Ligation and electronation states of cytochrome-c oxidase in relation to other oxidases and peroxidases

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Ligation states
Preparations of cytochrome-c oxidase (EC 1.9.3.1) isolated from bovine heart are usually heterogeneous. They can be considered as mixtures of two forms of the enzyme, 'slow' and 'fast', which differ considerably in their properties. Until recently preparations consisted mainly of 'slow' ('resting') oxidase. In this form haem $a_1$ is entirely high spin, reacts slowly with cyanide and is reduced slowly by dithionite. In addition, 'slow' oxidase shows a unique e.p.r. signal arising from the binuclear centre, the $g'=12'$ signal. Baker et al. [1] have shown that the appearance of the 'slow' form is prevented by maintaining a high pH (7.6–8.0) during the preparation of oxidase. 'Fast' oxidase as prepared can then be converted to 'slow' oxidase by incubation at low pH [1, 2]. 'Fast' oxidase lacks the $g'=12'$ e.p.r. signal and has a Soret absorption maximum that is red-shifted relative to that of 'slow' oxidase. This latter observation reflects the presence of a thermal mixture of the high spin and low spin forms of haem $a_3$. Haems $a$ and $a_3$ in fast oxidase are reduced essentially simultaneously by dithionite.

When turnover is started by the addition of reductant (e.g. ascorbate/N,N,N,N'-tetramethyl-2-p-phenylenediamine) to 'slow' oxidase under aerobic conditions Cu$_A$ and haem $a$ are initially fully-reduced. There is then a gradual conversion to the 'fast' form, with a time-course similar to that observed for the reduction of haem $a_1$ by the same reductant but under anaerobic conditions [3]. The 'slow' to 'fast' conversion can be monitored as (a) an increase in $\Delta A_{435-413 \text{ nm}}$, which reflects a change in haem $a_3$ from high spin to low spin [4]; and (b) a decrease in $\Delta A_{605-623 \text{ nm}}$, which reflects a decrease in the steady-state level of haem $a^{2+}$ [3].

The copper-containing laccases, isolated from the lacquer tree (Rhus vernicifera) and a fungus (Polyporus versicolor), show remarkably similar behaviour. Like cytochrome oxidase, these enzymes catalyse the four electron reduction of dioxygen to water using electrons donated by a variety of substrates [5, 6]. When turnover is started by addition of reductant (e.g. quinol) in the presence of oxygen a rapid bleaching of the blue colour of the laccase owing to reduction of the Type 1 Cu$^{2+}$ is observed. However, there is an overshoot in the level of reduction of this centre and the blue colour partially recovers as turnover proceeds [7]. The mechanism by which this occurs is largely understood. Laccase contains four copper ions in three distinct coordination environments [5]. The single Type 1 ion is the initial electron acceptor from the substrate, and a pair of anti-ferromagnetically-coupled Type 3 ions form a binuclear centre which is analogous to the Cu$_A$/haem $a_3$ binuclear centre in cytochrome oxidase and is at least partly the site of oxygen interaction. In addition, there is a single Type 2 ion which has high affinity for some anions, particularly $F^-$, which acts as an inhibitor. The simplest scheme places the Type 2 Cu$^{2+}$ on a linear pathway between the Type 1 Cu$^{2+}$ and the Type 3 pair, which acts as a two-electron acceptor. On isolation of the enzyme an OH$^-$ ion is bound to the Type 2 Cu$^{2+}$, and it is the elimination of this ion by reduction of the Type 2 Cu$^{2+}$ that is the initial rate-limiting step in the reduction of the Type 3 Cu$^{2+}$ pair [8].

