Kinetic evidence for the re-definition of electron transfer pathways from cytochrome *c* to O_2 within cytochrome oxidase

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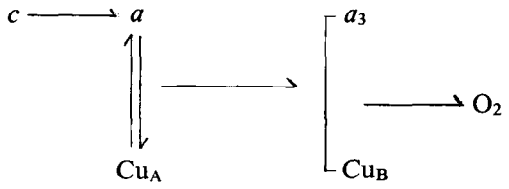
The reaction with O_2 of equimolar mixtures of cytochrome *c* and cytochrome *c* oxidase in high and low ionic strength buffers has been examined by flow-flash spectrophotometry at room temperature. In low ionic strength media where cytochrome *c* and the oxidase are bound in an electrostatic, 1:1 complex some of the cytochrome *c* is oxidised at a faster rate than a metal centre of the oxidase. In contrast, when cytochrome *c* and cytochrome *c* oxidase are predominantly dissociated at high ionic strength cytochrome *c* oxidation occurs only slowly ($t_{1/2} = 5$ s) following the complete oxidation of the oxidase. These results demonstrate that maximal rates of electron transfer from cytochrome *c* to O_2 occur when both substrates are present on the enzyme. The heterogeneous oxidation of cytochrome *c* observed in the complex implies more than one route for electron transfer within the enzyme. Possibilities for new electron transfer pathways from cytochrome *c* to O_2 are proposed.

Cytochrome c *oxidase* *O_2 reduction* *Electron transfer* *Flow-flash photolysis spectrophotometry* *Cytochrome* c *Ionic strength effect*

1. INTRODUCTION

One function of mitochondrial cytochrome *c* oxidase is to transfer electrons from cytochrome *c* to O_2. Pre-steady-state kinetic studies of the redox half-reactions of the enzyme with its two substrates, ferrocytochrome *c* and O_2, have led to the view depicted below for the path of electrons from cytochrome *c* to molecular O_2.



Cytochrome *c* is thought to donate electrons to cytochrome *a* which is in rapid equilibrium with the EPR detectable copper species, Cu_A. Cytochrome *c* oxidation is followed by intramolecular electron transfer from cytochrome *a* and Cu_A to the oxygen reaction site formed by the binuclear cytochrome a_3–Cu_B pair. This functional polarity between cytochrome *a* (and Cu_A) and cytochrome a_3 (and Cu_B) has been advanced in recent reviews of cytochrome *c* oxidase structure and function [1,2]. In this paper we report kinetic results on the O_2 reaction during a single turnover of cytochrome *c* in its complex with the oxidase that show that cytochrome *c* is oxidised prior to some component of the oxidase. Such a result leads us to suggest alternatives to the conventional pathway of electron transfer within this enzyme.

Abbreviations: TMPD, *N,N,N′,N′*-tetramethyl-*p*-phenylenediamine; EPR, electron paramagnetic resonance

2. EXPERIMENTAL

Bovine heart cytochrome *c* oxidase was prepared according to the method of Yonetani [3] as modified in this laboratory [4]. The cytochrome *c* was from horse heart as supplied by Sigma (Dorset) (type VI). The concentrations of the ox-

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idase and cytochrome *c* were determined spectrophotometrically using extinction coefficients of $\Delta\Delta E^{red-ox} = 27\ mM^{-1} \cdot cm^{-1}$ at 605–630 nm and $\Delta\Delta E^{red-ox} = 21.2\ mM^{-1} \cdot cm^{-1}$ at 550–540 nm, respectively. The protein solution was prepared for the flow-flash experiment by incubation with 3 mM sodium ascorbate and 250 nM TMPD for 20 h at 4°C in a large-volume Thunberg cuvette under an atmosphere of N_2 to obtain complete reduction. Prior to the experiment the N_2 atmosphere was replaced with CO. The solution of reduced, CO-bound oxidase was driven from the preparation vessel into a storage syringe of the flow device under a positive pressure of CO. Cytochrome *c* could be added to the oxidase solution via a microsyringe through a subaseal on the preparation cell. Solutions of varying O_2 content were prepared by mixing air-equilibrated or O_2-equilibrated buffer with N_2-equilibrated buffer in the desired ratio.

The flow-flash system consisted of a Gibson–Milne-type flow device which delivered the reaction mixture to a flow cell situated in an Applied Photophysics flash cavity. The optical pathlength was 4.3 cm. Complete photolysis of the oxidase–CO complex was obtained with a 90 J flash. The flow-flash system has been described in more detail in recent papers from this laboratory [5,6].

