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Received 15 June 1992

What form of cytochrome *c* oxidase reacts with oxygen *in vivo*?

The nature of the immediate donor of reducing equivalents to molecular oxygen has been a puzzle for many years. It had appeared to be resolved in favour of a single haem a species, termed cytochrome a_3 by Keilin & Hartree [1], following their abandonment of the 'copper enzyme' model [2] in favour of a version of Warburg's active haem species [3]. Discovery of more than one prosthetic metal in the terminal oxidase (see Wikström *et al.* [4] for review) led to reappraisals of this simple classical picture. More elaborate models involved several centres in O_2 reduction, working in a concerted way. The most complex [5] required that at least three and probably all four metal groups participate in transferring four electrons to an O_2 molecule initially bound at or close to the cytochrome a_3 haem. But a recent consensus seems to have emerged in which the species reacting with oxygen *in vivo* is the doubly reduced binuclear cytochrome a_3Cu_B centre. Thus Sarti *et al.* [6,7] use a 'bipolar' model in which formation of the oxygen-reactive species involves transfer of two electrons from Cu_A and cytochrome *a* to the binuclear centre, to give a 'mixed valence' form in which the binuclear centre is fully reduced and cytochrome *a* and Cu_A are both oxidized. This is based upon the well-established fact that carbon monoxide binding requires reduction of both cytochrome a_3 and Cu_B [4]. Yet the redox states of the other components in the O_2-sensitive form are not settled by this finding, as CO is not an electron acceptor. Indeed, it acts as a weak electron donor to the binuclear centre. And Babcock & Wikström [8], in their recent lucid and magisterial review of cytochrome oxidase activity in the cell, allowed themselves a similar rather counter-intuitive model of the oxygen-utilizing reaction step, in which the fully reduced enzyme plays no part in the physiological reaction and the role of cytochrome *a*, the first component of the enzyme complex to be discovered [9], is diminished to one of electron transfer to the actual oxygen-reactive centre.

One of the major problems with this enzyme has been to decipher the concerted and co-operative way in which electrons are transferred and coupled to proton movement across the membrane in which it is embedded. Cytochromes *a* and a_3 are located upon the same subunit, contain the same prosthetic group, and interact strongly both spectrophotometrically and in chemical reactivity [4,8]. It is hard to believe that they will not both be implicated in the key oxygen-utilizing and energy-conserving steps.

It is now accepted that a copper atom (Cu_A) located upon the other redox subunit, subunit II, is the immediate electron acceptor in the eukaryotic enzyme from cytochrome *c* and provides the gateway to the centres in subunit I [10]. But the behaviour of cytochrome *a* has long been a puzzle [8]. Its classical distinction from cytochrome a_3 depended upon its reduction under conditions in which cytochrome a_3 is oxidized but liganded and electron transfer to oxygen is slow [1]. When electron transfer is initiated with *N,N,N',N'*-tetramethyl-*p*-phenylenediamine in the absence of cytochrome *c* the flux depends upon the steady state reduction level of cytochrome *a*, once a small fraction of cytochrome *a* associated with unreactive enzyme has been subtracted [11]. Under these conditions the cytochrome, rather than Cu_A, may be the electron entry point. But in the presence of cytochrome *c*, cytochrome *a* behaves differently, both in the isolated enzyme and in cytochrome *c* oxidase-containing vesicles. Its steady state is especially sensitive to ΔpH and rather insensitive to $\Delta\psi$ [12,13], and although turnover is always almost directly proportional to the level of reduced cytochrome *c*, the reduction level of cytochrome *a* does not change significantly as flux changes. This is seen when controlled respiration is released by addition of ionophores [13] or when steady state flux is increased by raising the reductant concentration and hence the percent reduction of cytochrome *c* [14]. Cytochrome *a* can be monitored equally well at 605 nm and at 445 nm [13,14] and its redox changes are thus distinguished from events at the binuclear centre, including apparent spin state and related changes during the steady state. As respiration rates do not track cytochrome *a* reduction, it is doubtful whether the rate-limiting step in the ordinary reaction of the enzyme involves electron transfer from this component. It follows that at least one potentially rate-limiting electron transfer event to the binuclear centre may be from a component that more closely tracks the cytochrome *c* redox level than cytochrome *a*. That component is likely to be Cu_A.

