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The steady state behaviour of cytochrome *c* oxidase in proteoliposomes

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Electron transfer to oxygen catalysed by cytochrome *c* oxidase is accompanied by spectral changes at the binuclear a_3Cu_B centre, both in the soluble enzyme and in membranous systems, indicating spin or ligand state transitions of an iron that remains ferric. The other haem group, cytochrome *a*, does not change its spectral characteristics significantly during the steady state, but remains partially reduced until anaerobiosis. Cytochrome a_3 is fully oxidized in each of its major steady state forms, and reduced upon anaerobiosis to a single ferrous species. Although cytochrome *a* is normally the immediate electron donor to the binuclear centre, its redox state does not alter under conditions in which the flux through the enzyme is changing significantly. A second electron transfer pathway to the binuclear centre may therefore exist, possibly one in which direct reduction of the binuclear a_3Cu_B centre by Cu_A occurs. Both cytochrome *a* and Cu_A behave as simple electron transfer centres. The energy-conserving chemistry takes place at the binuclear centre in concert with the four-electron reduction of molecular oxygen.

Absorption spectroscopy; Cytochrome *c*; Cytochrome aa_3; Proteoliposomes, Spin state change

1. INTRODUCTION

Cytochrome *c* oxidase (ferrocytochrome *c*: oxygen oxidoreductase, EC 1.9.3.1), the terminal respiratory enzyme in all eukaryotes and some prokaryotes, delivers four electrons to oxygen in the overall reaction of Eq. (1):

$$4 \text{ cyt. } c^{2+} + O_2 + 4H^+ \rightarrow 4 \text{ cyt. } c^{3+} + 2H_2O \quad (1)$$

It is generally agreed that cytochrome *c* is not directly involved in the oxygen reaction. All four reducing equivalents must therefore reside on the enzyme transitorily. The enzyme form normally involved in reduction of oxygen, however, remains uncertain. Both fully reduced [1] and partially reduced [2] enzyme species can react with molecular oxygen. In the mitochondrial steady state the oxidase appears to occur largely in the $a^{3+}a_3{}^{3+}$ state with a small proportion in the $a^{2+}a_3{}^{3+}$ state [3,4]; but most such experiments have been carried out under conditions in which the oxidase is far from saturated with electrons. The low level of cytochrome *a* reduction is therefore not unexpected. Nothing substantial is known of the steady states of the two copper centres (but cf. [4,5]). Thus although the reaction is the most important step in energy conservation by aerobes, no consensus yet exists as to the pathway of electron transfer involved – either in the membrane-bound or detergent-solubilized forms of the enzyme [6,7]. Despite the greater antiquity of our general knowledge, it now lags behind that recently achieved in the study of photosynthetic electron transfer.

Copeland and coworkers [8–10] have suggested that two different conformational states of cytochrome *a* function during steady state turnover of the enzyme. Transitions between the two states are suggested to be involved in transmembranous proton pumping by this enzyme – the energy-conserving process coupled to oxygen reduction. Hill [11] as well as Han et al. [12] have developed a scheme for the initial reaction steps upon oxidation of fully reduced enzyme by oxygen. In this scheme, cytochrome *a* lies between the initial electron acceptor (the visible copper atom, Cu_A) and the terminal bimetallic centre (cytochrome a_3 Cu_B) that reacts with oxygen. The central position of cytochrome *a* in the scheme lends weight to the possibility of its involvement in the energy conservation process, as originally suggested by Wikström [13] and proposed again recently by Copeland [8]. More recently, Morgan and Wikström [4] have monitored the steady state behaviour of mitochondrial cytochromes *c* and *a*, concluding that the latter can behave as an intermediate whose reduction level reflects the flux through the enzyme. Reduction of the binuclear centre to a form that can react with oxygen requires the delivery of two electrons. Malmström [14] has proposed that two-electron reduction of the initially fully oxidized enzyme alters its conformation in such a way as to facilitate reduction of the a_3 Cu_B centre. Sherman et al. [9], using second-derivative spectroscopy, suggest that the form of cytochrome *a* present during the steady state is different from that at full

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Abbreviations: COV, cytochrome *c* oxidase-containing vesicles; DOPC, dioleylphosphatidyl-choline; DOPE, dioleylphosphatidylethanolamine; TMPD, *N,N,N′,N′*-tetramethyl-*p*-phenylenediamine; HEPES, *N*-(2-hydroxyethyl)-piperazine-*N′*-2-ethanesulphonate.