3. RESULTS AND DISCUSSION

Fig. 1 shows the oxidation by O_2 of cytochrome oxidase and cytochrome *c* in the stoichiometric complex at low ionic strength. In fig. 1a fractional absorbance change at 605 nm, the α-band maximum of fully reduced cytochrome *c* oxidase, in the presence and absence of one equivalent of ferrocytochrome *c* is plotted on a split time base out to 2.5 ms initially and then out to 3 s. In the absence of cytochrome *c* the absorption time course is identical to that originally reported in high ionic strength media [7]. The form of the overall time course is dependent upon O_2 concentration. At high levels such as that used in fig. 1 ($[O_2] = 340\ \mu M$) three exponential reaction phases may be discerned. The initial phase of decreasing absorption has the most O_2-sensitive rate and is proceeding at a pseudo-first-order rate of 20 000 s^{-1} under these conditions. This phase was

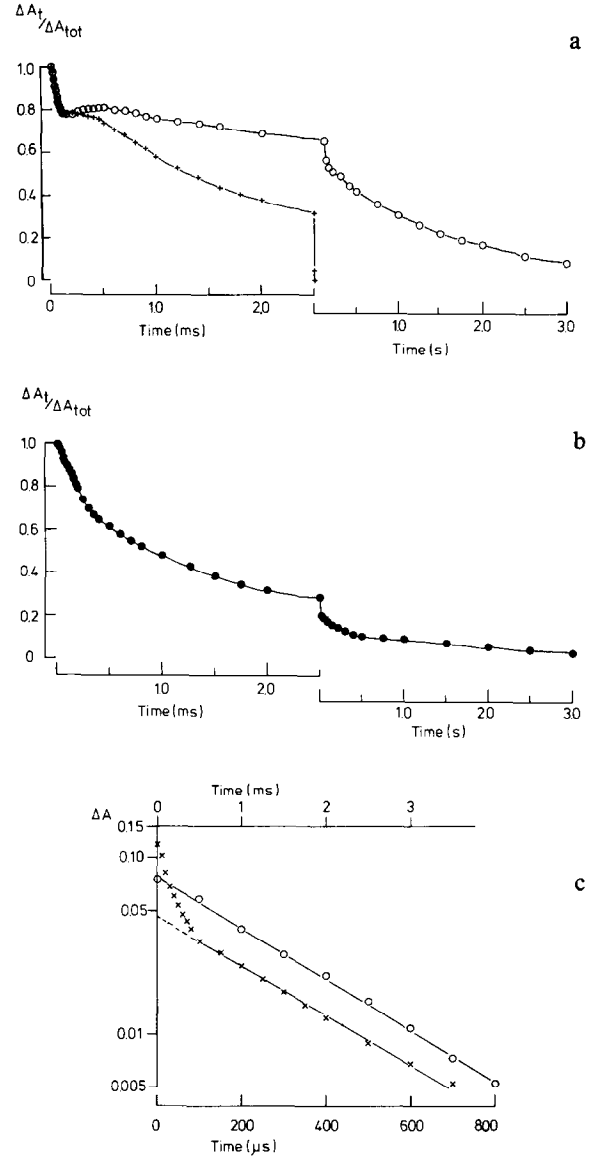

Fig. 1. The reaction of the fully reduced cytochrome *c*–cytochrome oxidase complex in a flow-flash experiment with O_2. The experiment was performed in 10 mM Tris–Mops buffer (pH 7.4) with 0.25% Tween-80 at 20°C. The reaction mixture contained 3.15 μM aa_3, 340 μM O_2, 500 μM CO, 1.5 mM ascorbate and 125 nM TMPD in the presence and absence of 2.65 μM cytochrome *c*. (a) Fractional absorbance at 605 nm plotted on a split time base in the presence (+) and absence (○) of cytochrome *c*. (b) Fractional absorbance at 550 nm on a split time base. (c) Semi-logarithmic plots of absorbance change against time at 550 nm. x, plot of overall absorbance change on the ms timescale. 0, the fast phase extracted from the overall plot and is on the μs timescale.