The binuclear centre can be reduced in two steps from the ground (ferric/cupric) state. If this happens before cytochrome *a* itself is reduced, although the O_2 reaction takes place at the same rate as with fully reduced enzyme [15], the following electron transfer steps are much slower than in the presence of reduced cytochrome *a*. The oxygenated complex of the enzyme (Com-

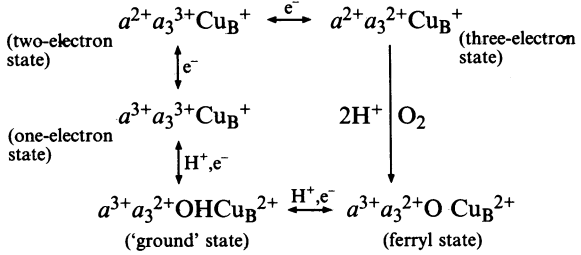
pound A) is detectable at room temperature under these conditions [16], and remains stable for several hundred microseconds [17], almost as if the enzyme were 'looking for' an extra reducing equivalent after the oxygen has bound. The reaction with fully reduced enzyme does not allow ready detection of the oxygenated form at room temperature [15]. Although a primary intermediate with a resonance Raman spectrum of an Fe–O_2 type is seen with a half-life of 20 μs [18], it is not clear that this is indeed Compound A [15,19]. It may be a species of a different kind. And it is transformed at a rate of between 25000 and 35000 s^{-1} into a form in which cytochrome *a* is oxidized [10,20].

The reduced binuclear centre itself (cytochrome a_3–Cu_B) contains a maximum of three available reducing equivalents, if ferrous iron can be oxidized to ferryl and cuprous copper to cupric. If nearby cytochrome *a* is also involved, three centres can now be oxidized almost simultaneously, cytochrome a_3 to a ferryl state, Cu_B to Cu^{2+}, and cytochrome *a* to a ferric state. It is therefore likely that the reaction steps reported for fully reduced enzyme by Greenwood & Gibson [5] are substantially correct for the enzyme *in vivo* [15]. The only significant contemporary modification of their scheme is the replacement of Cu_A by Cu_B as an essential component of the oxygen reduction process. Prior reduction of cytochrome *a* is preferred before reduction of cytochrome a_3 takes place to permit the O_2 reaction. At least one electron transferred to the binuclear centre will therefore not move *via* cytochrome *a*. No stable intermediates other than the initial oxygenated or related species and the 'final' ferryl enzyme are produced. The difficulty in identifying any intermediates with intact peroxy radicals (partially reduced forms with intact O–O bonds) in reactions of other haem proteins with peroxides makes this an unsurprising conclusion. Stable intermediates involve ferryl or similar Fe^{IV} states with radicals elsewhere, either on the porphyrin ring itself or on a neighbouring amino acid residue [21].

The 'mixed valence' (half-reduced) enzyme reacts with oxygen to produce a 607 nm form (Compound C) in high yield [15,17]. A similar species is produced when hydrogen peroxide is allowed to react with resting or pulsed enzyme samples [22]. What is its nature? Wikström [8,23] has identified it as a peroxy ('P') species, and produced a similar intermediate by a reverse reaction in intact mitochondria by adding ATP in the presence of ferricyanide [24]. The redox equilibria indicate that the latter species is two equivalents more oxidized than resting enzyme. If there is no oxidant loss during Compound C formation from the mixed valence state it too must be two equivalents more oxidized than the resting state, to which it decays rather slowly [25]. By analogy with other haem proteins we expect it to contain ferryl iron and a second oxidizing equivalent elsewhere in the molecule. It is too stable to be a simple peroxy complex [21], despite the arguments of Babcock *et al.* [26]. And it cannot normally be produced in the reaction between oxygen and reduced enzyme as its rate of formation is too slow. If Compound A converted at 4500 s^{-1} to give Compound C as part of a typical catalytic cycle, the steady state would contain a substantial population of both components. This is not observed. Although resonance Raman bands typical of ferryl and hydroxy intermediates are seen [27], these can be accommodated by the ferryl form proposed here, obtained from the three-electron reduced species, and from its immediate reduction product. The evidence of Ogura *et al.* [28] for bound O–O^- forms can be reinterpreted in terms of special states (e.g. hydrogen-bonded) of ferryl forms. Compound C production involves some substantial electron transfer events other than simple electron movement from cytochrome a_3 iron and Cu_B. These implicate the oxidase protein as well as the haem and copper atoms. As with the decays of peroxide compounds in peroxidases and catalases [21] such reactions can occur as side processes when the main pathway is blocked.

