Effects of Inhibitory Ligands on the Aerobic Carbon Monoxide Complex of Cytochrome *c* Oxidase

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1. In the presence of both CO and O₂, ox heart cytochrome *c* oxidase forms a 607 nm-peak intermediate distinct from both the cytochrome $a^{2+}a_3^{2+}$ CO and the cytochrome $a^{3+}a_3^{2+}$ CO ('mixed-valence') CO complexes. 2. This aerobic CO compound is stable towards ferricyanide addition, but decomposed on treatment with ferric cytochrome *a*₃ ligands such as formate, cyanide and azide. 3. Addition of formate or cyanide induces transfer of the reducing equivalent to cytochrome *a*, but addition of azide gives rise to a complex with α -peak at 598 nm, not identical with any azide complex of the free enzyme, but possibly a cytochrome a_3^{2+} NO complex produced by oxidative attack of partially reduced O₂ on the azide. 4. The results support the idea that although the initial reaction of oxygen is with cytochrome a_3^{2+} , the next step is not an oxidation of the ferrous cytochrome *a*₃, but a transfer of O₂ to a neighbouring group, such as Cu⁺, to give Cu²⁺O₂⁻ or similar complexes. 5. The aerobic CO complex is then identified as $a^{3+}a_3^{2+}$ COCu²⁺O₂⁻; a similar compound ('Compound C') is formed by photolysis of $a^{3+}a_3^{2+}$ CO (the 'mixed-valence' CO complex) in the presence of oxygen at low temperatures.

The CO complex of reduced, anaerobic, cytochrome *c* oxidase (cytochrome *a*₃) has an α -band maximum at 590 nm (Keilin & Hartree, 1939). Another type of complex, dependent on the presence of CO but formed under aerobic conditions, exists with an α -band maximum between 606 and 607 nm (Chance *et al.*, 1975; Nicholls, 1978). An intermediate of this type can be formed either by illumination and oxygenation of the 'mixed-valence' CO complex (Greenwood *et al.*, 1974) at low temperatures, or by saturating an aerobic solution of isolated cytochrome *aa*₃ with CO (Nicholls, 1978).

Both haem *a* and a copper atom seem to be associated with the site of oxygen binding, or attack, on cytochrome *aa*₃. Binding of CO to form the classical complex with α -peak at 590 nm apparently requires both metal centres to be reduced (Wilson & Miyata, 1977), although this point is disputed (Anderson *et al.*, 1976). Cytochrome *a*, and the associated e.p.r.-detectable copper atom, can be either reduced or oxidized without much effect on the 590 nm-peak species (Nicholls, 1963). The oxidation pathways of the fully reduced ($a^{2+}a_3^{2+}$) and 'mixed-valence' ($a^{3+}a_3^{2+}$) forms after photolysis of CO and admixture of O₂ seem to be different. The fully reduced enzyme gives rise first to 'oxyferro' enzyme* (Chance *et al.*, 1975) and ultimately to

oxidized (or the low-spin 'oxyferri') ferric enzyme (Gibson & Greenwood, 1963; Clore & Chance, 1978a), with only transient formation of a complex with 607 nm peak (Gibson & Greenwood, 1965); but the 'mixed-valence' species gives rise first to oxyferroenzyme and then to a complex with a 607–609 nm peak or 'Compound C', both at low temperatures (Chance *et al.*, 1975; Clore & Chance, 1978b) and at room temperature (Greenwood *et al.*, 1974). Although the latter authors identify the product as a low-spin oxyferricytochrome *aa*₃, it is clear from the published spectra that, unlike the corresponding classical low-spin ferric compound with maximal absorbance at 585 nm (Sekuzu *et al.*, 1959), the compound found by Greenwood *et al.* (1974) has a large contribution above 605 nm.

Chance *et al.* (1975) have assumed that this type of intermediate, 'Compound C', represents either a 'peroxy' form of the oxidase, presumably stabilized because the necessary reducing equivalents provided by the cytochrome *a* plus e.p.r.-detectable copper system are lacking in the mixed-valence enzyme, or a form of the enzyme in which the cytochrome *a*₃ haem

cytochrome *a*₃ with O₂ seen at low temperatures (Chance *et al.*, 1975), whereas 'oxyferri' refers to the presumably low-spin ferric forms of cytochrome *a*₃ which persist after reaction of reduced enzyme with O₂ at room temperature (Sekuzu *et al.*, 1959). The prefix 'oxy' in 'oxyferri' only indicates the method of formation and implies nothing about structure.

