figs. 1–6). The preparation of Hartzell and Beinert [17] seems to contain less endogenous reductant than the preparation of Kuboyama et al. [16] (Hartzell, C.R., personal communication). Bovine heart mitochondria were prepared by subtilisin digestion, homogenization, and differential centrifugation, essentially according to Tyler and Gonze [18], and used within 48 h of preparation.

Gases (N_2 and CO) were Linde Specialty gases from Union Carbide Co., Toronto. Cytochrome *c* (horse heart, Type VI), subtilisin, Triton X-114, morpholinopropane sulphonic acid, mannitol, cholic acid, Tween-80 and Tween-20

were from Sigma Chemical Co. Other reagents were from J.T. Baker Co. or from Fisher Chemical Co.

Incubation of the enzyme under CO or N_2 gas was carried out in Thunberg tubes with attached optical cuvettes (glass) for periods of two or three days at 4°C. These tubes were filled with the required gas at atmospheric pressure from an anaerobic tonometer system. Such incubation conditions secured full formation of the mixed-valence CO complex with preparations of the 'Kuboyama' enzyme (cf. Ref. 3). Split beam and dual wavelength spectrophotometry was then performed with an Aminco DW-2 instrument, with cell compartment modified to accommodate the Thunberg cuvette. Oxygen uptake was measured with a Clark electrode (Yellow Springs Instrument Co.) at 30°C, attached to a polarizing box and recorder. The oxygen electrode vessel was transparent and could be illuminated or darkened as described in the legend to Fig. 4. Saturated CO-containing buffer solutions (between 0.85 mM and 0.9 mM in CO) were assayed by titration into solutions of reduced myoglobin. Cytochrome *c* oxidase concentration was determined using a value of 27 $mM^{-1} \cdot cm^{-1}$ for E (reduced minus oxidized, 605—630 nm) as in Ref. 8.

Results

Fig. 1 illustrates the spectroscopic relationships in the visible region between the mixed-valence carbon monoxide complex [3] and the 607 nm complex obtained aerobically in the presence of carbon monoxide (cf. Refs. 7, 8). Under the conditions in Fig. 1, CO is present in considerable excess over O_2. The spectrum obtained after saturation of the aerobic system with CO is shown in trace (b), which illustrates the characteristic α and β peaks of the 607 nm compound. Reduction with a trace of dithionite followed by reoxidation of the reduced cytochrome *a* with ferricyanide, according to Nicholls [19], initially gives the mixed spectrum of trace (c), with peaks at both 607 and 590 nm. After a few minutes, however, the mixed-valence spectrum, trace (d), is seen. Reaeration produces the 607 nm species again, and depletes the 590 nm peak population, as in trace (e). Formation of the 607 nm peak therefore depends on the presence of both CO and O_2. Different preparations, however, show quite different 'occupancies' when treated aerobically with carbon monoxide. Submitochondrial particles require addition of micromolar levels of succinate for formation of the 607 nm species and its subsequent transformation into the mixed-valence complex [12,20]. Mitochondria, which contain some endogenous reducing power, rapidly form the mixed-valence compound on being treated with carbon monoxide in presence of ferricyanide to hold the remaining cytochromes in the ferric state (Fig. 2). Vigorous aeration of such preparations causes the replacement of the mixed-valence spectrum by that of some 607 nm complex (closed symbols, Fig. 2); if the latter is a steady-state intermediate, differences in its level in different preparations presumably reflect differences in its relative rates of formation and breakdown.

Such differences also exist in isolated oxidase preparations. Fig. 3 shows that an enzyme sample prepared by the Triton X-114 extraction method gives a much smaller proportion of 607 nm species on saturating a solution with CO (cf. trace (b) in Fig. 3 with trace (b) in Fig. 1). The compound can, however, be

