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Activation by reduction of the resting form of cytochrome *c* oxidase: tests of different models and evidence for the involvement of Cu_B

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(1) The reaction of the resting form of oxidised cytochrome *c* oxidase from ox heart with dithionite has been studied in the presence and absence of cyanide. In both cases, cytochrome *a* reduction in 0.1 M phosphate (pH 7) occurs at a rate of $8.2 \cdot 10^4$ $M^{-1} \cdot s^{-1}$. In the absence of cyanide, ferrocytochrome a_3 appears at a rate (k_{obs}) of 0.016 s^{-1}. Ferricytochrome a_3 maintains its 418 nm Soret maximum until reduced. The rate of a_3 reduction is independent of dithionite concentration over a range 0.9 mM–131 mM. In the presence or cyanide, visible and EPR spectral changes indicate the formation of a ferric a_3/cyanide complex occurs at the same rate as a_3 reduction in the absence of cyanide. A $g = 3.6$ signal appears at the same time as the decay of a $g = 6$ signal. No EPR signals which could be attributed to copper in any significant amounts could be detected after dithionite addition, either in the presence or absence of cyanide. (2) Addition of dithionite to cytochrome oxidase at various times following induction of turnover with ascorbate/TMPD, results in a biphasic reduction of cytochrome a_3 with an increasing proportion of the fast phase of reduction occurring after longer turnover times. At the same time, the predominant steady state species of ferri-cytochrome a_3 shifts from high to low spin and the steady-state level of reduction of cytochrome *a* drops indicating a shift in population of the enzyme molecules to a species with fast turnover. In the final activated form, oxygen is not required for fast internal electron transfer to cytochrome a_3. In addition, oxygen does not induce further electron uptake in samples of resting cytochrome oxidase reduced under anaerobic conditions in the presence of cyanide. Both findings are contrary to predictions of certain O-loop types of mechanism for proton translocation. (3) A measurement of electron entry into the resting form of cytochrome oxidase in the presence of cyanide, using TMPD or cytochrome *c* under anaerobic conditions, shows that three electrons per oxidase enter below a redox potential of around +200 mV. An initial fast entry of two electrons is followed by a slow ($k_{obs} \approx 0.02$ s) entry of a third electron. Above +200 mV, the number of electrons taken up in the initial fast phase drops as a redox center (presumably Cu_A) titrates with an apparent mid-point potential of +240 mV. The slow phase of reduction remains at the more positive redox values. (4) The results are interpreted in terms of an initial fast reduction of cytochrome *a* (and Cu_A at redox values more negative than +240 mV) followed by a slow reduction of Cu_B. Cu_B reduction is proposed to spin-uncouple cytochrome a_3 to form a cyanide sensitive center, and trigger a conformational change to an activated form of the enzyme with faster intramolecular electron transfer.

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Abbreviations: TMPD, *N,N,N′,N′*-tetramethyl-*p*-phenylenediamine; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulphonic acid.

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Introduction

Purified cytochrome oxidase, from mammalian mitochondria, has been shown to exist in several distinct oxidised forms [1,2] which have been distinguished on the basis of catalytic activity [2–6], conformation [7,8] spin state [9,10] and reactivity to various ligands [11,12]. A so-called 'resting' enzyme [3] is found in preparations of the oxidized enzyme as isolated by several different procedures [12]. This is a relatively inactive form of the enzyme, exhibiting a Soret maximum around 418–420 nm [13–15], a slow (1.8 $M^{-1} \cdot s^{-1}$) reactivity to cyanide [16], and a slow internal electron-transfer rate for the reduction of cytochrome a_3 [4]. On the basis of X-ray absorption studies [17,21], it has been suggested that this form of the enzyme contains a sulphur bridge between the a_3 iron and Cu_B metal atoms rendering the haem less reactive to external ligands such as cyanide, and possibly lowering the redox potential of cytochrome a_3 relative to that found in the active enzyme.