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reduction. If this were true, conclusions as to electron transfer pathways based upon experiments with fully reduced enzyme would be flawed. Understanding the enzyme's steady state is thus essential to unravel the complexities of its behaviour.

2. MATERIALS AND METHODS

Cytochrome *c* oxidase was isolated from beef heart according to the method of Kuboyama et al. [15], with Tween-80 substituting for Emasol, as before [16]. DOPC (dioleyl-phosphatidylcholine) and DOPE (dioleylphosphatidyl-ethanolamine) were products of Avanti Lipids (Alabaster, AL); sodium ascorbate, cytochrome *c* (type VI, horse heart), TMPD (*N,N,N′,N′*-tetramethyl-*p*-phenylenediamine) and valinomycin were from Sigma Chemical Co. (St. Louis, MO). Nigericin was from Calbiochem Corp (San Diego, CA).

Cytochrome *c* oxidase-containing vesicles (COV) were prepared as described previously (Wrigglesworth et al. [17]). 100 mg DOPE plus 100 mg DOPC were dissolved in chloroform, and the solvent was evaporated under nitrogen. The resulting lipid film was suspended in 5 ml 100 mM HEPES buffer, pH 7.4, with 1.5% sodium cholate, by vortexing; the suspension was sonicated for 7 min in pulsed mode at 30% duty cycle (Heat Systems, Ultrasonics W-375 sonicator) on ice under N_2, and centrifuged for 10 min at 20,000 × *g* to remove undispersed lipid and titanium particles. Cytochrome oxidase was added to a final concentration of 5μM cyt. aa_3 (50μL 500 μM (100 mg/ml) enzyme). Dialysis was then carried out at 4°C against 100 mM HEPES for 4 h, followed by 10 mM HEPES, 40 mM KCl, 50 mM sucrose pH 7.4 for 2 days (and 3 or 4 buffer changes). The final stock COV suspension contained ≈ 65 mM lipid and 5 μM cytochrome oxidase. Respiratory control ratios (respiration rates with valinomycin plus nigericin divided by the rates in absence of ionophores) varied from 7 to 10.

Absorption spectra were recorded using a Beckman DU-7 HS spectrophotometer interfaced with an Apple II-GS microcomputer. Steady state observations were made either in this system or in an Aminco DW-2 instrument linked to a Compaq 286 computer with Olis data acquisition hardware and software. Cytochrome aa_3 concentrations in COV were determined from difference spectra, fully reduced minus oxidized, using ΔE of 27 mM^{-1} at 605–630 nm. The ratio of outward-facing to total enzyme was determined by the ratio of the reduction at 605–630nm with ascorbate and cyt. *c* alone to that with TMPD present

3. RESULTS

Fig. 1 shows the steady states of cytochromes *c*, *a* and a_3 during aerobic oxidation of ascorbate by COV, in the controlled and uncontrolled (after addition of valinomycin and nigericin) states. Contributions of cytochromes *a* and a_3 are calculated using the relative extinctions in equations **(1)** and **(2)**. I assume that cytochrome *a* contributes 84% of the absorbance in the visible (a) region and 55% of that in the Soret region, with cytochrome a_3 contributing the remainder [18]. The higher than usual [3, 19, 20] contribution assumed for the Soret region reflects the shift in the Soret spectrum of oxidized cytochrome a_3 (see discussion of Fig. 3 below).