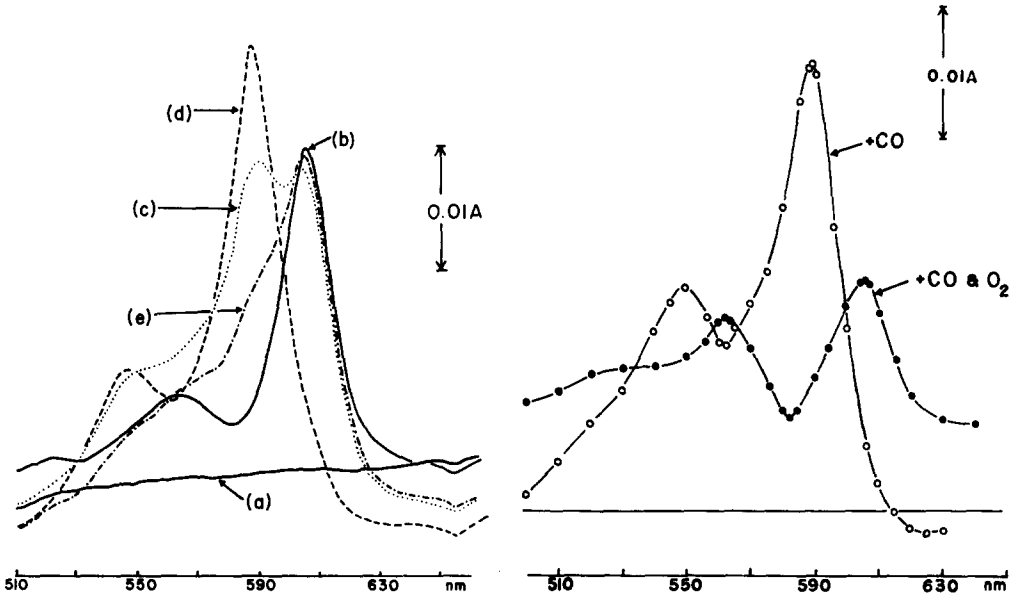


Fig. 1. Formation of the 607 nm species and its conversion into the mixed-valence CO complex of bovine heart cytochrome aa_3. 4 μM cytochrome aa_3, prepared by the cholate extraction procedure of Kuboyama et al. [16], was dissolved in 100 mM sodium phosphate buffer (pH 7.3), containing 0.1% Tween-20 at 30°C. The reference cuvette in each case contained ferric ('resting') enzyme. The sample cuvette was treated as follows: (a) ———, oxidized (baseline); (b) ———, saturated with CO under aerobic conditions, showing the spectrum of the 607 nm species; (c) · · · · · ·, reduced with $Na_2S_2O_4$ in the presence of CO and reoxidized with $K_3Fe(CN)_6$, plus a small amount of air, showing a 'mixed' spectrum; (d) - - - - - -, sample from (c) after 3 min (anaerobic), showing the spectrum of the mixed-valence form; (e) · — · — ·, sample from (d) after reaeration, showing the 'steady state' spectrum indicating the presence of mostly 607 nm species with some mixed-valence form.

Fig. 2. Effect of oxygen on the mixed-valence CO complex in intact bovine heart mitochondria. Mitochondria were suspended at 12 mg · ml^{-1} (protein) in 225 mM mannitol, 75 mM sucrose, 10 mM potassium morpholinopropane sulphonate plus 1 mM EDTA at pH 7.4 and 30°C, and treated with 0.1 mM $K_3Fe(CN)_6$ to oxidize all the cytochromes (as well as any residual myoglobin). The sloping baseline obtained in the Aminco DW-2 instrument has been redrawn as a horizontal line (———). The spectrum obtained after bubbling CO for 1 min into the aerobic sample cuvette (the mixed-valence enzyme) is shown by the open circles (○———○) and that obtained upon subsequent aereation (the 607 nm complex) by the closed circles (●———●).

induced to form more completely by successive addition of dithionite and ferricyanide (trace (c) in Fig. 3). Finally the mixed-valence complex is produced (trace (d), Fig. 3). The cycle between 607 nm compound and mixed-valence CO complex is illustrated in the inset. A pulse of oxygen (air) to the cuvette showing spectrum (d), now monitored by dual wavelength spectrophotometry, induces an immediate decline in absorbance at 590 nm and an increase at 607 nm, with a rate constant identical with that for the dissociation of a CO molecule from ferrous cytochrome a_3 (approx. 0.07 s^{-1}). A characteristic steady-state cycle ensues, terminated by the restoration of the mixed-valence spectrum. The duration of this cycle is quite prolonged compared to the duration of redox cycles of the enzyme in the absence of CO and in the presence of ascorbate plus cytochrome c, or even ascorbate plus N,N,N',N'-