One argument used by Babcock & Wikström [8] to exclude cytochrome *a* from the usual steady-state turnover is the decreased proton pumping stoichiometry observed in the reaction of vesicular enzyme with oxygen [29]. Oliveberg *et al.* [29,30] report that 2.0–2.5 protons per oxygen move from the 'M' to the 'C' face of the membrane when the partial oxygen reaction takes place, compared with the expected 4.0 protons in the overall reaction. Their measurements required corrections for scalar protons taken up in the reaction ($\approx$ 1.0 per O_2) and possible vectorial protons moved in the 'wrong' direction by a small amount of reduced enzyme inserted in the membrane 'M' face outwards. It is skilful to have measured even the few protons reported; they may not be the complete tally for comparison with steady state measurements. It is comparatively easy for experiments carried out under less than ideal thermodynamic conditions to reveal substoichiometric values for H^+ translocation. This may be a consequence of reaction 'slip' in enzyme or nearby lipid bilayer. Moreover, corresponding experiments with partially reduced enzyme showed no translocated protons at all. These results therefore provide rather limited support for an oxidase model in which reduced cytochrome *a* causes a reaction short-circuit when present at the same time as the O_2 reaction.

All these difficulties can be removed if cytochrome *c* oxidase only reacts rapidly with oxygen in the triply reduced condition $a^{2+}Cu_B{}^{+}a_3{}^{2+}$ and if the first stable oxidation product of that reaction is the $a^{3+}a_3O^{2+}Cu_B{}^{2+}$ form, in which cytochrome a_3 is one equivalent above the ground (ferric) oxidized state. Reduction of the ferryl species via either cytochrome *a* or the other copper atom (Cu_A) will regenerate the ground oxidized state. In reforming the oxygen-reactive species, two successive reductions, via Cu_A and cytochrome *a*, then occur, followed by a final (Cu_A-dependent) step in which the a_3 centre goes reduced, as illustrated in the following reaction scheme:



There are some consequences for cellular respiration and energy conservation which may be almost opposite to the conclusions drawn by Babcock & Wikström [8]. Only when the redox potential and cytochrome *c* steady state reach a level permitting reduction of cytochrome *a* will fast electron transfer to O_2 occur. At low oxygen levels, its uptake will always be fast and energy conservation efficient. At high oxygen levels but low levels of mitochrondrial reducing power, O_2 uptake will be kinetically diminished as cytochrome *a* fails to be significantly reduced. This seems to make more intuitive sense than an energetically inefficient side reaction which the alternative model postulates as taking place at high cytochrome *a* reduction levels [8]. But of course only further experimentation will tell us which is right.