* The terms 'oxygenated' or 'oxy' applied to cytochrome *c* oxidase are ambiguous. In this paper 'oxyferro' refers to the unstable and reversible initial reaction product of

remains ferrous but the e.p.r.-undetectable copper has been oxidized to a blue Type I Cu (Malmström *et al.*, 1975) state (Chance *et al.*, 1978). Neither model can account for the appearance of an essentially stable intermediate of this kind in the presence of CO and O₂ at room temperature (Nicholls, 1978, 1979a). Yet classical observations on the interaction between O₂ and CO in biological systems (Fenn & Cobb, 1932; Tzagoloff & Wharton, 1965) might have suggested the existence of such an intermediate. CO, in the presence of cytochrome *aa*₃ and O₂, is metabolized, presumably to CO₂ (Tzagoloff & Wharton, 1965). If the two gases were simply competing for a common binding site, ferrous cytochrome *a*₃ iron, the oxidation of one by the other would be difficult to understand. Only if an intermediate existed containing components of both species would such a process be expected. The 'aerobic CO complex' with 607 nm α -peak does have some of the characteristics required of such an

intermediate (Nicholls, 1979b). The experiments described in the present paper were designed to probe some largely spectroscopic aspects of its behaviour and, if possible, to clarify its relationship to 'Compound C' of Chance *et al.* (1975).

Materials and Methods

Ox heart cytochrome oxidase (cytochrome *aa*₃) was prepared by the method of Kuboyama *et al.* (1972), and stored at -75°C in 100 mM-sodium/potassium phosphate (pH 7.4)/0.25% Tween 80 solution as described by Nicholls & Hildebrandt (1978). The spectral properties were as given by Nicholls & Hildebrandt (1978), with very little unreducible enzyme (420 nm peak in dithionite-reduced or anaerobic enzyme), at least 45% of the haems capable of binding CO in the fully reduced enzyme, and a maximal catalytic-centre activity at 30°C in the storage medium of not less than 200 s⁻¹

Fig. 1. Effect of ferricyanide and O₂ on the 607 nm-peak CO compound

The CO complex was formed by bubbling CO through an aerobic sample of cytochrome *aa*₃ as described in the Materials and Methods section. The reference cuvette contained untreated enzyme (5.7 μ M-cytochrome *aa*₃). Spectra: — (a), oxidized versus oxidized baseline; — (b), plus CO versus oxidized; ···· (c), a few crystals of K₃Fe(CN)₆ were added to the sample cuvette from (b); ---- (d), the sample cuvette from (c) was aerated vigorously in front of a 200 W projector lamp as described in the Materials and Methods section. Cuvettes contained 2.6 ml samples of ox heart cytochrome *c* oxidase in 50 mM-sodium phosphate (pH 7.4)/0.5% Tween at 30°C. The Soret region is not shown because of the excess of ferricyanide present in (c) and (d).

(equivalents of cytochrome *c* per cytochrome *aa*₃ per second) provided that the enzyme is activated by a few minutes incubation with 0.1% asolectin (soybean phospholipids from Associated Concentrates, Long Island, NY, U.S.A.) and reductant, and 0.1% asolectin is added to the assay mixture (polarographic assay with ascorbate plus *NNN'*-tetramethyl-*p*-phenylenediamine as substrate, with various amounts of horse heart cytochrome *c*).

CO was from Niagara Welders Co. (Union Carbide Canada, Toronto, Ont., Canada). Ox hearts (beef steer) were purchased from Comfort and Tylee (St. Ann's, Ontario, Canada). Cytochrome *c* (type VI, horse heart), sodium ascorbate and *NNN'*-tetramethyl-*p*-phenylenediamine dihydrochloride were from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Sodium azide, sodium formate, potassium cyanide, potassium ferricyanide and sodium dithionite were from J. T. Baker Co. or from BDH (Poole, Dorset, U.K.).

Spectrophotometry was carried out with an Aminco DW-2 instrument. Catalytic activity was measured with a Yellow Springs Instruments Clark-type oxygen electrode coupled to a suitable polarizing box and 10mV recorder. Protein concentration was determined by the method of Gornall *et al.* (1949);

cytochrome *aa*₃ as isolated usually contained 8–9 μmol of haem *a*/g of protein, based on an absorption coefficient, $A_{605} - A_{630}$ (reduced minus oxidized) of $27\text{mm}^{-1}\cdot\text{cm}^{-1}$ for a two-haem cytochrome *aa*₃ unit (equivalent to a value of 13.5mm^{-1} per equivalent of haem *a*).