% redn at 605–630 nm =
= % redn cyt. *a** 0.84 + % redn cyt. a_3*0.16 (1)

% redn at 445–470 nm =
= % redn cyt. *a** 0.55 + % redn cyt. a_3*0.45 (2)

Using these relative contributions to the two absorbances it is found that cytochrome a_3 remains fully oxidized until the COV approach anaerobiosis, as with mitochondria [3] and isolated enzyme [19]. Addition of uncoupler changes the cytochrome *c* steady state from ≈ 12% reduced to ≈ 3% reduced. The corresponding cytochrome *a* steady state is almost unaffected by the big change in flux as controlling gradients are abolished.

If the ascorbate concentration is raised, the rate and steady state of cytochrome *c* increase proportionately (trace A, Fig. 2**i**). The flux is proportional to cytochrome *c* reduction over a wide range. This does not apply to cytochrome *a*. Its reduction reaches a maximum long before the enzyme has achieved maximal turnover (trace B, Fig. 2**i**). This applies in all the energy states of the COV. Fig. 2**ii** shows that the cyt. *a* reduction levels reach maxima in the controlled state (A), in the valinomycin-treated state (B), where ΔpH is present but $\Delta\Psi$ has been abolished, and in the uncontrolled state (C). Membrane energization exerts a two-fold action: (i) upon the equilibrium between cyt. *c* and cyt. *a* (E_o' of cyt. *a*), and, (ii) upon the maximal reduction of cyt. *a*, indicating control steps lying between cyt. *a* and the binuclear centre (Table I).

The spectrum of cytochrome aa_3 changes as the steady state continues. This can be conveniently monitored by using a relatively poor electron donor such as TMPD, rather than cytochrome *c*, as substrate. Nicholls and Hildebrandt [16] showed that the spectral change probably involved the bimetallic centre, and took place not only under uninhibited conditions but also in presence of low spin terminal inhibitors such as cyanide. Only if a ligand such as formate is present does the enzyme stay 'high-spin' throughout the steady state. Fig. 3 illustrates the phenomenon with COV. In the presence of ascorbate and TMPD, cytochrome *a* becomes progressively less reduced ($A_{445\,nm}$ declines) and the ferric haem (cyt. a_3) peak undergoes a red shift ($A_{415\,nm}$ declines and $A_{430\,nm}$ increases). Second derivative spectra (shown in the inset) are similar to those of Copeland [8]. The shift in the oxidized Soret peak (attributed to cyt. a_3) occurs at the same time as the decrease in cytochrome *a* reduction. On anaerobiosis both centres are fully reduced, but appropriate deconvolution shows that cytochrome *a* does not change its spectral form after cytochrome a_3 has become ferrous [18].

4. DISCUSSION

In cytochrome *c* oxidase-containing vesicles, cytochrome *a* responds to redox input from either cytochrome *c* or TMPD and to membrane energization, in

