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Single electron reduction of 'slow' and 'fast' cytochrome-*c* oxidase

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Evidence is presented that single electron reduction is sufficient for rapid electron transfer (k>20 s^{-1} at pH 8.0 in 0.43 M potassium EDTA) between haem *a*/Cu_A and the binuclear centre in 'fast' oxidase, whereas in 'slow' oxidase intramolecular electron transfer is slow even when both Cu_A and haem *a* are reduced (k≈0.01 s^{-1}). However, while a single electron can equilibrate rapidly between Cu_A, haem *a* and Cu_B in 'fast' oxidase, it seems that equilibration with haem a_3 is relatively slow (k≈2 s^{-1}). Electron transfer between cytochrome *c* and Cu_A/haem *a* is similar for both types of enzyme (k≈2.4×10^5 $M^{-1}\cdot s^{-1}$).

Cytochrome-*c* oxidase; Cytochrome *c*; Electron transfer; Binuclear centre; Photoreduction; Bovine heart

1. INTRODUCTION

Cytochrome-*c* oxidase (E.C. 1.9.3.1), the terminal component of the mammalian electron transfer chain, is a redox-linked proton pump [1,2]. It contains four redox-active metal centres. Two of these, haem a_3 and Cu_B, form a binuclear centre which is the site of interaction between the enzyme and dioxygen. The other two centres, haem *a* and Cu_A, are involved in accepting electrons from cytochrome *c* [3].

It has been suggested that rapid electron transfer between haem *a*/Cu_A and the binuclear centre can only occur when the enzyme is in the appropriate conformation, and that the switch to this conformation from one without an efficient electron transfer pathway is induced by reduction of haem *a* or Cu_A [4–8]. Hence, two-electron reduction would be required for rapid intramolecular transfer. This idea has led to the proposal that the input and output states, which are obligatory in any viable pump mechanism, might correspond with the two conformations [6,8].

The question of whether such a 'two-electron gate' exists in cytochrome oxidase is complicated by heterogeneity in oxidase preparations. It is known that the 'slow' form of the enzyme shows slow intramolecular electron transfer (k≈0.01 s^{-1}) even when both haem *a* and Cu_A are reduced by excess dithionite whereas much faster rates are observed for the 'fast' form (k>0.2 s^{-1}) [9–11]. Hence, the question is only relevant for 'fast' oxidase, which appears to be the native form of the enzyme.

In previous, preliminary, work we have found some indications that two-electron reduction is not a requirement for rapid electron transfer from haem *a*/Cu_A to the binuclear centre in 'fast' oxidase [12]. We now present the results of further experiments which confirm this view. In these experiments we have generated singly-reduced cytochrome oxidase using the technique of Flash-Induced chemical photoREduction (FIRE) [13]. A similar technique has been used recently to examine the kinetics of the intermolecular electron transfer from cytochrome *c* to oxidase [14,15]. The present work, however, examines the kinetics of intramolecular transfer of a single electron between the redox centres of cytochrome oxidase by comparing two oxidase preparations, one of which is completely in the 'slow' form while the other is completely in the 'fast' form.

A preliminary account of some of this work has already been reported [16].

2. MATERIALS AND METHODS

'Fast' cytochrome-*c* oxidase (defined as showing no slow phase of cyanide binding) was prepared from bovine heart as previously described (preps. C and E in [11]). The 'slow' oxidase (defined as showing no fast phase of cyanide binding) was a kind gift from Dr J.M. Wrigglesworth and was prepared by the method of Kuboyama et al. [17]. The stock concentration of both preparations was about 250 μM. Tween 80 was present in both cases (0.1% in the 'fast' oxidase and 0.25% in the 'slow').

PMS (5-methyl phenazinium methosulphate) was obtained from Lancaster Synthesis Ltd (Morecambe, UK). Horse heart cytochrome *c* came from Sigma (type VI). All other reagents were supplied either by BDH (Poole, UK) or Sigma (Poole, UK).

The single-beam apparatus used for the optical measurements and the Xenon flash apparatus were essentially as described before [18]. Two fibre-optic bundles (1 cm diameter) placed about 2.5 cm from the arc (length 3 mm) were used to collect the light and guide it, perpendicular to the measuring beam, to opposite sides of the 10 mm path length cuvette (external and internal widths, 12 mm and 3 mm, respec-

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tively). To reduce flash artefacts BG 1 filters were placed between the lamp and the fibre-optic bundles and the photomultiplier was protected with a WG 360 or GG 495 filter as appropriate.