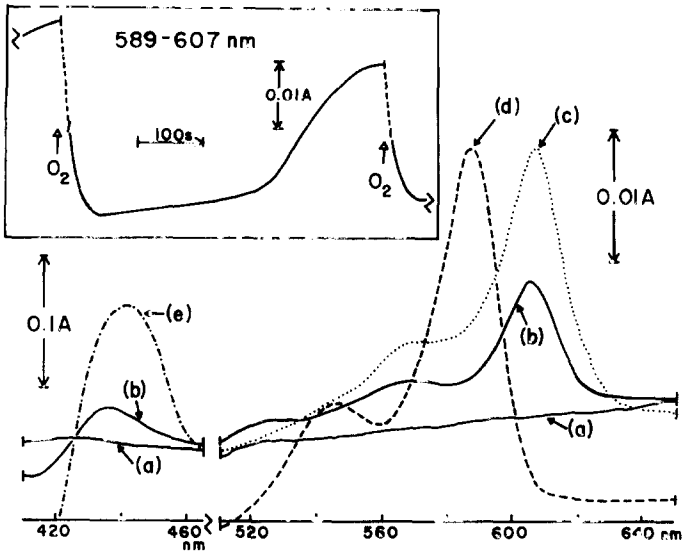


Fig. 3. Spectra obtained during the catalytic cycle between the mixed-valence CO complex and the 607 nm compound of cytochrome *c* oxidase. 4 μM cytochrome aa_3, prepared by the Triton X-114 extraction procedure of Hartzell and Beinert [17], was dissolved in 100 mM sodium phosphate buffer (pH 7.3), containing 0.1% Tween-20. The reference cuvette contained oxidized enzyme and the sample cuvette was treated as follows: (a) ———, oxidized (baseline); (b) ——–, saturated with CO (aerobic), indicating partial formation of the 607 nm species; (c) · · · · · ·, reduced with a trace of $Na_2S_2O_4$, reoxidized with a slight excess of $K_3Fe(CN)_6$ plus a small amount of air, and scanned immediately to demonstrate the full formation of the 607 nm band; (d) - - - - - -, the sample in (c) after 6 min (anaerobic), showing formation of the mixed-valence CO complex; (e) · — · — ·, the Soret band of an enzyme sample partially reduced by dithionite ($a^{2+}a_3^{3+}$), showing the difference from the 607 nm compound (cf. traces (b) and (c)). The inset shows the kinetics of the transition between the two species whose spectra are shown in (d) and (c), monitored by dual wavelength spectrophotometry at 589—607 nm. The anaerobic cuvette (d), containing mixed-valence enzyme, was treated with O_2 by stirring air into the solution. Oxidation of the CO mixed-valence complex is followed by a 'steady state', the spectrum during which is seen in trace (c), and then a return to the mixed-valence state upon exhaustion of the added O_2.

tetramethyl-*p*-phenylenediamine dihydrochloride. The rate of the process involved is therefore slow. That oxygen is indeed being used up by the system is shown by the oxygen electrode tracing in Fig. 4. Using a level of enzyme similar to that employed spectroscopically and 100-fold greater than normally employed in the catalytic assay, a CO-induced uptake becomes evident (cf. traces (a) and (b) with trace (c)). In the polarographic system, cytochrome *c* somewhat accelerates the rate of CO-induced oxygen uptake (addition of cytochrome *c* is indicated at arrows marked '*c*'). The maximal turnover (electron equivalents per cytochrome aa_3 per second) was about 0.05 s^{-1} in presence of 0.8 mM CO, about 0.1% of the rate with cytochrome *c* and ascorbate. Under conditions where light greatly accelerates the usual catalytic reaction, CO oxidation is little affected by illumination (cf. traces (a) and (b)). However, like the normal catalytic reaction, the CO-induced O_2 uptake is blocked by cyanide and by formate (traces not shown). These ligands promote the dissipation of the 607 nm complex (Ref. 8 and Fig. 5A below).

Fig. 5 compares the spectroscopic effects of small amounts of CO on the ferric and ferrous enzymes. Formation of the 607 nm complex is slow in the presence of excess O_2 (Fig. 5A). The apparent rate is about 0.1% that of the