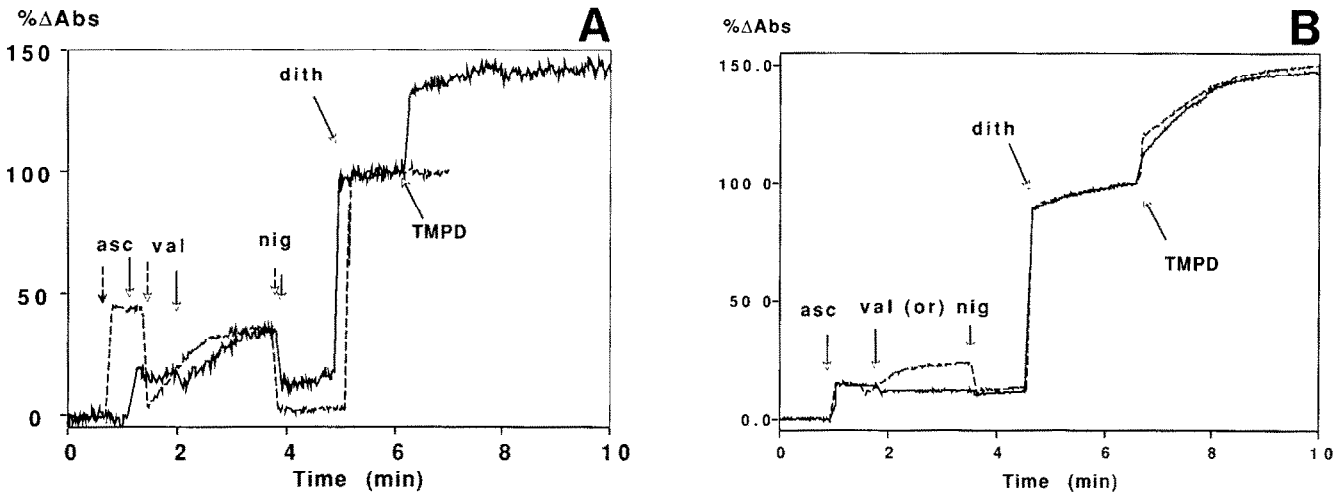

Fig. 1. Steady state reductions of cytochromes *c*, *a* and a_3 during the proteoliposomal steady state: effect of nigericin + valinomycin. Dialysed COV (containing 1.0 μM total, 0.65 μM externally-facing, cyt. aa_3) plus 1.0 μM cyt. *c* in 1.0 ml 2 mM HEPES, 8 mM KCl, 10 mM sucrose, 4 mM potassium phosphate pH 7.4 at 30°C. Respiration was initiated by addition of 20 mM ascorbate. (A) Reduction at 550–540 nm (dashed line) and 605–630 nm (continuous line). Valinomycin (3 μg) and nigericin (0.3 μg) were added at the points indicated to abolish the ΔpH and $\Delta\Psi$ gradients across the proteoliposomal membrane. Full reduction of outward-facing oxidase was achieved by addition of dithionite (dith) and of internally-facing oxidase by addition of TMPD. (B) Reduction at 445–470 nm (dashed and continuous lines). Valinomycin and then nigericin (dashed line) or nigericin and then valinomycin (continuous line) were added as in Fig. 1A. Full reduction of outward-facing oxidase was achieved by addition of dithionite (dith) and of internally-facing oxidase by addition of TMPD (fast and slow phases show reductions of internally-facing cytochromes *a* and a_3). N.B. Percentage reductions of the three cytochromes were estimated separately using Eqs. **(1)** and **(2)** for the two *a* haems and ΔE of 20 mM^{-1} for cytochrome *c* at 550–540 nm.

the form both of ΔpH and of $\Delta\Psi$, as shown by Gregory and Ferguson-Miller [21] and by Papa et al. [22]. Present and previous results show that it is not necessary to postulate spectral changes in reduced or oxidized cytochrome *a*, responding to the redox state of the binuclear centre. As cytochrome oxidase cycles through its various states cytochrome *a* remains spectrally constant as cytochrome a_3 varies. Nevertheless the cytochrome *a* redox potential is modulated both by reduction of the binuclear centre, and by membrane energization. Both processes decrease the attributed cytochrome *a* redox potential, from a value of $\approx$ +330 mV to values 60–100