In fungal laccase 15–65% of the molecules in a given preparation are found to have a F$^-$ ion bound to the Type 2 Cu$^{2+}$ instead of an OH$^-$ ion [9]. Both OH$^-$ and F$^-$ are displaced from the enzyme by reduction of Type 2 copper [5]. Hence, both OH$^-$ and F$^-$ are lost from the enzyme during rapid turnover or immediately after a cycle of reduction and re-oxidation, leaving the enzyme fully active [9, 10]. An exactly analogous phenomenon is observed with oxidase. During turnover [3] or after redox cycling ('pulsing' [11]) 'slow' oxidase is converted to the fully-active 'fast' form. Given this similarity between laccase and cytochrome oxidase it seems reasonable to suppose that there is an inhibitory ligand present in the 'slow' form of cytochrome oxidase as well. The recent finding that 'fast' enzyme ligated with formate, a known inhibitory ligand of oxidase [12], has properties that are the same as those of 'slow' oxidase [2, 13] adds further weight to this proposal, and, at the same time, gives us a valuable aid to understanding the nature of the 'slow' form. For instance, it is possible to perform equilibrium redox titrations of formate-ligated oxidase...
(in the presence of high concentrations of formate under anaerobic conditions) whereas this is not possible with 'slow' oxidase.

Kojima & Palmer [14] have published titration data for formate-ligated oxidase, where magnetic c.d. (m.c.d.) was used to monitor the redox poises of the two haems (see also [15]). A feature of the titration curves is a partial reversal in the reduction of haem $a_1$ as the potential decreases over the range 280–180 mV. This appears to result from co-operativity between Cux and haem $a_1$, combined with anti-co-operativity between haem $a$ and the binuclear centre. The net result of this is that the mid-point potential of the binuclear centre as a whole is close to that of Cux/haem $a$. Therefore, the model proposed by Bickar et al. [16] for 'slow' oxidase, in which the mid-point potential of the binuclear centre is much lower than that of Cux/haem $a$ is unlikely to be correct. Nevertheless, mid-point potentials for Cux in the presence of oxidized haem $a_1$ and for haem $a_1$ in the presence of oxidized Cux are significantly lowered by formate, indicating that the ligand cannot be displaced by a single electron, unlike the displacement of OH$^-$ or F$^-$ from laccase. This implies that the substrate with Cux/haem $a$ reduced and the binuclear centre oxidized might be particularly stable unless an excess of reductant is present, to rapidly donate a third electron to the enzyme whenever Cux (the presumed initial electron acceptor [17]) is transiently oxidized.

The identity of the ligand in 'slow' oxidase is not known. However, oxidase is similar to laccase in that an active turnover form reverts spontaneously to an inactive resting form without the addition of any obvious free ligand. Yeast cytochrome-c peroxidase also shows similar behaviour. In this case the enzyme as isolated contains a penta-co-ordinated high-spin ferric protohaem. However, upon aging, particularly at low pH, it is converted, by ligation by a weak field ligand (possibly H$_2$O), to an unreactive hexa-co-ordinated high-spin state [18]. This contrasts with low-spin plant peroxidases, e.g. horseradish paraperoxidase, which as isolated has cyanide bound to the haem iron. Once the cyanide is released, e.g. by addition of HgCl$_2$, and removed they remain high-spin [19]. It is clear then that the ligand in 'slow' oxidase either must always be present in buffer solutions or must copurify with the enzyme. Hydroxide (and bicarbonate as well) seem unlikely because the pH-dependency of the rate of conversion of 'fast' oxidase to 'slow' is the reverse of what would be expected. The possibility that it might be a $\mu$-oxo bridge between Cux and haem $a_1$ arising from an oxygen atom retained after turnover [20] appears to have been discounted by the failure to find any evidence for the retention of such an atom [21]. Scott et al. [22] have suggested that a sulphur-containing bridging ligand detected by Powers et al. [23] using x.e.a.f.s. might actually be a chloride ion. However, again this possibility appears to be specifically discounted by the finding that chloride-ligated oxidase, though possible, clearly does not have the same properties as 'slow' oxidase [2]. Given the similarity between formate-ligated and 'slow' oxidase we have suggested that a carboxylate-containing ligand might be involved. Yet another possibility is that a haem A farnesyl side-chain could be involved [24].