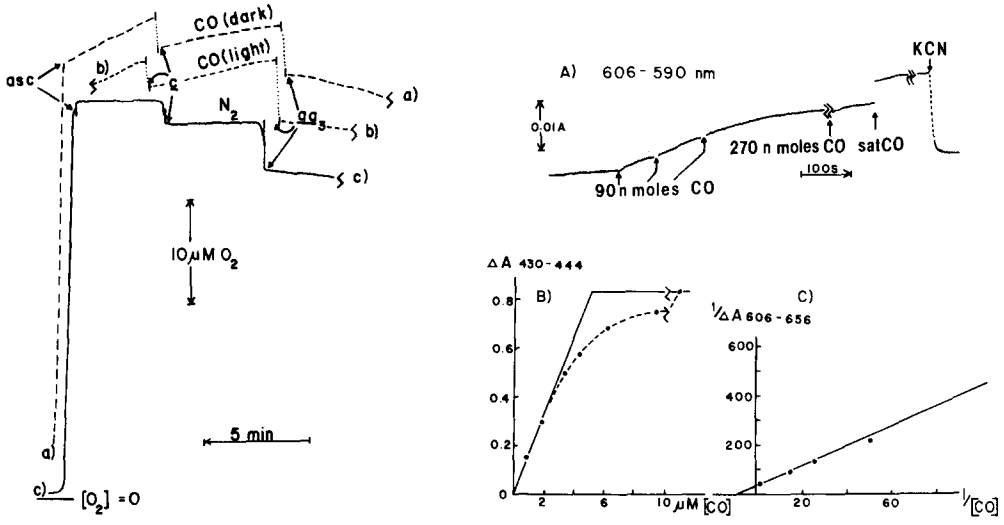
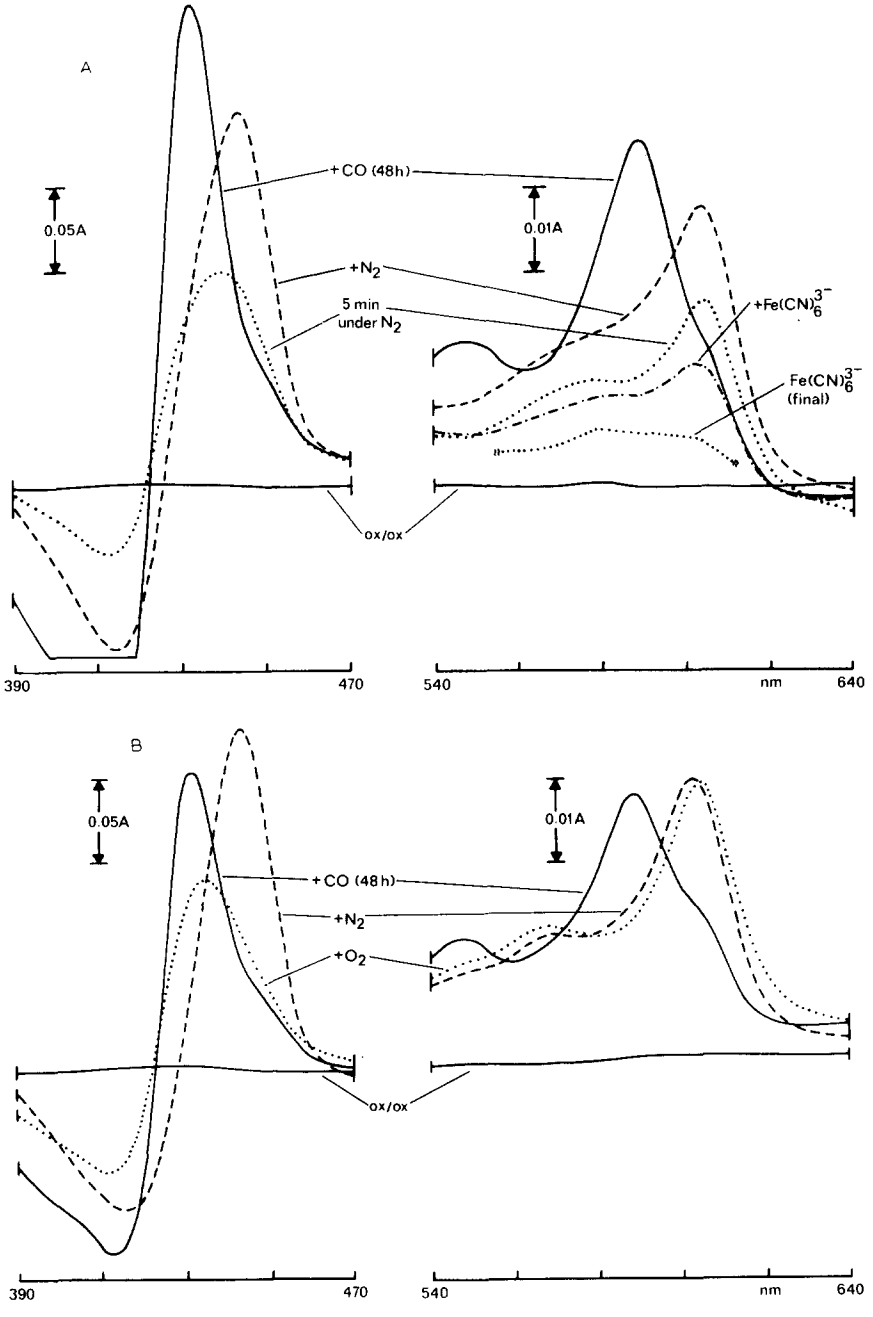