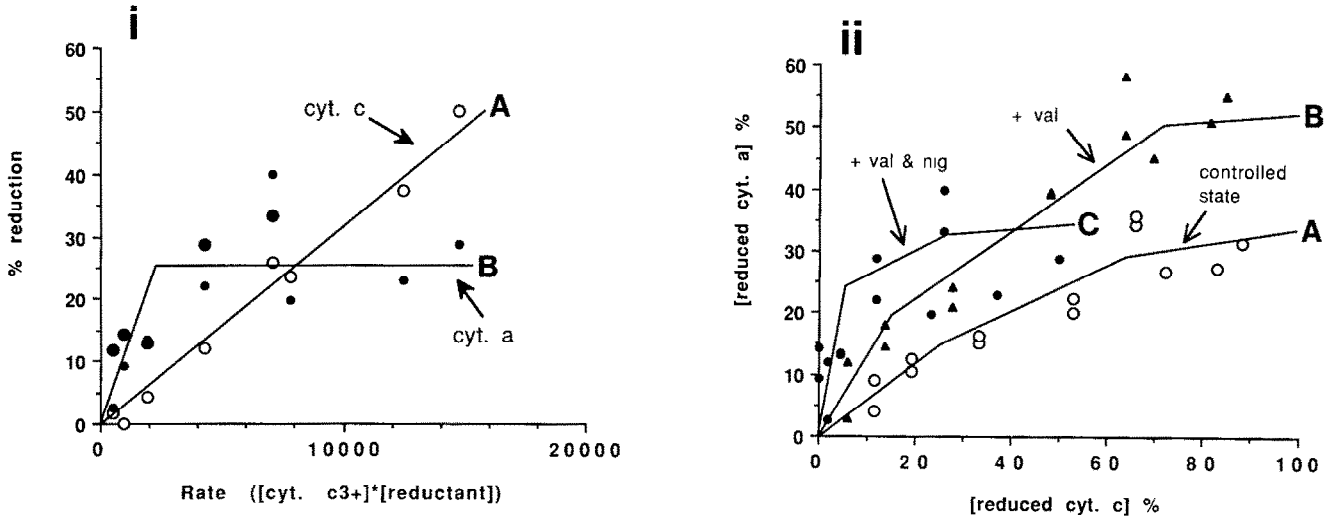

Fig. 2. The relationships between cyt. *c* steady state, cyt. *a* steady state, and respiration rate in proteoliposomes. (i) The percentage reductions of cyt. *c* and of cyt. *a* plotted as a function of rates of respiration in the presence of ionophores. N.B. Enzyme turnover is indicated as the product of the concentrations of ascorbate and ferricytochrome *c* (actual turnovers as measured polarographically varied from 10 to 70 sec^{-1} with RCRs of 7 to 10). (ii) Effect of ionophores on the steady states of cyt. *a* and *c*. ○. controlled, no ionophores; ▲: control by ΔpH alone (in the presence of 1 μg/ml valinomycin); ●: uncontrolled state (+0.3 μg/ml nigericin and 1 μg/ml valinomycin). Dialysed COV containing 0.43 μM total, 0.32 μM externally-facing, cyt. aa_3 plus 1.5 μM cyt. *c* in 1.0 ml 1 mM HEPES, 4 mM KCl, 5 mM sucrose, 45 mM potassium phosphate, pH 7.4, at 30°C. Respiration was initiated by addition of ascorbate (from 1 to 20 mM). Other conditions as in Fig. 1.

