A New Carbon Monoxide-Induced Complex of Cytochrome *c* Oxidase

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Cytochrome *c* oxidase isolated from ox heart forms a complex in the presence of millimolar concentrations of CO with absorption bands at 606, 565 and 435 nm (difference spectrum), distinct from both ferrocycytochrome *a* and the classical 590 nm carbonmonoxyferrocycytochrome *a*₃. This species, which closely resembles Compound C, the derivative formed on photolysis and oxygenation of mixed-valence cytochrome *a*³⁺*a*₃²⁺CO, may represent a cytochrome *a*₃²⁺CO complex in which the associated ('invisible') copper is still oxidized.

The photochemical experiments of Warburg and Keilin, culminating in the direct observation of the CO complex of cytochrome *a*₃ (Keilin & Hartree, 1939), seemed to establish conclusively the existence of not more than two forms of this complex, the fully reduced form (*a*²⁺*a*₃²⁺CO) and the 'mixed-valence' form (*a*³⁺*a*₃²⁺CO), both with α -bands at 590 nm and γ -bands at 430 nm (see Wikström *et al.*, 1976). The 'mixed-valence' species, obtained by reoxidizing one haem and probably one copper atom in the fully reduced form, has been used to simplify the reaction sequence that takes place between reduced enzyme and oxygen (Greenwood *et al.*, 1974; Chance *et al.*, 1975).

The reaction between enzyme and CO has, however, always involved anomalies. Tzagoloff & Wharton (1965), confirming earlier physiological studies, found that cytochrome *c* oxidase (cytochrome *aa*₃) oxidizes CO to CO₂ in the presence of O₂. Moreover, of all the oxidase ligands available, CO is the only one that consistently induces reduction or autoreduction of the enzyme, a reaction that can be used to form the mixed-valence species directly (Greenwood *et al.*, 1974; Tzagoloff & Wharton, 1965), but that also complicates titrations (Wever *et al.*, 1977) and redox-potential measurements (Wilson & Miyata, 1977). Such titrations and potentiometry have nevertheless provided strong evidence that CO binding to form the classical photodissociable 590 nm complex involves the obligatory reduction of both cytochrome *a*₃ haem and the associated e.p.r.-invisible copper, according to eqn. (1):

The present paper describes experiments suggesting that at least one intermediate may occur in the process of eqn. (1), the existence of which can throw light on the nature of intermediates seen in the reaction with oxygen (Chance *et al.*, 1975).

Experimental

Ox heart cytochrome *c* oxidase (cytochrome *aa*₃) was prepared by the method of Kuboyama *et al.* (1972). Its spectral properties are also described by Nicholls & Hildebrandt (1978). Spectrophotometry was carried out with an Aminco DW-2 instrument, and photolysis achieved where necessary with a 200 W Kodak projector lamp and focusing lens. Purified CO was a Linde sample (Union Carbide Canada, Toronto, Ont., Canada). Similar results are obtained using BOC (London S.W. 19, U.K.) CO (P. Nicholls & J. M. Wrigglesworth, unpublished work). All reactions were carried out at 30°C. Cytochrome *aa*₃ concentration is obtained from $A_{605} - A_{630}$ (reduced minus oxidized) equal to $27 \text{ mm}^{-1} \cdot \text{cm}^{-1}$.

Results

If CO is bubbled through a solution of cytochrome oxidase for about 2 min, a 'saturated' solution is obtained of about 1.2 mM concentration (Wilson & Miyata, 1977). If such a solution is used as a sample, with untreated oxidase as reference, a difference spectrum is obtained with an α -band at 606–607 nm, a β -band at 565 nm and a Soret (γ) band at 435–436 nm. Such an α -peak might derive from a small amount of reduced cytochrome *a*, although its position can clearly be seen to lie slightly to the red side of the usual 604–605 nm reduced enzyme peak. But ferrocycytochrome *a* does not show any β -band, and its split Soret peak (442, 446 nm) is clearly distinct from that in the CO-induced spectrum. Fig. 1 shows the differences between the several complexes obtained.