Photodissociation of CO intermediates was achieved by illuminating the cuvette or other vessel within 3cm of the lens of a Kodak projector lamp system (150W Q-I lamp).

Results

Fig. 1 shows the spectrum in the visible region of the 'aerobic CO' complex induced by saturating a previously aerobic solution of cytochrome *c* oxidase with CO. Titration of CO-saturated solutions prepared at 25°C into known amounts of myoglobin showed them to contain 0.85mm-CO (cf. the value of 1.2mm reported by Wilson & Miyata, 1977). At the same time, oxygen-electrode measurements at 30°C showed that the oxygen concentration was decreased nearly tenfold, from 240 to 30 μM . As previously reported (Nicholls, 1978), a complex with a characteristic 'liganded ferrous' spectrum (Lemberg, 1969) is obtained, with an α -band close to 606.5nm and a

Fig. 2. Action of formate on the 607 nm-peak CO compound

(a) Kinetics of formate decomposition of the 607 nm-peak CO complex compared with formate binding to resting ferric enzyme. In trace (a) (—), 5.6 μM -cytochrome *aa*₃ was treated aerobically with CO to give approx. 60% full formation of CO complex, and 16mM-sodium formate was then added at the indicated point. In trace (b) (---), a control experiment was carried out in the absence of CO. Conditions were as in Fig. 1. Dual-wavelength measurements were carried out at 433–413 nm. (b) Spectra of the 607 nm-peak CO compound and of the product with formate. The CO complex was prepared aerobically as in Fig. 1 (—); 90mM-sodium formate was then added and the sample scanned immediately (---). A final spectrum was obtained after the sample cuvette had stood at 30°C for 16 min (---). Conditions were as in Figs. 1 and 2(a).

Fig. 3. Comparison between the reaction products obtained with formate from the ferric cytochrome *c* oxidase and from the 607 nm-peak CO derivative

In each cuvette 5.6 μ M-cytochrome *aa*₃ was present in 50 mM-sodium phosphate (pH 7.4)/0.5% Tween 80 at 30°C. (a) —, Oxidized versus oxidized baseline; (b) —, 16 mM-sodium formate added to sample cuvette; (c) ----, CO added to sample cuvette, followed by 16 mM-sodium formate to both cuvettes.

Fig. 4. Effect of cyanide on the 607 nm-peak CO compound

The CO complex was prepared aerobically as before (—); 1.9 mM-KCN was then added and the sample scanned immediately (----). A final spectrum was obtained after the sample cuvette had stood at 30°C for 25 min (····). Cuvettes contained 2.6 ml samples of 5.6 μ M-cytochrome *c* oxidase in 50 mM-sodium phosphate/0.5% Tween 80 at 30°C. The reference cuvette contained oxidized enzyme.

β -band at 565nm. The reducing equivalent(s) probably required may derive from the oxidation of CO to CO₂ (Tzagoloff & Wharton, 1965). This 'aerobic CO complex' is not the 'mixed-valence' CO complex, formed reductively when the oxidized enzyme is placed under a CO atmosphere in fully anaerobic conditions (Greenwood *et al.*, 1974; Nicholls, 1979b), although its spectrum is qualitatively similar to the spectrum of that complex and shifted 16nm to the red. Nor is it a form of ferrous cytochrome *a*, as ferricyanide has no effect on it (Fig. 1). Re-aeration of the system in the light (see the Materials and Methods section) destroys the compound with 607nm peak and gives rise to the 'oxyferri' enzyme (Sekuzu *et al.*, 1959). In the experiment shown in Fig. 1, the difference absorption coefficient, measured at 606–656nm, was about 7mm⁻¹. Titration with increasing amounts of CO

suggests that this represents at least 60% of the maximal change possible. As CO cannot form complexes with ferric haems, and as the absorption spectrum of the aerobic CO complex is analogous to that of the mixed-valence CO complex, as well as involving the disappearance of the 655nm band of ferric cytochrome *a*₃ (Fig. 1), the new complex thus appears to be a ferrous cytochrome *a*₃ species; it is, however, vulnerable to decomposition by ligands specific for the ferric cytochrome *a*₃ haem. It was previously shown that the 607nm-peak species is not formed from cyanocytochrome *aa*₃ (Nicholls, 1978). Fig. 2 shows that formate added after formation of the aerobic CO complex induces its decomposition. A decrease in absorbance at 433–413nm (Fig. 2a) indicates either a low-to-high-spin transition in the ferric state (Nicholls *et al.*, 1976) or a ferrous to ferric change at the cytochrome *a*₃ haem. CO causes