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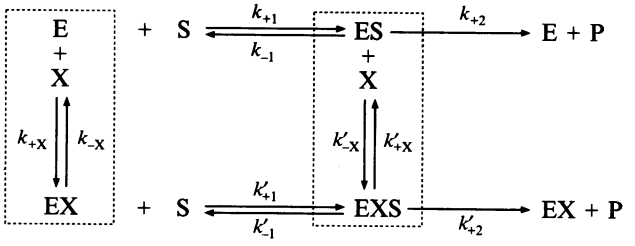
Received 6 July 1992

The steady-state rate equation for the general modifier mechanism of Botts and Morales when the quasi-equilibrium assumption for the binding of the modifier is made

Two recent contributions (Segel & Martin, 1988; Topham & Brocklehurst, 1992) deal with the derivation of the steady-state rate equation for the general modifier mechanism of Botts & Morales (1953), in which the quasi-equilibrium assumption for the binding of the modifier X to E and ES is made. This mechanism is shown in Scheme 1 where the broken lines denote the equilibria assumed and E, S, X and P are the free enzyme, the substrate, the modifier and the product, respectively. The considerations of these authors are based on the following two,



Scheme 1

different steady-state rate equations for the reactions in Scheme 1:

$$v = \left(\frac{k_{+2} + k'_{+2}\dfrac{[X]}{K'_X}}{1+\dfrac{[X]}{K'_X}} [E]_T[S] \right) \Bigg/ \left(K_m^s \frac{1+\dfrac{[X]}{K_X}}{1+\dfrac{[X]}{K'_X}} + [S] \right) \quad (1)$$

where:

$$K_m^s = \frac{k_{-1}}{k_{+1}} \left[\left(\frac{k_{-1}+k_{+2}}{k_{-1}} \right) k_{+1} + \left(\frac{k'_{-1}+k'_{+2}}{k'_{-1}} \right) k'_{+1} \frac{[X]}{K'_X} \right] \Bigg/ \left(k_{+1} + k'_{+1} \frac{[X]}{K_X} \right) \quad (2)$$

or

$$K_m^s = (k_{-1}+k_{+2})/k_{+1} \quad (3)$$

In eqns. (1–3), [S] and [X] are the concentrations of substrate and modifier, respectively, $[E]_T$ is the total enzyme concentration, K_X and K'_X are the dissociation constants of the complexes EX and EXS, respectively, i.e. $K_X = k_{-X}/k_{+X}$ and $K'_X = k'_{-X}/k_{+X}$. In the following, we show that eqns. (2) and (3) are erroneous and we give the correct expression for K_m^s in eqn. (1). To improve clarity, we label eqn. (2) as the equation of Topham & Brocklehurst and eqn. (3) as the equation of Segel & Martin.

The steady-state concentrations of the enzyme species in Scheme 1 may either be obtained by using the method of Cha (1968) or from the expressions obtained by the strict application of steady-state assumptions with the condition:

$$k_{+X}[X], k_{-X}, k'_{+X}[X], k'_{-X} \gg k_{+1}[S], k_{-1}, k_{+2}, k'_{+1}[S], k'_{-1}, k'_{+2} \quad (4)$$

The result is given by eqns. (5–8) in the work of Topham & Brocklehurst (1992). Dividing the above mentioned equations, (5) and (7) as well as (6) and (8), side by side, we have:

$$k_{+X}[X][E] = k_{-X}[EX] \quad (5)$$

$$k_{+X}[X][ES] = k'_{-X}[EXS] \quad (6)$$

Eqns. (5) and (6) show that condition (4) is necessary and sufficient for the quasi-equilibrium of the binding reactions of X to E and ES. From eqns. (6) and (8) of Topham & Brocklehurst (1992) and the equation:

$$v = k_{+2}[ES] + k'_{+2}[EXS]$$

one obtains, after some rearrangement, eqn. (1), in which K_m^s is given by:

$$K_m^s = \frac{k_{-1}}{k_{+1}} \left[\left(\frac{k_{-1}+k_{+2}}{k_{-1}} \right) k_{+1} + \left(\frac{k'_{-1}+k'_{+2}}{k_{-1}} \right) k_{+1} \frac{[X]}{K'_X} \right] \Bigg/ \left(k_{+1} + k'_{+1} \frac{[X]}{K'_X} \right) \quad (7)$$

instead of the eqn. of Topham & Brocklehurst. Only if the microscopic reversibility condition:

$$k_{+1}k'_{+X}k'_{-1}k_{-X} = k_{+X}k'_{+1}k'_{-X}k_{-1}$$