Most resting forms of the enzyme appear to contain a heterogeneous mixture of species [8,12]. A transition from resting to a more catalytically active ('pulsed') species of enzyme takes place during turnover and this is accompanied by a shift to the red of the Soret maximum [9]. The transition is promoted by the interaction of cytochrome *c* with the enzyme [22,23] and dioxygen is reported to enhance internal electron-transfer rates [24]. The pulsed form of the oxidase was originally defined on the basis of a fast turnover of the fully reduced enzyme following the simultaneous addition of both O_2 and ferrocytochrome *c* [3]. Reduction and reoxidation of the cytochrome a_3/Cu_B centre appears to be a necessary step for conversion [15]. A redox cycle involving cytochrome *a* is not a requirement [25], neither is dioxygen a required oxidant [26]. On the other hand, Scholes and Malmström [27] report that a two electron reduction of the resting enzyme is sufficient to cause a conformational transition to the pulsed form and only molecules with both cytochrome *a* and Cu_A reduced can undergo the conformational change. It was suggested that reduction of these metal centres triggers an immediate transition of the enzyme to a more 'open' form in which electron flow can be inhibited by cyanide within a few seconds. The puzzling observation that the rate of cyanide binding to this half-reduced form of the enzyme, as monitored by spectral changes, is still slow (several hundred seconds) was explained by the authors as being due to an initial fast binding of cyanide to form a spectroscopically silent complex which subsequently converts to the optically visible cytochrome a_3 cyanide species in a slow reaction (see also Ref. 9).

An alternative explanation of the resting to pulsed transition has been presented by Bickar et al. [23]. This is based on the assumption that resting oxidase is capable of rapid electron transfer between cytochromes *a* and a_3 but the redox potential of cytochrome a_3 in the resting form is significantly below that of the same cytochrome in the pulsed form. The redox equilibrium in the resting form would thus favour reduced cytochrome *a* and oxidised cytochrome a_3. A kinetic treatment of the model shows that these assumptions are capable of explaining the increased rate of cytochrome a_3 reduction seen with ferrocytochrome *c* as reductant and why O_2 also accelerates the rate of internal electron transfer. The cause of the lower redox potential of cytochrome a_3 in the resting enzyme is unknown but it was suggested that this could reflect, in part, the difference in liganding of the a_3 haem in the two states as indicated by the X-ray absorption studies [17,21].

A further proposal by Mitchell et al. [37] seeks to explain the redox contact between the a/Cu_A and the a_3/Cu_B centres by means of hydrogen transfer using oxygen as a hydrogen conductor. In an O-loop model derived from this concept, intramolecular electron conduction to the a_3/Cu_B centre would be suppressed in the absence of oxygen. An apparent dependence of intramolecular electron flow on oxygen has been reported [24,36]. The lack of activity in the resting form of the enzyme would be a consequence of the loss or modification of the putative hydrogen-carrying couple.

The present work describes experiments aimed at testing these different models and further characterising the sequence of events during the transition of the relatively inactive resting form to a

more catalytically active form, in particular the change in population density of the different states. We have attempted to determine at which stage during the reduction of the four metal centres the resting-to-pulsed transition occurs, and the oxygen dependence of this transition.

Materials and Methods

Ox heart cytochrome *c* oxidase was prepared by the method of Kuboyama et al. [46] with Tween 80 substituting for Emasol. The final preparations (approx. 200 μM with a haem *a*/protein ratio between 8.5 and 10 nmol/mg) were dialysed against potassium phosphate (0.1 M, pH 7.0) containing 0.5% Tween 80 and stored at −75 °C until required. In the subsequent text, this preparation is termed the 'resting' form of the enzyme and is characterised by slow intramolecular electron transfer to the Cu_B/a_3 center. Cytochrome *c* (horse heart, type VI), sodium ascorbate and *N,N,N′,N′*-tetramethyl-*p*-phenylenediamine (TMPD) were from Sigma Chemical Co., Poole, Dorset, U.K. 4-(2-hydroxyethyl)-1-piperazineethanesulphonic acid (Hepes) was obtained from Research Organics Inc., Cleveland, OH, and sodium dithionite from BDH, Poole, Dorset, U.K. Dithionite solutions were made freshly as required in anaerobic phosphate buffer and generally used within a few minutes of preparation. TMPD solutions were also made fresh in anaerobic buffer and maintained under argon. In experiments with varying $TMPD^+$/TMPD ratios, a solution of TMPD was exposed to air and left to autoxidise to the required ratio (usually a few hours) and then resealed under argon. Reduced cytochrome *c* was prepared by ascorbate treatment of ferricytochrome *c* followed by separation on a Sephadex G-25 column.

Optical spectra were recorded in a Cary 210 spectrophotometer and stored in digitalised form for treatment on an Apple II computer. Double-wavelength spectra were recorded using a Perkin-Elmer 356 spectrophotometer. The stopped-flow spectrophotometer was from Applied Photophysics (London N1 6DR, U.K.), and EPR spectra were obtained using a Varian E-4 EPR spectrometer fitted with an Oxford Instruments (Osney Mead, Oxford, U.K.) liquid helium cryostat. Conditions for EPR spectra were: microwave power, 20 mW; microwave frequency, 9.192 GHz; modulation amplitude, 10 G; scan time, 2 min; time constant 0.3 s; temperature, 11 K.