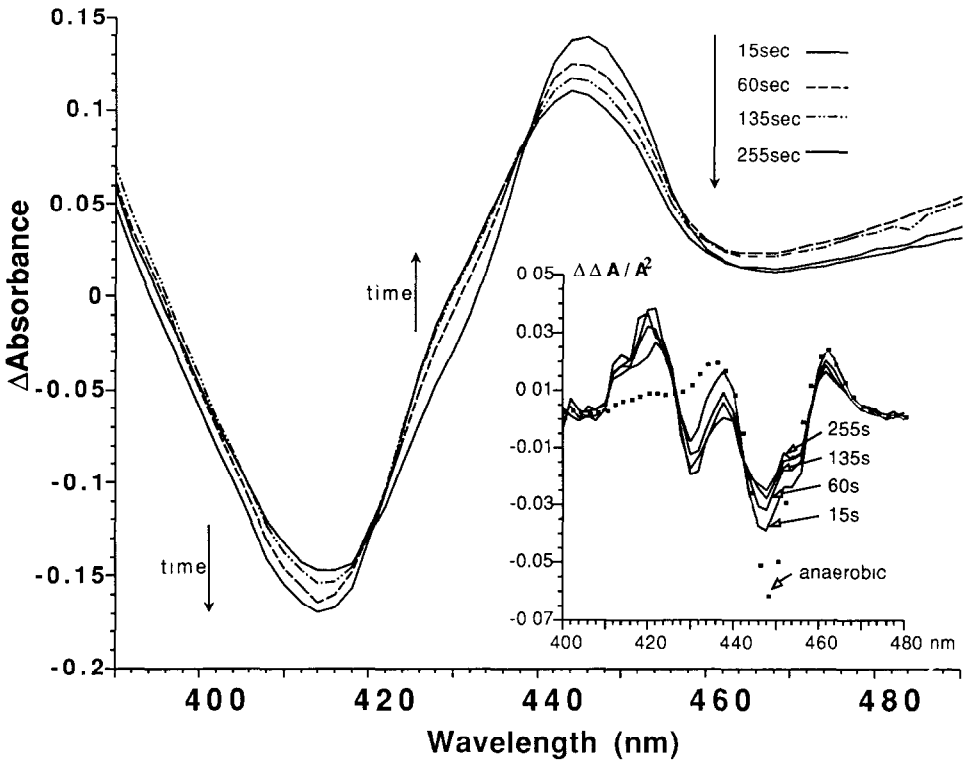

Fig. 3. Steady state difference spectra of vesicular cytochrome oxidase as a function of time during turnover: the shift in the Soret peak of oxidized cytochrome a_3. Dialysed COV (≈ 4.8 μM cyt. aa_3) in 10 mM HEPES, 40 mM KCl, 50 mM sucrose pH 7.4 plus 0.06% lauryl maltoside. Respiration was initiated by addition of 10.8 mM ascorbate plus 485 μM TMPD. Difference spectra were obtained in the Soret region after the indicated times. Note: Satisfactory spectra in the visible region cannot be obtained because of steady state production of $TMPD^+$ (Würster's Blue) in the dispersed membranes. Each spectrum represents the difference between the spectrum at the time indicated and that at time zero (before reductant addition). *Inset*: the inset (lower right) shows the corresponding 2nd derivative spectra obtained numerically using a 4 nm offset. The small closed squares show the 2nd derivative spectra for anaerobic COV (reduced-oxidized), on a five-fold reduced scale, for comparison.

mV more negative. In accordance with the 'neo-classical' analysis [19,20] the spectral effects accompanying these redox potential effects are minimal.

Ferric cytochrome a_3 exists in several different states which depend upon the states of cytochrome *a* and of the neighbouring Cu_B atom. Cytochrome *a* reduction induces at least one transition in the binuclear centre so that the ferric cytochrome a_3 spectrum is red-shifted. This change occurs in both free enzyme [16] and in COV (this paper). It may be related to similar spectral shifts that occur as the enzyme changes from a 'slow' to a 'fast' or 'pulsed' state [23]. It is sufficient to account for all spectral changes that occur during steady state turnover. Neither substantial reduction of the binuclear centre nor changes in the reduced cytochrome *a* spectrum are needed.

In the isolated enzyme, and in these cytochrome *c* oxidase-containing vesicles, the steady state reduction of cytochrome *a* reaches a maximum when the flux is still increasing. Somewhat different results have been obtained with intact rat liver mitochondria, where the steady state of cytochrome *a* in the absence of uncouplers was closer to that expected for a simple redox intermediate [4]. In the presence of FCCP, however, the reduction of cytochrome *a* was biphasic and suggested saturation at about 20% total reduction. In the presence of both azide and FCCP, over 90% reduction of cytochrome *a* was achieved long before a maximal rate was attained. Under uncoupled conditions, therefore, the mitochondrial and COV data are qualitatively consistent. Control patterns in coupled mitochondria, accessibility of reductant, and incomplete occupancy of cytochrome *c* binding sites on the mitochondrial oxidase may all contribute to complications (cf. 24). The steady

Table I

Reduction of cytochrome *a* by cytochrome *c* during the steady state of cytochrome *c* oxidase in COV

COV control state	% redn cyt. *c* @ 50% max redn. cyt. *a*	% redn cyt. *a* (maximal)	E_m (cyt. *a*) apparent*
Controlled**	35	28	+275 mV
+ valinomycin	30	52	+282 mV
+val and nig***	6.5	30	+328 mV

*Assuming E_m for cytochrome *c* = +260 mV under these conditions
**In the absence of ionophores (rate ≈ 15% that of uncontrolled rate)
***Valinomycin plus nigericin abolish both ΔpH and $\Delta\Psi$.
pH 7.4, 50 mM phosphate buffer, 30°C. Other conditions as in Figs. 1 and 2.