**Electronation states**

We have found that 'fast' cytochrome oxidase as isolated often contains partially-electronated forms of the enzyme that are 'stable' in the presence of dioxygen. There are three possibilities here: singly-electronated (which we term the E state, 'E' for electronated'); doubly-electronated (P state, 'P' for 'peroxy', using Wikström's notation [25]); and triply-electronated (F state, 'F' for 'ferryl' [25]) [2].

P appears to be an adduct with peroxide, and can be formed by adding H$_2$O$_2$ to the fully-oxidized 'fast' enzyme (O state). However, further reaction with P with H$_2$O$_2$ is possible to form F. In addition, electronation of F can occur to complete the 'catalatic' turnover of the enzyme and regenerate O.

The rate of this reaction appears to be peroxide-independent (but may involve superoxide generated in the reaction of H$_2$O$_2$ with P). This follows from the observation that the proportion of O present at steady state is negligible over a wide range of peroxide concentration ($0.2-2.0$ mM) F, which has a characteristic absorption peak (relative to the O state) at 580 nm, is formed quantitatively [26]. The mechanism by which peroxide reacts with P to form F is unknown, although it is clear that the rate of this reaction is pH-dependent, and that this may be the reason for the apparent pH-dependency of the spectrum of P (relative to O) reported by Vygodina & Konstantinov [26]. Addition of catalase after mixtures of P and F have been formed leads to the slow decay of both states [2, 26].

'Fast' oxidase catalyses a carbon monoxide-oxygenase cycle in which CO acts as a two-electron
donor directly to the binuclear centre to form P when oxygen is present [27]. The rate-limiting step in this cycle appears to be release of peroxide from the enzyme, and so P, which has a characteristic absorption maximum (relative to O) at 607 nm, is formed essentially quantitatively. A state, which appears identical to P, can also be quantitatively formed by the addition of ethyl peroxide to oxidase. In this case further reaction of ethyl peroxide with the P-like state occurs at a low rate, if at all [28].

It is clear that cytochrome oxidase can show peroxidatic activity and, indeed, Miki & Orii [29] have demonstrated proton translocation by reconstituted oxidase catalysing this reaction using cytochrome c as electron donor. However, there is a clear difference between the peroxidatic cycle of oxidase and that of peroxidases in that the product of the reaction of peroxidases with hydrogen peroxide is analogous to F rather than P, i.e. the O=O bond is cleaved and one oxygen atom is lost as H₂O while the other is retained in an oxyferryl complex. The source of the other electron needed for this reaction depends on the peroxidase in question, e.g. in horseradish peroxidase it is a π electron from the porphyrin ring [30]. When ferric yeast cytochrome-c peroxidase is exposed to H₂O₂ an oxyferryl complex in conjunction with a protein free radical is produced [31]. The reaction cycle may then be completed by sequential donation of two electrons from cytochrome c. Another mode of action, however, for cytochrome-c peroxidase is for the haem to be reduced by cytochrome c before the reaction with H₂O₂. In this case the oxyferryl complex is formed, but not the free radical [32]. The 'primed' reduced state is therefore analogous to the E state of cytochrome oxidase. An even clearer analogy can be drawn between oxidase and the dihaem cytochrome-c peroxidase from Pseudomonas aeruginosa where the reaction with H₂O₂ cannot take place unless a second, high-potential haem, which supplies the extra electron, is already reduced [33].

The E state of cytochrome oxidase has not been investigated until now. Our strategy for the generation of E is to add substoichiometric levels of reductant to the fully-oxidized enzyme in the presence of oxygen. If low levels of reductant are used then the number of double hits (forming P) is small compared with the number of single-hits (forming E). Fig. 1 shows the predicted yields of E and P (and F) for varying 'loads' of electrons. To obtain these theoretical electron loading curves we used the Poisson distribution, i.e. we made the assumption that the probability of electron donation to a given molecule is the same irrespective of its electronation state. If the probability of electron donation to E were less than that to O then the E:P ratio would be greater for a given electron load.