For experiments involving the addition of anaerobic oxidised enzyme to anaerobic solutions of TMPD or ferro-cytochrome *c*, the enzyme sample (usually around 30 μl) was made anaerobic 30 s prior to its addition, using glucose (2 mM) in the presence of trace amounts of glucose oxidase and catalase. Control experiments showed that the sample went anaerobic within 10–20 s and there was no reduction of the cytochrome oxidase over this time period. Sufficient dilution occurred on addition of the sample to the final volume (3 ml) to prevent any further significant action of the glucose oxidase system. Partial reduction of the enzyme in the glucose oxidase system was found to require several hours and concentrations of glucose greater than 10 mM. Control experiments were also performed where the oxidase sample was made anaerobic by repeated cycles of evacuation and flushing of N_2 over a period of several hours and gave the same results as the glucose oxidase system. The disadvantage of the gas-flushing method for small concentrated samples of enzyme was that some increase in the oxidase concentration took place due to evaporation.

The individual reduction changes of cytochromes *a* and a_3 were calculated by an iterative procedure from the wavelength changes at 444–462 nm and 605–623 nm assuming cytochrome *a* contributes 50% of the absorbance change at 444 nm and 85% at 605 nm. The rate constants calculated from these fits were found to be relatively insensitive to small (10%) changes in the assumed values, but for the enzyme with a shifted Soret maximum to 428 nm (a form of the pulsed enzyme) a best fit to the reduction changes was found to occur using a 33% contribution of cytochrome *a* to the absorbance changes at 444 nm according to Anatalis and Palmer [24]. The extinction coefficient for $TMPD^+$ was measured under anaerobic conditions over the range of redox values used in the experiments. Below redox potentials of around 200 mV the $TMPD^+$/TMPD system was unstable in the presence of oxygen. Under anaerobic conditions, however, a constant value of 6.05 ± 0.05 $mM^{-1} \cdot cm^{-1}$ (630–650 nm) was found between

100 mV to 200 mV, and measurements were confined to this redox range. Above 200 mV the value fell to around 4 $mM^{-1} \cdot cm^{-1}$ at 250 mV, a value in agreement with that reported by Nicholls and Chanady [42] in an aerobic system. We attribute this apparent fall in extinction coefficient to be due to instability and breakdown of the oxidation product Wurster's blue at the more positive redox potentials. Other wavelengths and extinction coefficients used were: cytochrome oxidase at 605–630 nm, reduced minus oxidised, ($\Delta\varepsilon = 27$ mM); cytochrome *c* at 550–535 nm, reduced minus oxidised, ($\Delta\varepsilon = 23.5$ mM); Cu_A at 830–740 nm, reduced minus oxidised ($\Delta\varepsilon = 1.0$ mM). Corrections were made where appropriate for the small extinction changes from cytochrome oxidase at 630–650 nm and 550–535 nm.

Results

Oxidised cytochrome oxidase, as isolated by most methods of preparation, has been shown to be reduced by dithionite in a biphasic manner [22,28]. Values of around 0.1–0.4 s^{-1} for the rate constant for the slow second phase have been reported [22]. We have followed the spectral changes following dithionite addition to the oxidised enzyme (Fig. 1) and have analysed the spectral changes at 444 nm and 605 nm, as described in the methods section, to present the spectral changes in terms of the redox changes of the individual cytochromes *a* and a_3 (Fig. 1, inset). By this analysis, it is possible to distinguish clearly the two phases of oxidase reduction as a fast reduction of cytochrome *a* followed by a slower reduction of cytochrome a_3. The value for k_{obs} for a_3 reduction in these resting oxidase preparations turns out to be 0.016 ± 0.005 s^{-1}. This value is close to the very slow phase of heterogeneous reduction of cytochrome oxidase observed using dithionite by Jones et al. [22] and for phenazine methosulphate by Halaka et al. [28], both of which involved half-times of the order of 50 s or more.