Fig. 4. Polarographic measurements of CO oxidation by oxygen catalysed by cytochrome aa_3. An oxygen electrode vessel (final volume 4.2 ml) was filled with 50 mM sodium phosphate 0.25% Tween-80 medium at pH 7.4 that had been purged by bubbling either CO (traces (a) and (b), - - - - - -) or nitrogen (trace (c), ———) until the O_2 level was approx. 20% atmospheric (about 40 μM). Under these conditions the CO concentration is approx. 0.9 mM. Oxygen uptake * at 30°C is followed using 4.4 μM cytochrome aa_3 (the preparation of Kuboyama et al. [16]) before and after additions of cytochrome *c* (c) and of the usual reductant, 7 mM ascorbate (asc). (a) - - - - - -, respiration rate in the dark **; (b) - - - - - -, respiration rate in the light ***; (c) ———, reaction in the absence of CO. Notes: * Time proceeds from left to right. ** The reaction prior to ascorbate addition was completely inhibited by 1 mM cyanide and partially inhibited by 10 mM formate (not shown). The oxygen electrode vessel was kept dark by enclosing it in several layers of black cloth. *** Experiments with 44 nM cytochrome aa_3 and ascorbate showed a 4-fold increase in rate in the light, compared to the rate in the dark and in presence of CO. The transparent oxygen electrode vessel was illuminated with room light (two 100-W fluorescent tubes at a distance of 1.0—1.5 m).

Fig. 5. Rate and extent of formation of the 607 nm species compared with the titration behaviour obtained on adding carbon monoxide to fully reduced cytochrome aa_3. (A) Time course of the reaction of CO with aerobic resting enzyme to form the 607 nm compound. 5 μM cytochrome aa_3 (prepared according to Kuboyama et al. [16]) in 50 mM sodium phosphate 0.5% Tween-80 (pH 7.4) was treated with successive additions of CO-saturated buffer as shown. After maximal formation was obtained by saturating the cuvette with bubbled CO reversal of the reaction is induced by addition of 1.6 mM KCN (cf. Ref. 8). (B) Titration of fully reduced enzyme with carbon monoxide, showing the near-stoichiometric binding. 5.8 μM cytochrome aa_3 (prepared according to Kuboyama et al. [16]) was dissolved in buffer, as in (A), reduced with sodium dithionite, and then treated with successive amounts of CO-saturated dithionite-treated buffer; measurements were made at 430—444 nm in the Aminco DW-2 instrument at 30°C, pH 7.4. (C) A double reciprocal plot of the extent of the aerobic spectroscopic response of ferric cytochrome aa_3 to additions of CO, under conditions identical with those in Fig. 5A. The reciprocal of ΔA, the difference in absorbance between 606 and 656 nm, was plotted as shown, and the resulting curve indicates a much lower 'apparent affinity' for CO than that shown in (B).

combination reaction with ferrous enzyme, i.e. 90 $M^{-1} \cdot s^{-1}$ compared with 80 000 $M^{-1} \cdot s^{-1}$ [21]. The amount of CO required (with the enzyme prepared according to Kuboyama et al. [16]) for half maximal formation of 607 nm compound, about 125 μM (Fig. 5C), is also much greater than that required for the liganding of ferrous enzyme, about 0.4 μM (Fig. 5B). The extent of 607 nm compound formation in the aerobic steady state is however clearly a function of CO concentration (Fig. 5C). For reasons of this kind, it was suggested that the 607 nm complex is actually a new form of CO complex, perhaps with the

associated Cu_U atom in a different redox state [8,9]; similarly, it was possible that compound C formation at low temperatures involved rebinding of the CO molecule photodissociated when compound A (oxyferrocytochrome a_3) is produced [6,22]. On the other hand, the 607 nm compound does not appear to be photosensitive (Greenwood, C., personal communication), and nor does com-