The lower spectrum shown in Fig. 2, which is mostly that of E (relative to O), was generated by

![Electrons added](image)

**Fig. 1**

Theoretical electron loading curves for cytochrome oxidase

O₂ → O → E → P → F → 2H₂O

oxidized 'electronated' 'peroxy' 'ferryl'

Fraction of oxidases receiving n electrons = \( \frac{1}{n!} \times e^{-x} \)

where x = Electrons added

Oxidases present

- E
- P
- F

**Fig. 2**

Flash-induced photoreduction (FIRE) of 'fast' and formate-ligated cytochrome-c oxidase

'Fast' oxidase was prepared and preincubated with catalase to remove P and F as described elsewhere [2]. Formate-ligated oxidase was prepared by incubating stock enzyme solution (200 μM, pH 8) with 90 mM formate for 1 h at 20°C. Oxidase was diluted to 6 μM in 0.2 M Bis-Trispropane sulphate, pH 8.0, containing 1 mM potassium EDTA and 16 μM PMS. A baseline scan was taken and the sample was then given a train of 50 flashes at 100 ms intervals. This was followed by a 2 s delay after which it was re-scanned. A white Xenon flash filtered with BG-1 filters was used [41].

![Flash-induced photoreduction](image)
using Flash-Induced photoreduction (FIRE) to produce the radical semiquinoid form of 5-methylphenazinium methosulphate (PMS), which then directly reduces the oxidase [34]. The electron load was estimated to be less than 0.3 e-/oxidase by reference to the upper spectrum in Fig. 2, which was produced in an identical experiment but using formate-ligated oxidase instead of 'fast' oxidase. This spectrum, as expected (see previous section), has the characteristics of the redox difference spectrum of haem a. Given the electron load, the amount of P produced through double hits is estimated to be less than 15% of the amount of E.

The principal difference between the spectrum of E and the spectra of P and F is the absorption maximum at 444 nm in the Soret region rather than 437 nm or 435 nm, which are maxima for P and F, respectively. E has some of the features of the redox difference spectrum of haem a: the distinct α-band at 606 nm; the peak in the Soret region; and a slight peak at about 520 nm. However, the trough in the Soret region has a minimum at 414 nm, whereas haem a has a minimum at 430 nm. In addition, E has a trough centred at 660 nm which is caused by the loss of the '655 nm' charge-transfer band of high-spin haem a₁ (which is present in O, but not P and F). One possible explanation is that the electron in E can only equilibrate between Cuᵢ and haem a, as suggested by Malmström and co-workers [35], but that the reduction of haem a triggers a change in haem a₁ from high-spin to low-spin. However, if this were the case then the height of the peaks at 444 nm and 606 nm should roughly correspond to those in the haem a spectrum obtained using formate-ligated oxidase, which they clearly do not.

A better demonstration of this point is shown in Fig. 3(a) where oxidase was again reduced using FIRE, but this time via cytochrome c. When high concentrations of an electron donor (0.5 mM potassium EDTA) are used together with PMS, rapid reduction of cytochrome c can be achieved (90% complete in <25 ms, 99% in <100 ms) whereas the rate of electron donation from reduced cytochrome c to oxidase is low because of the high ionic strength. This allows us to use the change in A₅₅₀ owing to cytochrome c to calibrate both the electron load and the rise in A₆₀₅ owing to the formation of E. The results agree with the experiments carried out in the absence of cytochrome c and give extinction coefficients for E (relative to O) in 'fast' and 'slow' oxidase (and formate-ligated oxidase, which is not shown) of about 8 and 16 mm⁻¹ cm⁻¹, respectively.

Fig. 3 shows that the rate of equilibration of the electron in E must be rapid. Once again we have reduced oxidase using FIRE via cytochrome c, but this time we have reduced the ionic strength so

We conclude that the electron in E can equilibrate throughout the enzyme and, in accord with the mid-point potentials measured under anaerobic conditions [36], is shared mostly between Cuᵢ and the two haems, while Cuᵢ remains essentially oxidized. The E state spectrum should, therefore, resemble the redox difference spectrum of haem a plus haem a₃. In addition, the '655 nm' charge-transfer band should be absent when either haem a₁ or Cuᵢ is reduced. However, the observed decrease in A₄₃₂ from the trough in the haem a redox spectrum, is too small. To explain this we must again assume that a spin-state change is induced in haem a₃ whenever haem a is reduced. The conversion of haem a₁ from high spin to low spin (e.g. by binding of cyanide) is characterized by a red-shift in the Soret band, which gives rise to a difference spectrum with a trough at 412 nm and a peak at about 432 nm.