3. EXPERIMENTAL RATIONALE

3.1. *Generation of singly-reduced oxidase*

Singly-reduced cytochrome oxidase was generated by adding substoichiometric amounts of reductant (up to a maximum of 0.2 e^-/molecule) to fully-oxidized enzyme. On the initial assumption that the probability of an oxidase molecule receiving an electron is the same irrespective of whether it is already partly-reduced, we would expect 10% double hits at the maximum electron load [16]. However, because of antico-operative interactions between the redox centres in cytochrome oxidase [19] we might in fact expect the probability of a partly-reduced oxidase molecule receiving an electron to be lower than that of a fully-oxidised molecule, hence the number of double hits would be less. The effect of double hits on the results will be considered later.

The reductant, cytochrome c^{2+}, was added by FIRE using PMS/EDTA as the FIRE system [13]. At the flash intensity used (lamp output 2.7 J/flash) about 0.15 μM cytochrome c^{2+} was produced per flash (90% complete in <25 ms, 99% in <100 ms). In addition, a high ionic strength was used so that the reduction of the cytochrome oxidase by the cytochrome c^{2+} would be relatively slow and so easily observable.

3.2. *'Slow' oxidase versus 'fast' oxidase*

'Slow' oxidase appears during the preparation of cytochrome oxidase, particularly when the pH falls below about 8 [11,12,20]. Ligation of the enzyme by formate produces enzyme with similar properties, so it seems likely that 'slow' oxidase has simply acquired an inhibitory ligand, as yet unknown, during its preparation [16,21]. Both 'slow' and formate-ligated oxidases can be converted to 'fast' oxidase by redox-cycling.

There is little question that intramolecular electron transfer between haem a/Cu_A and the binuclear centre is slow in 'slow' oxidase even if both haem a and Cu_A are reduced (k≈0.01 s^{-1}, Refs [9–11]. By analogy with formate-ligated enzyme, it is likely that this arises from the lowered midpoint potentials of Cu_B and haem a_3, and a low rate constant of dissociation of the ligand. Further, there seems little doubt that intramolecular electron transfer is fast in 'fast' oxidase under the same conditions (k>0.2 s^{-1}). Our aim, therefore, is to use 'slow' oxidase as a control to test the idea that two-electron reduction is a requirement for rapid intramolecular electron transfer in 'fast' oxidase, since if this is true then the response of both types of enzyme to one-electron reduction should be similar.

4. RESULTS AND DISCUSSION

4.1. *Single-electron of 'slow' cytochrome oxidase*

Fig. 1, left panel, shows the time-courses of cytochrome c reduction and reoxidation at 550 nm, and haem a reduction at 605 nm after flash-reduction of 'slow' oxidase via cytochrome c. The spectral contributions by cytochrome c at 605 nm and haem a at 550 nm were removed by matrix deconvolution [22] using the relative extinction coefficients shown in the insert. These were obtained by reference to redox spectra of the pure components. The data for cytochrome c were taken from [13] and the data for haem a were obtained by FIRE of formate-ligated oxidase in the absence of cytochrome c (see Fig. 2 in [16]). The time-courses are consistent with equilibration of the electrons only between haem a, Cu_A and cytochrome c, as expected for