Fig. 5. Spectra of the 607nm-peak aerobic CO complex and its reaction products with formate, azide and cyanide. Spectra of 5.6 μ M-cytochrome *aa*₃ were obtained in the visible region in the presence of K₃Fe(CN)₆. (a) —, Oxidized versus oxidized baseline; (b) —, plus CO; (c) ····, after addition of 16mM-sodium formate; (d) ----, after addition of 0.77mM-sodium azide; (e) ----, after addition of 0.4mM-potassium cyanide (sample (c) in reference cuvette). Ferricyanide at 10mg/ml was added in samples (b), (c) and (e), and at 3mg/ml in sample (d). Other conditions were as in Fig. 4.

formation of the new CO complex (Nicholls, 1978), as shown in trace (a); subsequent addition of formate (trace a) induces its decomposition, with the appearance of the same formate complex of oxidized enzyme as that which is formed directly by untreated enzyme (trace b). Fig. 2(b) shows the spectra obtained during this process. The CO-induced species is rapidly decomposed by formate, and this process is followed by a slower change in the Soret region, to give the final spectrum. The last step is probably a low-to-high-spin state change accompanying formate binding by ferric cytochrome a_3 that had previously not reacted. The disappearance of the CO-induced spectrum is also accompanied by the appearance of reduced cytochrome a with absorption bands at 605 and 445 nm.

Fig. 3 attempts to disentangle the two overall phenomena involved, namely the binding of formate to ferric cytochrome a_3 and the reduction of cytochrome a . The reaction of formate with oxidized enzyme in the absence of CO gives rise to blue shifts in both Soret and visible regions of the spectrum (cf. Nicholls, 1976). If this change is 'subtracted' from the process in the CO-containing cuvette by adding formate to the reference cuvette, it can be seen that the product obtained by addition of formate to the aerobic CO species is indeed ferrocycytochrome a , distinguished in the Soret region by its broad double peak, by the loss of an oxidized species with Soret band at 428 nm, and in the visible region by the appearance of a reduced peak at 605 nm without an appreciable β -band contribution (Nicholls *et al.*,

Fig. 6. Effect of azide on ferric cytochrome aa_3 and on the 607 nm-peak CO complex

(a) Dual-wavelength measurements at 433–413 nm. Addition of 0.8 mM- NaN_3 induces an apparent low-to-high-spin state transition, whereas 80 mM- NaN_3 produces an opposite effect. (b) As in (a), but in the presence of 0.8 mM-CO. (c) Split-beam measurements in the Soret region. The spectra shown are the oxidized versus oxidized baseline (—), the products obtained with 0.8 mM- NaN_3 (· · · ·) and 120 mM- NaN_3 (—), and the products obtained with CO (----) and with CO plus 0.8 mM- NaN_3 (— · —).

1976). Moreover, unlike the starting complex with 607 nm peak (Fig. 1), this product is sensitive to ferricyanide (see also Fig. 5 below).

Cyanide has an effect on the 607 nm-peak complex analogous to that of formate. As cyanide binding to ferric enzyme involves a high-to-low-spin spectral change (van Buuren *et al.*, 1972), the changes are less easy to follow in the Soret region, but the spectra obtained are shown in Fig. 4 (cf. Fig. 2*b*). A rapid collapse of the 607 nm-peak is accompanied by a slight blue shift in the Soret region and the appearance of shoulders at 445 nm and 605 nm. This is followed by the slow development of the full ferric cyanide spectrum without further redox changes (25 min trace in Fig. 4).