Fig. 3(b) shows that the rate of equilibration of the electron in E must be rapid. Once again we have reduced oxidase using FIRE via cytochrome c, but this time we have reduced the ionic strength so
as to increase the rate of electron transfer from cytochrome c to oxidase. Unfortunately, under these conditions (50 mM EDTA) there is an additional (approx. 10% more) reduction of cytochrome c with a $t_{1/2}$ of about 50 ms. However, the yield after 25 ms is almost identical to that obtained with 0.5 M EDTA. It is clear that there is no overshoot in $A_{465}$ that might be caused by transient over-reduction of haem $\alpha$, even though the electron transfer from cytochrome c to oxidase is now much faster (complete in <40 ms). We conclude from these experiments that the rate of equilibration of the $E$ state must be at least 100 s$^{-1}$.

**The oxidase steady state**

To calculate the electron loading curves in Fig. 1 we assumed that the probability of electron donation to any given oxidase molecule was the same. This assumption is justified since we now know that Cu is almost completely oxidized in $E$ (although perhaps with a slightly lower mid-point potential than in O, P or F, because of the anti-co-operative interaction with haem $\alpha$ [36]). By assuming a constant rate of donation, the electron loading curves can be directly converted to theoretical time-courses for O, E, P and F during the approach to steady-state turnover. These time-courses apply as long as all intramolecular electron transfers and all reaction steps at the binuclear centre are fast in comparison with the rate of electron donation to the enzyme. It is clear then that O, E, P and F should be present at equal concentrations provided turnover is low. This is exactly what is seen for an analogous cycle of reactions, i.e. the cycle of $S$ states in the oxygen-evolving system of chloroplasts [37].

The $O$ state is included in the cycle of reactions shown in Fig. 1. This seems reasonable for cytochrome oxidase since the fully-reduced enzyme is rapidly and completely reoxidized by oxygen (provided excess reductant is not present) [38]. For laccase the situation is different. Here the equivalent of F in oxidase contains a bound oxygen radical [5]. However, rapid reduction of the bound radical cannot occur unless the Type-2 copper is first reduced. The enzyme then returns to the equivalent of the $E$ state rather than the $O$ state. Hence, when fully-reduced laccase is reoxidized by oxygen the intermediate with reduced Type-2 copper and a bound oxygen radical is rapidly formed [10]. In addition, when F$^-$, which inhibits the reduction of the Type-2 copper, is present, the steady-state level of the oxygen radical increases whereas a decrease would be expected if the cycle were analogous to that of cytochrome oxidase [39].

The oxidase steady state is often analysed by assuming that any increase in $A_{465}$ relative to that of oxidized enzyme simply represents reduction of haem $\alpha$. This is based on the notion that the rate of electron transfer from Cu/haem $\alpha$ to Cu/haem $\alpha_1$ is the rate-limiting step in the turnover of the enzyme. There are, however, at least three potential errors in this: (a) under low turnover conditions we expect 25% of the enzyme to be in the $E$ state and so some reduced haem $\alpha$ must always be present; (b) under the same conditions we also expect 25% of the enzyme to be in the $P$ state, which has a significant $A_{465}$; and (c) a single rate of intramolecular electron transfer to the binuclear centre has no meaning since the rate must depend on the electron state of the enzyme [40]. We anticipate that an approach to analysis of the oxidase steady state based on the arguments presented here, starting from low turnover conditions and then moving to more rapid turnover, may be more useful in determining the actual rate-limiting step (or steps) in the reaction cycle of cytochrome oxidase.

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