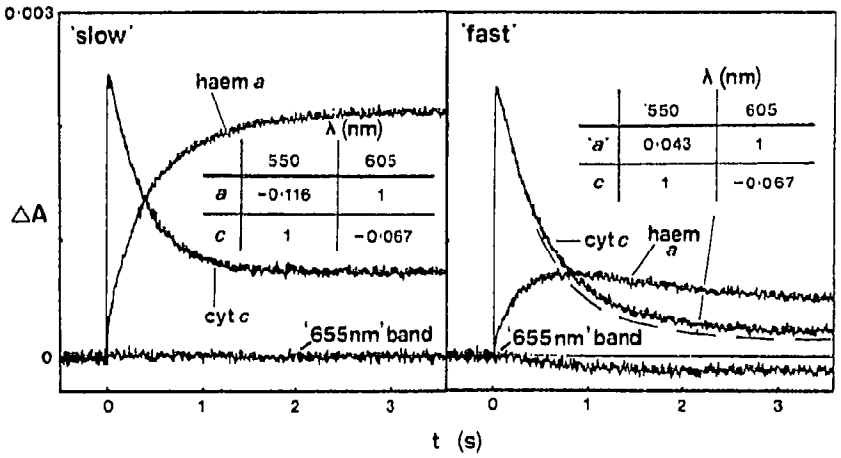

Fig. 1. FIRE of 'slow' and 'fast' cytochrome-c oxidase via cytochrome c. Stock oxidase was diluted to a final concentration of about 8 μM in 0.43 M potassium EDTA, pH 8.0, containing 0.09% lauryl maltoside, 400 U/ml catalase, 17 μM cytochrome c and 60 μM PMS. Transients resulting from single flashes were measured at various wavelengths. Each sample of oxidase received 10 flashes at 15s intervals. The time-courses (averages of 10 transients) are deconvolutions of the contributions by cytochrome c^{2+} at 550 nm and haem a^{2+} at 605 nm using the relative extinction coefficients given in the insert in the left panel. The dashed line is explained in the text, as are the time-courses labelled '665 nm band'.

'slow' oxidase. After 3.5 s, cytochrome *c* and cytochrome oxidase are close to redox equilibrium, at which point 0.39 μM cytochrome c^{2+} and 1.04 μM haem a^{2+} are present (using extinction coefficients of 18.5 $mM^{-1} \cdot cm^{-1}$ for cytochrome *c* at 550 nm [23] and 20.5 $mM^{-1} \cdot cm^{-1}$ for haem *a* at 605 nm [24]). From the redox poise of cytochrome *c* (using $E_{m,cyt.c} = 260$ mV [25]) a value of 305 mV can be obtained for the E_m of haem *a*, which is in good agreement with published values (e.g. [18,26,27]). Between 5 and 10% of the cytochrome *c* reoxidised is unaccounted for in the reduction of haem *a*. This can be accounted for in the reduction of Cu_A, which must therefore have an E_m of 230–250 mV, again in good agreement with published values (e.g. [18]).

It might be noted that the kinetics of cytochrome *c* reoxidation and of haem *a* reduction do not quite match. We attribute this to the energetically-favourable ($K \approx 17$) reverse dismutation via cytochrome *c* of a small fraction of doubly-reduced oxidase with fully-oxidized enzyme. If the total electron load is kept below 0.1 e^-/oxidase then this discrepancy is not seen and the time-course of haem *a* reduction is closer to exponential. However, for the purely practical reason that we had a limited quantity of 'slow' oxidase, we used loads up to 0.2 e^-/oxidase so as to obtain a reasonable signal to noise ratio.

4.2. *Single-electron reduction of 'fast' cytochrome oxidase*

Fig. 1, right panel, shows the time-courses of cytochrome *c* reduction and reoxidation at 550 nm, and 'haem *a*' reduction at 605 nm after flash-reduction of 'fast' oxidase via cytochrome *c*. Again, the spectral contributions by cytochrome *c* at 605 nm and haem *a* at 550 nm were removed by matrix deconvolution using the relative extinction coefficients shown in the insert in the left panel. It is clear that the time-course of haem *a* reduction is completely different to that obtained with 'slow' oxidase. First, the apparent initial rate of reduction is only about 60% of that found with 'slow' oxidase even though the initial rates of reoxidation of cytochrome *c* are similar ($k > 1.7 \times 10^5$ $M^{-1} \cdot s^{-1}$ from initial rates; see Fig. 3 for a better estimate) and second, after reaching a maximum after about 0.8 s there is then a partial reoxidation of haem *a*. In addition, the extent of reoxidation of cytochrome *c* is much greater than that found with 'slow' oxidase.

Fig. 1 also shows the time-course of the absorbance changes at 660 nm minus the average changes at 630 and 690 nm (average of 10 transients in each case) for both enzyme types. As expected, there is no change in the absorbance of this wavelength triplet after flash-reduction of 'slow' oxidase. However, with 'fast' oxidase, after a short lag, there is a decrease in the absorbance consistent with the disappearance of the '655 nm' charge transfer band ($\varepsilon \approx 0.8$ $mM^{-1} \cdot cm^{-1}$) associated with high-spin ferric haem a_3 [27]. This is also clear from kinetic spectra constructed from single transients at multiple wavelengths (see example in Fig. 2) and might indicate that haem a_3 has been reduced or has changed spin state.