Trace (e) shows the spectrum of the new complex. Upon reduction by sodium dithionite, it gives rise to the spectrum in trace (d), a species containing some ferricytochrome *a*₃ and some carbonmonoxyferrocycytochrome *a*₃. Eventually all the cytochrome *a*₃

Fig. 1. Difference spectra of three CO-induced cytochrome aa_3 derivatives

Approx. $3.7\ \mu\text{M}$ -cytochrome aa_3 was dissolved in 50 mM-sodium phosphate buffer, pH 7.4, containing 0.5% Tween-80, at 30°C , in both sample and reference cuvettes. The following additions were then made to the sample cuvette (2.7 ml final volume): (a) none ($a^{3+}a_3^{3+}$ minus $a^{3+}a_3^{3+}$); (b) dithionite, followed by CO, followed by ferricyanide reoxidation to give the 'mixed-valence state' ($a^{3+}a_3^{2+}\text{CO}$ minus $a^{3+}a_3^{3+}$); (c) dithionite, followed by CO, to give the fully reduced CO complex ($a^{2+}a_3^{2+}\text{CO}$ minus $a^{3+}a_3^{3+}$); (d) CO, followed by dithionite and scanned immediately, giving a partial formation of fully reduced CO complex ($a^{2+}a_3^{2+}\text{CO}/a^{2+}a_3^{3+}$ minus $a^{3+}a_3^{3+}$); this is the product obtained on reduction of the complex in (e), and on leaving (d) becomes identical with (c); (e) saturation with CO alone [$a^{3+}a_3^{3+}(\text{CO})$ minus $a^{3+}a_3^{3+}$]. Note the different scales in Soret and visible regions.

present is reduced and combines with CO (trace c). If this complex is reoxidized the mixed-valence species is obtained (trace b). The latter is clearly distinct from the complex formed initially, even though upon standing under anaerobic conditions, CO-saturated cytochrome c oxidase gradually gives rise to the mixed-valence form (Tzagoloff & Wharton, 1965).

Fig. 2 compares the spectrum of the new complex in the visible region with the spectra of the intermediates obtained after photolysis and oxygenation (Chance *et al.*, 1975; Greenwood *et al.*, 1974). Again the spectrum after bubbling CO (trace f) has an α -peak distinct from that of the fully reduced enzyme (partial trace e) as well as from the so-called 'oxy' (low-spin) species produced on aerating the fully reduced CO complex (trace d). It is, however, very similar to the intermediate obtained on illuminating and aerating the mixed-valence state, the 'Compound C' of Chance *et al.* (1975), shown in trace (c). Both the new complex [$a^{3+}a_3^{3+}(\text{CO})$] and

Compound C resemble the mixed-valence species ($a^{3+}a_3^{2+}\text{CO}$) whose spectrum is given in trace (b), but with both α - and β -bands shifted some 15 to 17 nm to the red. The extra 'shoulder' at 575–595 nm seen with Compound C may be due to residual cytochrome $a^{3+}a_3^{2+}\text{CO}$ or to low-spin ('oxy') cytochrome $a^{3+}a_3^{3+}$, both of which have absorption bands in this region. The major difference between Compound C and the new complex is that the former is unstable ($t_{1/2} \sim 20\text{s}$) and the latter stable ($t_{1/2} > 500\text{s}$) at 30°C .

Formation of the new CO complex requires 'free' ferricytochrome a_3 to be available. It is not formed from the cyanide complex of the enzyme. Unlike the usual CO complexes ($K_d \sim 0.3\ \mu\text{M}$) its formation requires a high concentration of CO ($K_d \sim 0.2\text{ mM}$). The spectrum is increased in intensity in presence of ferrocyanide, but under these conditions a transformation into the spectrum of ferrocycytochrome a (605, 445 nm) seems to occur. A similar spectrum, of decreased intensity, is seen with

Fig. 2. Difference spectra comparing several cytochrome aa_3 complexes in the visible region