When analogous experiments are carried out in the presence of ferricyanide (Fig. 5), only the decomposition of the 607 nm-peak compound and the appropriate 'spin-state' changes are seen. Formate now induces a visible spectrum identical with that obtained directly (Fig. 3), the difference between the cyanide and formate products is that expected from the difference between their ferric products (Nicholls *et al.*, 1976), and azide, the third ligand examined, also produces a spectrum like that obtained with ferric enzyme (Wever *et al.*, 1973). Unlike the effects of the other two (typical high- and low-spin) ligands, however, the effect of azide in the absence of oxidant cannot be simply analysed in terms of: (a) formation of ferric cytochrome *a*₃ and ferrous cytochrome *a*

from (liganded) ferrous cytochrome *a*₃ and ferric cytochrome *a*; and (b) binding of the added ligand to the ferric cytochrome *a*₃ produced.

Fig. 6 shows the results obtained in the Soret region (cf. Fig. 2). As shown by Wever *et al.* (1973), azide addition has opposite effects at low (0.8 mM) and high (80 mM) concentrations; the former (a concentration expected to bind over 90% of active enzyme, as estimated from *K*_i values of 40–50 μM under the same conditions), induces a slight 'low-to-high-spin' transition, whereas the latter (a concentration with no further qualitative effect on catalysis) induces a change in the opposite direction (Fig. 6*a*).

When added to a cuvette containing the aerobic CO complex, azide, in the absence of ferricyanide, only induces a partial disappearance of the 433–413 nm absorbance (Fig. 6*b*); further additions of azide do not alter this situation. The resulting difference spectrum in the Soret region is unlike the spectra with azide at either low or high concentrations, but closely resembles the original CO-induced spectrum blue-shifted by 3 or 4 nm (Fig. 6*c*). In addition, azide, unlike cyanide, does not completely block CO-induced changes if added before CO treatment commences (Fig. 7). The azide–ferric complex is susceptible to attack by cyanide, in a reaction more rapid than that of cyanide with resting ferric enzyme (van Buuren *et al.*, 1972). It is also sensitive to attack by CO, which, over a period of about ½ h, induces a Soret spectral shift analogous

Fig. 7. Effect of CO on the ferric cytochrome *aa*₃-azide complex

Cytochrome *aa*₃ (5.6 μM) as in Fig. 4 was treated with 0.8 mM- NaN_3 (· · · ·). Saturation of the solution with CO gave the indicated spectra after 5, 15 and 30 min (—). Subsequent addition of ferricyanide partially altered the spectrum in the visible region (----).

to that seen on adding azide to the aerobic CO complex (Fig. 7, left-hand side) and the appearance of a spectrum in the visible region with α - and β -bands at 598 and 562 nm respectively (Fig. 7, right-hand side). Like the aerobic CO species, and unlike ferrous cytochrome *a*, this species is not destroyed by ferricyanide (dashed line, Fig. 7).

Fig. 8 compares the CO-induced and CO-plus-azide-induced spectra in more detail in the visible

region. Although in the presence of ferricyanide the product obtained by adding azide to the 607 nm-peak compound (Fig. 5) is identical with the azide complex with ferric enzyme (Fig. 8, line - - - -), the spectrum obtained in the absence of ferricyanide on adding azide to both cuvettes (Fig. 8, line - - - -) is certainly not identifiable with that of ferrous cytochrome *a* (cf. Fig. 5). Like the original species (Fig. 1), this compound is itself insensitive to ferricyanide. Its spectrum

Fig. 8. Effect of azide on the 607 nm-peak complex

Cytochrome aa_3 ($5.6 \mu\text{M}$) was present in each cuvette as in Fig. 4. Spectra were obtained in the visible region. (a) —, Oxidized versus oxidized baseline; (b) —, CO bubbled through sample cuvette (the 607 nm-peak species); (c) ····, spectrum obtained after addition of 0.8 mM-azide; (d) - - - -, spectrum of the azide complex of fully oxidized enzyme; (e) - - - -, product of azide reaction with 607 nm-peak complex versus oxidized azide complex [i.e. (c) minus (d)].

Table 1. Comparison between difference spectra of cytochrome aa_3 CO-induced complexes obtained under anaerobic ('mixed-valence'), aerobic and aerobic (plus azide) conditions

The reference cuvette contained oxidized enzyme in all cases ('resting' ferric spectrum). Millimolar absorption coefficients for the reduced minus oxidized species at the indicated wavelength are shown in parentheses. The medium contained 50 mM-sodium phosphate, pH 7.4, with 0.5% Tween 80, at 30°C. (See the Materials and Methods section.)