previously assigned to the oxidation of cytochrome a_3 [7]. The first phase is followed by a second phase of increasing absorption occurring with a maximal observed rate of 6000 s^{-1}. This phase has been assigned to the oxidation of Cu_A as supported by its correlation with the kinetics of absorption change at 830 nm, a band which is assigned to the EPR-detectable Cu_A species [7–9]. The last phase at 605 nm of decreasing absorption has a rate limit of 750 s^{-1} and has been attributed to the oxidation of cytochrome *a* [7]. The overall reaction is complete in about 6 ms. When one equivalent of ferrocytochrome *c* is present the absorption change at 605 nm is identical to that in the absence of cytochrome *c* up to 150 μs. The second phase seen previously in the absence of cytochrome *c* is interrupted and the third phase only goes to an extent 20% of that seen after 6 ms when cytochrome *c* was absent. The remainder of absorbance change at 605 nm occurs on a time scale of seconds and is complete after about 5 s. In fig. 1b the time scale of cytochrome *c* oxidation is observed at 550 nm under the same conditions as that shown in fig. 1a. Cytochrome *c* is oxidized to an extent of 80% in the first 5 ms. The remainder of the cytochrome *c* is oxidized on the time scale of seconds paralleling the slow absorption changes in the oxidase shown in fig. 1a. A semi-logarithmic plot of absorbance change against time of the ms reaction at 550 nm shows it to be composed of two exponential processes (see fig. 1c). The first phase extends to 50% at a rate of 6000 s^{-1} whereas the second half of the reaction goes at a rate of 500 s^{-1}. Therefore, cytochrome *c* oxidation is occurring in a heterogeneous manner over the first 5 ms. These results show that in the electrostatic cytochrome *c*–cytochrome oxidase complex containing 5 reducing equivalents, a portion of the total cytochrome *c* (i.e., 40%) is able to donate an electron to O_2 prior to a metal centre of the oxidase.

When this experiment is repeated at high ionic strength a different result is obtained. Fig. 2 shows the same set of absorption changes as in fig. 1 only now obtained at high ionic strength. The experiment was done at a lower O_2 concentration than that reported in fig. 1 and so the three separate phases are not as well resolved. Nevertheless, the absorption change of the oxidase as monitored at 605 nm is nearly entirely coincident in the presence or absence of cytochrome *c* on the time scale of

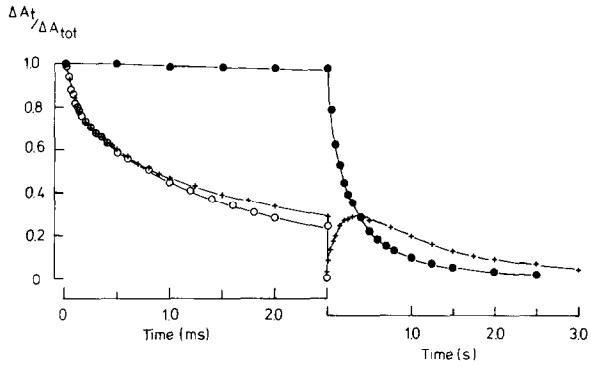

Fig. 2. The reaction of an equimolar mixture of cytochrome *c* and cytochrome oxidase with O_2 at high ionic strength. The experiment was done in 0.1 M $NaPO_4$ (pH 7.4) with 0.25% Tween-80 at 20°C. The reaction mixture contained 1.8 μM aa_3, 135 μM O_2, 500 μM CO, 50 nM cytochrome *c*, 1.5 mM ascorbate in the presence (+,○) and absence (○) of an additional 2.5 μM cytochrome *c*. The records were made at 605 nm (+,○) and 550 nm (●).

milliseconds and both time courses are close to zero after 5 ms. In contrast, there is virtually no absorption change at 550 nm on the time scale of milliseconds indicating that no cytochrome *c* is oxidized under these conditions as fast as the components of the oxidase. Cytochrome *c* is only oxidized on a time scale of seconds. Its oxidation corresponds with a transient re-reduction of the oxidase as indicated by the increase in absorption at 605 nm.

Therefore, when cytochrome *c* is bound to the oxidase electron transfer from cytochrome *c* can occur at a rate which is kinetically consistent with the known maximal turnover of the oxidase [10]. This is the first report measuring the rate of electron transfer from cytochrome *c* to O_2 under the conditions of the pre-steady-state experiment which meets this criterion.