Approx. $3.7 \mu\text{M}$ -cytochrome aa_3 was present in each cuvette, dissolved in 50mM-sodium phosphate, pH 7.4, plus 0.5% Tween-80 at 30°C . The following additions were then made to the sample cuvette: (a) none ($a^{3+}a_3^{3+}$ minus $a^{3+}a_3^{3+}$); (b) dithionite, followed by CO, followed by ferricyanide reoxidation to give the CO complex with peak indicated at 590 nm ($a^{3+}a_3^{2+}\text{CO}$ minus $a^{3+}a_3^{3+}$); (c) the complex obtained in trace (b) (mixed-valence state), illuminated and oxygenated; equivalent to 'Compound C' (Chance *et al.*, 1975); (d) dithionite, followed by CO, illuminated and oxygenated; equivalent to 'oxycytochrome aa_3 ' (Sekuzu *et al.*, 1959); (e) dithionite alone, to indicate position of fully reduced peak at 605 nm ($a^{2+}a_3^{2+}$ minus $a^{3+}a_3^{3+}$); (f) CO alone, bubbled to give approx. 1.2mM-solution, with peak indicated just below 607 nm [$a^{3+}a_3^{3+}(\text{CO})$ minus $a^{3+}a_3^{3+}$]. Note that trace (e) is displaced downwards; other traces may involve slight baseline shifts.

Keilin-Hartree submitochondrial particles. In the latter case, addition of small amounts of NADH or succinate increases the amount of 607nm complex present, and under anaerobic conditions the mixed-valence state ($a^{3+}a_3^{2+}\text{CO}$) finally forms.

Discussion

Why has the new complex not been seen before? The answer may lie in the close proximity of its α -peak to that of reduced cytochrome a , together with the strong classical evidence assigning a 590 nm peak to all forms of the cytochrome $a_3^{2+}\text{CO}$ complex (Keilin & Hartree, 1939; Wikström *et al.*, 1976). However, now that we know of the need for two-electron reduction in formation of the tight CO complexes (eqn. 1) the conceptual possibility of a

cytochrome $a_3^{2+}\text{COCu}^{2+}$ complex of low affinity may seem less unreasonable.

The state of the invisible copper in the complex is uncertain. The simplest formulation (eqn. 2) would place the Cu_V atom in the tervalent oxidation state:

The effects of small quantities of reducing agents, however, suggest that a single reduction step may be involved (eqn. 3):

In the absence of added reductants, the electron may derive from an endogenous donor (Wever *et al.*, 1977) or a second molecule of CO.

The 16 nm shift in the α -peak on oxidoreduction of the copper atom, implied by either eqn. (2) or eqn. (3), may be attributed either to CO–Cu interactions or to structural changes in the haem pocket. Using model compounds of haem *a*, Lemberg *et al.* (1961) have shown that the position of the α -band of CO complexes can lie between 588 and 607 nm. For example, the CO complex of pyridine haem *a* haemochromogen has a band at 589 nm, whereas that of the CO complex of 4-methylimidazole haem *a* haemochromogen lies at 605 nm. The CO complex of haem *a*-apomyoglobin has its α -band at 603 nm.

Whenever cytochrome *aa*₃–CO complexes are photolysed and react with oxygen, intermediates appear with absorption bands between 605 and 610 nm, either transitorily if fully reduced cytochrome *a*²⁺*a*₃²⁺CO is used as starting material (Gibson & Greenwood, 1965; Clore & Chance, 1978*a*) or as a relatively stable end product, below 0°C, if mixed-valence cytochrome *a*³⁺*a*₃²⁺CO is used as starting material (Chance *et al.*, 1975; Clore & Chance, 1978*b*). The present comparisons (Fig. 2) suggest that these intermediates do not involve peroxy forms or Type-I copper spectra, but reflect rebinding of the CO molecules released into the haem pocket immediately the O₂ molecule bound in Compound A [cytochrome (*a*)*a*₃²⁺O₂] has been reduced (Chance *et al.*, 1975). The unstable Compound C may then be related to the stable 607 nm CO complex according to a reaction sequence such as that of eqn. (4):

or

The spectrum of 'Compound C' thus reflects its relationship to the stable 607 nm CO complex. The oxygenated 'Compound A' is probably the only intermediate occurring in the reaction of oxygen with unliganded cytochrome *a*₃. The existence of the 607 nm CO complex, on the other hand, suggests a number of new lines of investigation concerning the analogous reaction with O₂.

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