pound C (Barlow, C.H. and Chance, B., personal communication). Nor does the 607 nm compound show any modified EPR spectrum which might be expected if the Cu_U atom is oxidized (Seiter, C., personal communication) and likewise compound C shows only the EPR signal of Cu_D [10]. The experiments in Fig. 6 were therefore carried out to probe the role of CO in formation of this compound. After incubation for 48 h under CO, the mixed-valence CO complex is formed as described by Greenwood et al. [3] and shown in the continuous spectra in Fig. 6 A and B. Evacuation of the Thunberg cuvette, followed by flushing with nitrogen, induces the appearance of the dashed line spectra (Fig. 6, A and B). In this spectrum (presumably that of cytochrome $a^{3+}a_3^{2+}$) the characteristic single Soret peak of ferrocytochrome a_3 is seen at 445 nm, but the sharp 603 nm α-band is not the same as that obtained by the difference method (e.g. cytochrome $a^{2+}a_3^{2+}$ minus cytochrome $a^{2+}a_3^{3+}$) using reduced enzyme [23,24]. A slow decay of this species to give some ferrocytochrome a (dotted spectrum, Fig. 6A) is not rapidly accelerated by ferricyanide (Fig. 6A) but a related transition is rapidly achieved on admission of air (dotted spectrum, Fig. 6B). The latter induces an immediate collapse and blue shift of the Soret band together with a red shift of the α-peak and an intensification of the β-band. This O_2-induced species is spectrally indistinguishable from the 607 nm compound usually obtained in the presence of both O_2 and CO (Fig. 1) and may also be identical with the similar low temperature compound C of Chance and coworkers [2,6,22]. Evidently it does not always contain the elements of carbon monoxide, as the spectrum of its immediate precursor (Fig. 6) shows no sign of the presence either of the mixed-valence CO complex or of fully reduced carbon monoxide cytochrome aa_3. One of these two species should have been seen had any CO remained in the Thunberg cuvette.

Discussion

The cycling of the oxidase between the mixed-valence and 607 nm forms (Figs. 1 and 3) strongly suggests a steady-state role for the latter in the oxida-

Fig. 6. Formation and reactivity of CO-free mixed-valence cytochrome aa_3. (A) Stability and reactivity of the mixed-valence enzyme with ferricyanide. 3.75 μM cytochrome *c* oxidase (bovine heart) (prepared according to Kuboyama et al. [16]) in 50 mM sodium phosphate buffer plus 0.25% Tween-80 (pH 7.4) was incubated in a Thunberg tube with attached cuvette under 100% CO gas at 4°C for two days. The reference sample (oxidized) was incubated aerobically, and is identical in all cases. (a) ———, (ox/ox), oxidized enzyme in both the sample and reference cuvettes; (b) ———, CO-incubated enzyme (mixed-valence complex), in the sample cuvette; (c) - - - - - -, the CO-incubated enzyme sample was evacuated twice and flushed each time with nitrogen to give the CO-free mixed-valence species; (d) · · · · · ·, the sample after standing 5 min, and flushed a third time with N_2 gas; (e) · — · — ·, after the addition of a few crystals of $K_3Fe(CN)_6$ to the sample; (f) · · · · · ·, after 7 min in the presence of ferricyanide, showing the very slow oxidation of the mixed-valence form, even in the absence of CO. The Aminco DW-2 spectrophotometer was scanned at 2 nm · s^{-1}, with the Soret region checked again after recording the visible region absorption. Experiments at 30°C. (B) Reaction of the ligand-free mixed-valence cytochrome aa_3 with oxygen to form the 607 nm species. 3.75 μM cytochrome *c* oxidase (prepared according to Kuboyama et al. [16]) in 50 mM sodium phosphate buffer plus 0.25% Tween-80 (pH 7.4) was incubated in a Thunberg tube under 100% CO gas at 4°C for two days as in (A). The reference sample (oxidized 'resting' enzyme) was incubated aerobically. (a) ———, (ox/ox), oxidized vs. oxidized enzyme; (b) ———, CO-incubated enzyme (mixed-valence complex), in the sample cuvette; (c) - - - - - -, the CO-incubated enzyme sample was evacuated twice and flushed with N_2 to give the presumed ligand-free mixed-valence form (this sample remained stable for up to 300 s); (d) · · · · · ·, the sample shown in (c) was treated with air to form the 607 nm species shown. The Aminco DW-2 spectrophotometer was scanned at 2 nm · s^{-1} (traces (a)—(c)) or 10 nm · s^{-1} (trace (d)). Experiments at 30°C.

tion of CO. Under the same conditions, oxygen is being taken up (Fig. 4) and CO is converted to CO_2 [12,13], a process which ceases as the spectrum changes to that of the mixed-valence CO complex [12]. The level of 607 nm compound in the steady state is dependent on CO concentration (Fig. 5).