It appears, in accordance with the conventional view of electron transfer during the O_2 reaction of the oxidase as outlined above, that cytochrome *c* is able to precede cytochrome *a* in its reaction with O_2. How may this happen? We suggest two possible electron transfer pathways in fig. 2 to accommodate this result. The scheme begins with the fully reduced cytochrome *c*–cytochrome oxidase complex, as produced by the flash photolysis of its CO-bound counterpart, binding O_2. This produces

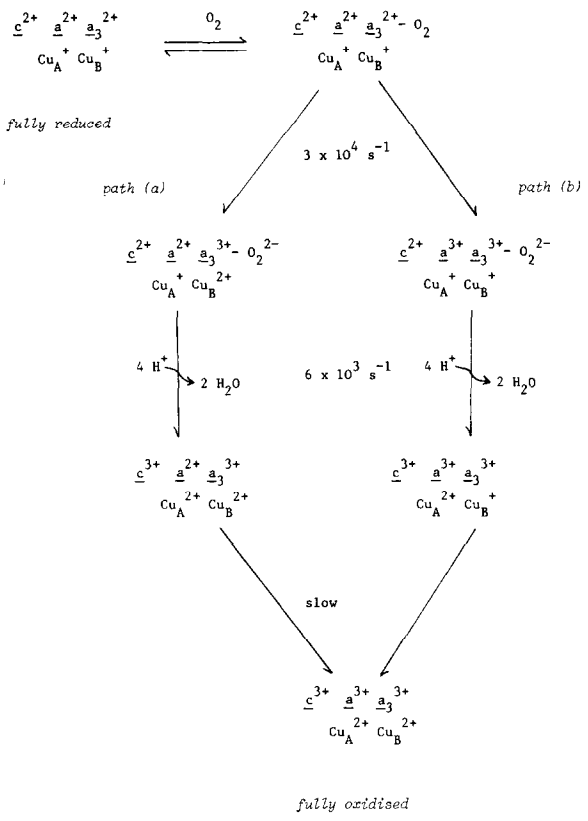

Fig. 3. Electron transfer pathways within the cytochrome *c*–cytochrome oxidase complex which account for the 'rapid' oxidation of cytochrome *c*.

an oxyferrocytochrome a_3 species termed Compound A [11]. Following O_2 binding a two-electron-transfer step is proposed to occur. Along path (a) of fig. 3 the two electrons come from the cytochrome a_3–Cu_B pair. A bridged peroxo intermediate has been suggested for the structure of the intermediate produced [11]. We suggest that within this intermediate electron transfer may occur directly from cytochrome *c* to O_2. This would define an electron transfer path from *c* to oxygen which bypasses cytochrome *a*. The observation of heterogeneous cytochrome *c* oxidation would be accounted for by having this activated species decay on the same time scale as the fast cytochrome *c* oxidation and thereby cause the rest of the cytochrome *c* to go via cytochrome *a* at the conventional rate (i.e., at 500 s^{-1}). Alternatively, as shown in path (b) of fig. 3 the initial O_2-sensitive phase of absorption change could be the result of a two-electron transfer from cytochrome a_3 and cytochrome *a*. If cytochrome *a* is oxidized during the initial phase of the reaction, then cytochrome *c* could be oxidized at a rate apparently faster than that traditionally assigned to the oxidation of cytochrome *a*. Only a portion of the cytochrome *a* would be oxidized rapidly as reflected in the heterogeneous oxidation of cytochrome *c*. The rest of the cytochrome *a* would be transferring electrons at the conventional rate of about 700 s^{-1} and would thus be in a steady-state with cytochrome *c* on the millisecond time scale and would only go oxidized, following the cytochrome *c*, on the time scale of seconds. Alternative (b) calls for the reassignment of the absorption changes seen during the transient state reaction of reduced cytochrome oxidase with oxygen and implies that more than one conformational state of the fully reduced enzyme exists. We have recently suggested alternative (b) so as to reconcile kinetic difference spectra generated by the O_2 reaction of the fully reduced enzyme at high ionic strength in the absence of cytochrome *c* with those difference spectra from static ligand binding studies and kinetic-reductive experiments [12–14]. We suggested that 40% of the cytochrome *a* was oxidised along with cytochrome a_3 during the initial phase of the reaction of the fully reduced enzyme with O_2. This value is in agreement with the amount of rapidly oxidized cytochrome *c* observed in this work. Since this pattern of reactivity appears to be a feature in the room-temperature O_2 reaction of the isolated, reconstituted enzyme [15] and in the low-temperature reaction of the isolated [16] and membrane-bound species [11], we suggest that it is an intrinsic feature of the native enzyme and not an artefactual consequence of isolation. Such a structural feature may be of importance in the suggested functional role of the oxidase as a proton pump [17].

In any case, the observation that cytochrome *c* oxidation proceeds at such a fast rate shows that the conventional view of the electron transfer pathway from cytochrome *c* to O_2 needs amending. Maximal rates of electron transfer from cytochrome *c* to O_2 require the presence of both substrates at their binding sites on the enzyme.

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