As noted before we would expect a small fraction of double hits in these experiments. In the case of 'fast' oxidase we expect the rapid formation of the 'peroxy' form of oxidase since oxygen is present. This form of the enzyme is characterized by a redox spectrum with a peak at 606 nm with an extinction coefficient of about 10 $mM^{-1} \cdot cm^{-1}$. There may therefore be a slight contribution by 'peroxy' oxidase to the absorbance changes at 605 nm. However, no significant variation of the individual time-courses from single flashes with total electron load (0.02–0.2 e^-/oxidase) was observed. A potentially more serious problem with 'fast' oxidase is the dismutation of singly-reduced enzyme via cytochrome *c* to form 'peroxy' oxidase and perhaps even 'ferryl' oxidase. This could explain both the disappearance of the '655 nm' band, which is absent in both 'peroxy' and 'ferryl' oxidase, and the apparent reoxidation of haem *a*. However, this explanation is unlikely to be correct because only a slight blue shift of the Soret maximum is observed (from 447 nm at 1 s to 446 nm at 3.5 s) which is much smaller than would be expected given that 'peroxy' and 'ferryl' oxidase have peaks at about 437 nm and 435 nm, respectively [16]. In addition, since 'peroxy' oxidase has a high electron affinity (E_m for the singly-reduced to 'peroxy' couple is about 1 V [28]), essentially complete reoxidation of cytochrome *c* would be expected if significant dismutation had occurred.

We have previously shown that the redox spectrum

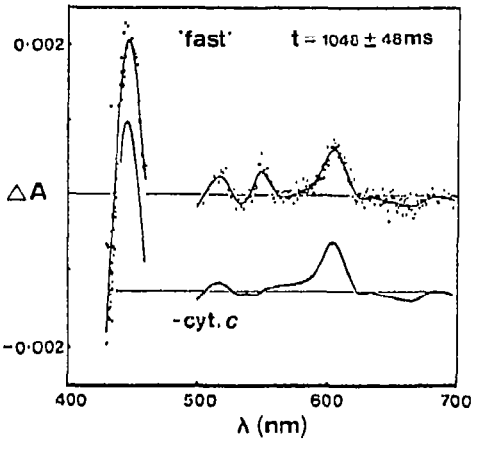

Fig. 2. FIRE of 'fast' cytochrome-*c* oxidase: a sample kinetic spectrum. Conditions are described in the legend to Fig. 1. Each point is derived from a single transient measured at the indicated wavelength. Note that the high basal absorbance due to oxidized cytochrome *c* prevented measurements below 430 nm. The continuous spectrum was obtained by interpolation and smoothing (using program REGSPL2 in [29] with PP = 0.01). The lower spectrum, which is offset on the *y*-axis, was obtained by subtracting a pure redox spectrum of cytochrome *c* equivalent to 0.025 μM. Note that the pure cytochrome *c* spectrum was processed with the interpolation/smoothing program before subtraction.

obtained about 1 min after FIRE of 'fast' oxidase (<0.3 e^-/molecule) in the absence of cytochrome *c* is characterised (a) by Soret and alpha maxima at 445 nm and 605 nm, respectively, and (b) by the presence of a '655 nm' trough (see Fig. 2 in [16]), which are all features that are observable 3.5 s after FIRE of 'fast' oxidase in the presence of cytochrome *c*. The dashed line in Fig. 1, right panel, shows the time-course of cytochrome *c* reduction and reoxidation deconvoluted using relative extinction coefficients based on this spectrum rather than the redox spectrum of haem *a* (values in insert). Note that the deconvoluted time-course of 'haem *a*' reduction is essentially unaffected by this change. If we assume that any increase in ΔA_{605} is due principally to haem a^{2+} then we find there to be 0.090 μM cytochrome c^{2+} and 0.25 μM haem a^{2+} at 3.5 s. Again, from the redox poise of cytochrome *c*, a value of 305 mV can be obtained for the E_m of haem *a*, which is the same as that obtained for 'slow' oxidase.