Complex (formal composition)	Visible-region peaks				Soret-region peaks			
	α -peak (nm) (A_{mM})	β -peak (nm) (A_{mM})	Maximum (nm) (A_{mM})	Minimum (nm) (A_{mM})	α -peak (nm) (A_{mM})	β -peak (nm) (A_{mM})	Maximum (nm) (A_{mM})	Minimum (nm) (A_{mM})
'Mixed-valence' anaerobic CO complex	$a^{3+}a_3^{2+}\text{CO}$	589 (10.0)	546 (2.4)	430 (67)	412 (5)			
Aerobic 607 nm CO complex	$a^{3+}a_3^{3+}(\text{CO})$	607 (7.8)*	566 (2.0)†	438 (24)†	414 (33)*			
Aerobic 607 nm CO complex plus azide	$a^{3+}a_3^{3+}(\text{CO}, \text{N}_3^-)$	598 (4.5)*	563 (2.4)*	435 (26)*	413 (29)*			

* Maximal value achieved.

† Referred to maximal value of aerobic CO-complex α -peak.

closely resembles those of the anaerobic 'mixed-valence' CO complex and of the aerobic 607 nm-peak compound, with α - and β -bands located almost exactly midway between the corresponding bands of the two CO-induced species. Table 1 summarizes the band positions and maximal difference absorbances seen for the three species in these and similar experiments.

As reduction of cytochrome *a* does not accompany the observed reaction of azide with the 607 nm-peak complex, it is likely that all three species listed in Table 1 are liganded forms of ferrous cytochrome a_3 . The mixed-valence CO complex is presumably to be written as $a_3\text{Fe}^{2+}\text{COCu}^+$, although even in this case the redox state of the associated copper is uncertain (Greenwood *et al.*, 1974; Anderson *et al.*, 1976; Wilson & Miyata, 1977). The nature of the other two intermediates and their significance for an understanding of the catalytic mechanism are discussed below.

Discussion

Chance *et al.* (1975, 1978) have suggested that Compound C, produced by illumination and oxygenation of the mixed-valence cytochrome $a^3+a_3^{2+}\text{CO}$ complex at low temperature, is either a 'peroxy' form of cytochrome a_3 or a 'blue' (Type I) form of the associated (e.p.r.-undetectable) copper (eqn. 1):

The similarity of the room-temperature spectrum of the 607 nm-peak aerobic CO complex (Nicholls, 1978; Fig. 1 of the present paper) to that of the anaerobic mixed-valence complex shifted about 17 nm to the red, and the uniqueness of its formation only in the presence of both CO and oxygen, suggests, on the other hand, that CO or a closely related ligand is still associated with the cytochrome a_3 haem not only in the aerobic complex at room temperature but also in the low-temperature species produced photolytically. Whether the two are identical is uncertain; if the mixed-valence species is indeed a form containing two reducing equivalents ($\text{Cu}_D^{2+}a^3+a_3^{2+}\text{COCu}_U^+$), then several other oxidation states preserving ferrous cytochrome a_3 are possible, e.g. $a_3^{2+}\text{COCu}^{2+}$, $a_3^{2+}\text{COCu}^{3+}$, $a_3^{2+}\text{COCu}^{2+}\text{O}_2^{2-}$, and $a_3^{2+}\text{COCu}^{2+}\text{O}_2^-$.

Although Compound C cannot therefore represent a true intermediate in the normal oxygen reaction with cytochrome a_3 , its behaviour may give some clues to the real enzymic process. Instead of reaction (1) we should write (assuming that cytochrome *a*

remains ferric, and e.p.r.-detectable copper remains cupric, throughout the reaction sequence):

where (CO) represents the photodissociated CO molecule trapped at low temperature in the 'haem pocket' (Sharrock & Yonetani, 1977; Yoshikawa *et al.*, 1977). When the initially bound O_2 molecule has been reduced by the neighbouring Cu_U^+ atom (Cu_U = 'e.p.r.-undetectable' copper), the displaced CO molecule may be capable of rebinding to the cytochrome a_3 haem (eqn. 2b). Any subsequent reactions, including cytochrome a_3 oxidation and loss of CO, would be expected to be slow, especially at low temperatures (eqn. 2c). Thus, although the oxygen molecule may first bind to the cytochrome $a_3\text{Fe}^{2+}$ centre, to give the 'oxyferro' species, as is very strongly suggested by the spectrum and properties of Compound 'A' (Chance *et al.*, 1975; Clore & Chance, 1978a), the oxidation that follows may not begin with the cytochrome a_3 iron. The substrate-binding site (O_2 -carrying centre) would then be distinct from the catalytic site (electron-transferring centre). If the enzyme is fully reduced ($\text{Cu}_D^+a^2+a_3^{2+}\text{Cu}_U^+$), reaction (2b) will probably be followed by:

where Cu_U = e.p.r.-undetectable and Cu_D = e.p.r.-detectable copper species; and then a faster reaction of the peroxide or equivalent species with the protected cytochrome a_3 group, as in eqn. (2e), where Cu_U^{3+} = 'percupric copper' (cf. Malmström *et al.*, 1975) or cupric copper with an associated hydroxyl radical:

The species with 607 nm band is a transient form in the reaction of fully reduced enzyme with oxygen (Gibson & Greenwood, 1965; Clore & Chance, 1978a). Reaction (2e) must be much faster than reaction (2c). When reduced cytochrome *a* is present the final rather slower step involves its oxidation (eqn. 2f):

The ferric ligand reactions described in the present paper suggest that there is some kind of equilibrium between ferrocycytochrome a_3 in the

aerobic CO complex and ferrocyanochrome *a* (see also Nicholls, 1979a), according to eqn. (3) where the transfer of an electron to cytochrome *a* results in the release of CO into a 'pocket', as indicated by the parentheses:

As less cytochrome *a*²⁺ appears than cytochrome *a*₃²⁺CO disappears (see, e.g., Fig. 2b), the electron transferred to cytochrome *a* may be shared with Cu_D. The product from eqn. (3) may be trapped by the ligand, either cyanide or formate. However, if the ligand reacting with ferric cytochrome *a*₃ is subjected to oxidative attack, like azide, one may have a subsequent reaction involving the oxidizing equivalents associated with the Cu atom (eqn. 3), as in eqn. (4):

This reaction would then be analogous to that taking place when azide attacks catalase compound I (Nicholls, 1964; Nicholls & Schonbaum, 1963). The product in the latter case is the NO-ferrocatalase complex. In the present case, the complex with 598 nm peak (Fig. 8) is most simply explained as the NO-ferroenzyme (Wainio, 1955).

The reactions of ligands with the aerobic CO complex also give some clues about the otherwise unusual behaviour of the reduced cyanide complex, *a*²⁺*a*₃²⁺HCN, towards O₂ (Keilin & Hartree, 1939; Storey, 1970). Unlike the fully reduced CO complex, this species readily reacts with O₂, but the product is not fully oxidized, but is the mixed-valence form, cytochrome *a*²⁺*a*₃³⁺HCN. Displacement of HCN by O₂ (eqn. 5a) would be expected to be followed by return of HCN when the O₂ was transferred to Cu (eqn. 5b):

Attack of the O₂⁻ on the cyanoferrocyanochrome *a*₃ centre would then give rise to the inert *a*₃³⁺HCN species and a product at the level of peroxide (eqn. 5c):

The resulting complex would be unreactive, as reported (Nicholls *et al.*, 1972).

The stability of the aerobic CO complex to ferri-cyanide (Fig. 1) shows, however, that in the presence

of both O₂ and CO it can readily be regenerated if decomposed. It is therefore a candidate for the role of steady-state intermediate responsible for CO oxidation to CO₂ by the enzyme (Fenn & Cobb, 1932; Tzagoloff & Wharton, 1965). Its catalytic behaviour is described in more detail elsewhere (Nicholls, 1979b). But for its formation in the absence of other added reductants we may tentatively write:

Whether there is more than one form of the aerobic CO complex, with different ligand states of the copper atom, is not known. The present analysis, however, differs from that of Chance *et al.* (1975) in two key ways:

(a) the ligand responsible for the stabilization of the "607 nm species" ('Compound C') is CO itself, as with no other reducing system is an intermediate with 607 nm peak observed in the presence of O₂; and

(b) the persistence of ferrous cytochrome *a*₃ after attack by O₂ must imply that, although involved in the initial O₂-binding step, it is not involved in the initial electron transfer, which must be attributed to another nearby group, such as the e.p.r.-undetectable copper atom.

It may finally be noted that if such mixed CO/O₂ complexes exist, so may complexes with more than one molecule of oxygen, such as *a*₃²⁺O₂Cu²⁺O₂⁻. How stable such a form would be is unclear, but its existence might help to account for the reported multiplicity of 'oxyferri' species of the enzyme (Orri & King, 1976).

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