Like many substrates of other enzymes that give Michaelis complexes with a low turnover, carbon monoxide acts as an inhibitor towards the more effective substrate systems such as ascorbate plus cytochrome *c*. It is unusual in that it acts competitively not towards ferrocytochrome *c* but towards oxygen, the terminal acceptor. This effect is understandable if oxygen has two binding or reaction sites at each ferrocytochrome a_3 centre, the first being the ferrocytochrome a_3 iron, and the second a group to which the 'activated' O_2 is transferred, perhaps the EPR-undetectable copper atom. If cytochrome a_3 remains reduced after this initial transfer step, it will provide a binding site to which CO may return and then be oxidized by the adjacent 'activated' O_2.

If the sequence of reactions monitored by Chance and coworkers [2,6] is represented as in Eqn. 1, then the slow 'CO-oxidizing' pathway may be schematically indicated as in Eqn. 2.

$$\begin{array}{ccccc} a_3^{2+}COCu^{+} & & & & a_3^{3+}Cu^{2+} \\ \text{CO}\uparrow\downarrow & & & & \uparrow \\ a_3^{2+}Cu^{+} & \xrightarrow{O_2} & a_3^{2+}O_2Cu^{+} & \longrightarrow & a_3^{2+}Cu^{2+}O_2^{-} \\ \text{(mixed-valence enzyme)} & & \text{(Comp. A)} & & \text{(Comp. C)} \end{array} \qquad (1)$$

$$a_3^{2+}Cu^{2+}O_2^{-} \longrightarrow a_3^{2+}CuO^{+} \longrightarrow a_3^{2+}Cu+ \qquad (2)$$

(Comp. C) CO CO_2 ? CO CO_2 (mixed-valence enzyme)

Although CO seems to 'steer' the formation of the 607 nm compound (Eqn. 1) by preserving the enzyme in its mixed-valence state (Eqn. 2) and not allowing decay to a ferric ground state, contrary to previous suggestions [7,20] it appears not to be an intrinsic part of the 607 nm compound itself. The ligand, if any, that stabilizes ferrocytochrome a_3 in compound C must be a group on the enzyme itself, perhaps an interhaem bridging group [25] such as the imidazolate ion which has also been proposed as a link between iron and EPR-undetectable Cu atom [26,27].

The fully reduced enzyme, cytochrome $a^{2+}a_3^{2+}$, does not give rise to a stable 607 nm species on reaction with oxygen [2,22], although transient forms with absorption bands in this region are seen both at room temperature [5] and in frozen solutions [9]. It is under these conditions that a new EPR (Cu) signal has recently been seen [11] clearly distinguishable from that of the EPR-detectable Cu atom. Yet neither compound C itself [10] nor the steady-state 607 nm compound (Seiter, C., personal communication) show any such new Cu signal. The complex indicated as '$Cu^{2+}O_2^{-}$' in Eqns. 1 and 2 may therefore be a diamagnetic species; or this type of model may be wrong. But whatever its detailed molecular structure, compound C, the semi-stable product of the reaction of oxygen with the mixed-valence oxidase, does seem to be a 'waiting' form of the enzyme in which the cytochrome a_3 haem is protected from

further oxidation until more reducing equivalents are made available. This can occur either by reduction of cytochrome *a* and the EPR detectable copper or, more slowly, by interaction with a molecule of CO.

The idea of the 607 nm intermediate as essentially identical with compound C [2] and a 'normal' product of the reaction between mixed-valence cytochrome *c* oxidase and dioxygen is now closer to the concepts of Greenwood et al. [3] and Chance and coworkers [4,6]. It differs from the former in assigning a ferrous rather than a ferric state to the cytochrome a_3 haem, and from the latter in assigning the 607 nm peak to the ferrous haem rather than to a hitherto invisible blue copper species.

Acknowledgements

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