Our working model to explain the observations for both 'fast' and 'slow' oxidases is outlined in the simplified reaction scheme shown in Fig. 3. For 'slow' oxidase a single species, E'', is formed in which the electron can only equilibrate between Cu_A and haem *a*. For 'fast' oxidase the time-course of 'haem a^{2+} can be described by two sequential first order processes, corresponding to the reduction and partial reoxidation phases, respectively. As noted above, the product of the partial reoxidation appears to be the same as that observed about

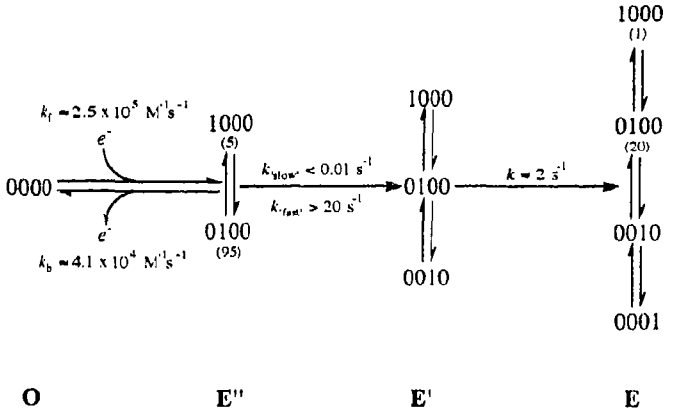

Fig. 3. A minimal reaction scheme for pH 8.0 and high ionic strength. In each substate the order of the redox components is Cu_A, haem *a*, Cu_B and haem a_3, with '0' and '1' representing oxidized and reduced components, respectively. The possibility of double-hits is ignored. The rate constants, k_f and k_b were calculated from the observed first order rate constant for the reoxidation of cytochrome *c* from the data for 'slow' oxidase. The rate constant for the step E' → E for 'fast' oxidase was obtained by fitting (using the simplex method [29]) the analytical solution for two sequential first order processes to the time-course for 'haem *a*' reduction and partial reoxidation. Note that the fit was constrained to the observed rate of reoxidation of cytochrome *c*. A value of 4.1 $mM^{-1} \cdot cm^{-1}$ for the extinction coefficient of E at 605 nm was also obtained. The values in parentheses, where shown, are the approximate percentages of each substate obtained by assuming (a) that only haem a^{2+} contributes to ΔA_{605} and (b) that the relative midpoint potentials of Cu_A and haem *a* do not change.

1 min after FIRE of 'fast' oxidase in the absence of cytochrome *c*, i.e. E, in which the single electron has equilibrated with all the redox centres [16]. The question then remains as to the identity of the intermediate product. We have found, on the basis of curve-fitting with the rate of reduction constrained to the observed rate of reoxidation of cytochrome *c*, that the time-course of 'haem *a*' reduction/reoxidation cannot be simulated using a model in which the intermediate between O and E is E''. Instead, we find that another intermediate, E', with an extinction coefficient ($14.5 > \varepsilon_{605} > 11.4$ $mM^{-1} \cdot cm^{-1}$) less than E'' ($\varepsilon_{605} = 19.5$ $mM^{-1} \cdot cm^{-1}$) but greater than E ($\varepsilon_{605} \approx 4.1$ $mM^{-1} \cdot cm^{-1}$), is required. Hence, in E' there appears to be rapid equilibrium between haem a/Cu_A and another redox centre which we presume to be Cu_B. Since E'' is not observable we conclude that the rate of equilibration of Cu_B with haem a/Cu_A to form E' must be at least tenfold greater (> 20 s^{-1}) than the rate of electron transfer from cytochrome *c* to the enzyme (approx. 2 s^{-1}). Another possibility for intermediate E' is that equilibration is rapid between haem a/Cu_A and haem a_3, but this is not supported by the observations. In this case we would expect to see (a) no lag in the disappearance of the '655 nm' band and (b) a high initial ratio between the Soret (444–462 nm) and alpha (605–630 nm) peaks, i.e. a value similar to the value of 4.6 found for the ratio in fully-reduced minus fully-oxidized 'fast' oxidase rather than the actual value of about 2.6 (see e.g. Fig. 2).

We conclude that two-electron reduction is **not** a requirement for rapid electron transfer from haem a/Cu_A to the binuclear centre in 'fast' oxidase. Nevertheless, equilibration of a single electron shared between Cu_A, haem *a* and Cu_B with haem a_3 appears to be a relatively slow process ($k \approx 2$ s^{-1}). This slow equilibration could be envisaged as the release or rearrangement of a ligand (or ligands) from haem a_3 in a process analogous to that suggested for the conversion of 'slow' oxidase to 'fast', i.e. electron transfer (presumably between Cu_B and haem a_3) is rapid but the equilibrium is unfavourable unless the ligand is displaced [16]. An alternative possibility is that it represents equilibration along an intrinsically slow electron transfer pathway, although this seems unlikely given the proximity of Cu_B and haem a_3.

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