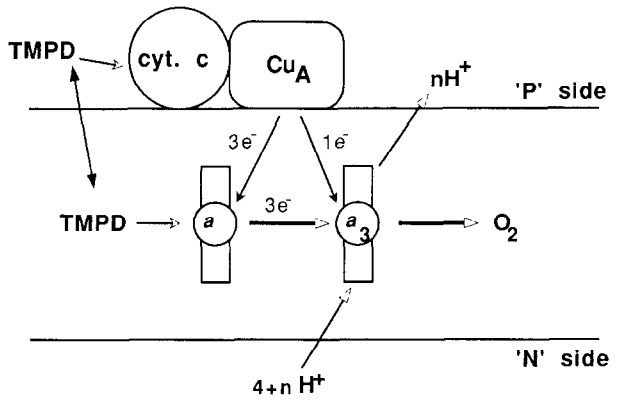

Fig. 4. Scheme for electron transfer through cytochrome oxidase in the membrane or in detergent-dispersed solution. Cytochrome *c* reduces the oxidase by transferring electrons to Cu_A. TMPD may reduce cyt. *a* directly. Two or three of the electrons required to reduce a molecule of O_2 reach the binuclear centre via cyt. *a*. One electron (rate-limiting in steady state?) *may* travel directly from Cu_A. Whether cyt. a_3 can be reduced directly or via Cu_B is uncertain. Under some but perhaps not all conditions cyt. *a* and Cu_A are in rapid electron exchange equilibrium.

state behaviour of the oxidase in COV is easier to monitor and thus interpret than that in the mitochondria which they are presumed to model, although there may still be significant functional differences between the two systems. The simplest explanation of the present data is that the enzyme component whose reduction controls the flux is reduced progressively as cyt. *c* is reduced, but is not cytochrome *a*. The most plausible candidate for such a centre is the 'visible' copper Cu_A. Estimates in mitochondria [4] and with isolated enzyme [25] suggest that Cu_A remains highly oxidized during the usual steady state. Control by its redox state rather than that of cytochrome *a* implies that steady state electron transfer from cytochrome *c* to O_2 may involve direct passage of electrons from Cu_A to the binuclear centre. Alternatively an oxidase catalytic cycle must be constructed with another rate-determining step which does not alter the $[a^{2+}]:[a^{3+}]$ ratio. If such a step did not involve either Cu_A or cyt. *a*, then there should be substantial occupancy of a binuclear centre state during steady state that does not correspond to any ground state. There is no current evidence for such a state.

I therefore propose the model of electron transfer shown in Fig. 4. Initial reduction of cytochrome aa_3 by cytochrome *c* occurs at Cu_A. Cytochrome *a* is either reduced indirectly via Cu_A or directly by reagents penetrating the hydrophobic regions of protein and bilayer, such as TMPD [25,26]. Cyt. *a* transfers both its electron and that of Cu_A to rereduce the binuclear centre when oxygen is added to the fully reduced enzyme, as in the flow-flash experiments of Hill [11] and Han et al. [12]. The further reduction of that centre, required to regenerate an O_2-reactive species, may be associated with direct electron transfer from Cu_A in the steady state where flux increases without corresponding change in the cytochrome *a* redox state (Fig. 2). In this way the kinetics of the partial reaction and of the steady state can be reconciled. During turnover the Soret band of the oxidized haem (cytochrome a_3) is red-shifted. This is associated with reduction of either cytochrome *a* or Cu_B, and linked to the corresponding changes in cytochrome *a* in the presence of ligands such as azide [16,27]. Both cytochrome *a* and Cu_A are therefore simple electron transfer centres. The complex coupled biochemistry occurs at the binuclear a_3Cu_B centre, which catalyses both oxygen reduction and proton pumping [6,14].

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