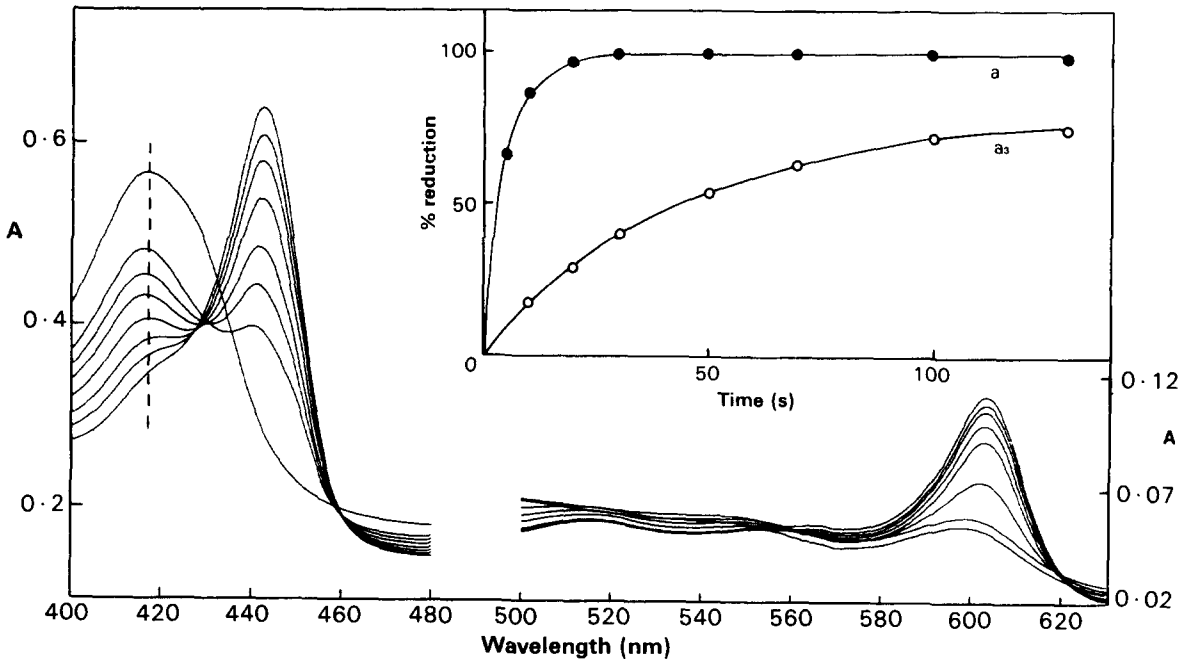

Fig. 1. Spectral changes following the addition of dithionite to resting cytochrome oxidase. Sodium dithionite (0.9) mM was added to a solution of cytochrome oxidase (3.6 μM) in potassium phosphate (0.1 M, pH 7.0). Spectral scans were taken at 1 min intervals (50 s scan time from high to low wavelength) up to 4 min and then at 8, 10 and 15 min, and can be identified in the figure as spectra with increasing absorbance at 444 nm and 605 nm compared to the spectra of the oxidised enzyme. The unchanging position of the 418 nm peak with time is indicated with the dotted line. Inset: Rates of reduction of cytochrome *a* (●— — —●) and a_3 (○— — —○) calculated from a continuous monitor of the spectral changes at 444 nm and 605 nm as described in the methods section.

The fast phase of reduction at 605 nm measured by stopped-flow spectrometry was found to be 0.294 ± 0.006 s^{-1} for k_{obs}, giving a value for the second-order rate constant for cytochrome *a* reduction by dithionite of $8.2 \cdot 10^4$ $M^{-1} \cdot s^{-1}$ in close agreement with values for the fast phase of oxidase reduction under similar conditions of ionic strength and detergent concentration as reported previously [22].

In the present experiments, not all preparations of the resting enzyme showed a slow monophasic reduction of cytochrome a_3. In several samples, all prepared by the same method, a biphasic reduction of cytochrome a_3 with an initial fast phase of $k_{obs} > 0.2$ s^{-1} could be seen following dithionite addition. However, k_{obs} for the slow phase of reduction did not significantly change in value. What did change was the relative proportion of the fast and slow phases. In most preparations, the proportion of molecules showing a fast cytochrome a_3 reduction varied between 10 and 20%. The relative proportions of the fast and slow phases of cytochrome a_3 reduction by dithionite was also altered by the presence of cytochrome *c* (to approx. 70% fast phase with 10 μM cytochrome *c*) and by changes in pH (to approx. 50% fast phase at pH 6.5), again with no significant change in the observed rate constant for the remaining slow phase of reduction. The results suggest that the heterogeneity in oxidase preparations seen in this and other studies arises from differences in the relative proportion of the enzyme preparation in the resting state, incapable of fast intermolecular electron transfer to cytochrome a_3. This proportion can be affected if reduction is carried out in the presence of cytochrome *c* [23] or at a more acid pH [40].

We have previously shown [29] that reduction of the resting ferric enzyme by ascorbate plus TMPD, under anaerobic conditions, gives a similar pattern of redox change for the two haems as that found when dithionite is used. A fast reduction of cytochrome *a* is followed by a much slower reduction of cytochrome a_3. From Fig. 2, it can be seen that during turnover, with ascorbate plus TMPD as reductant, the proportion of oxidase

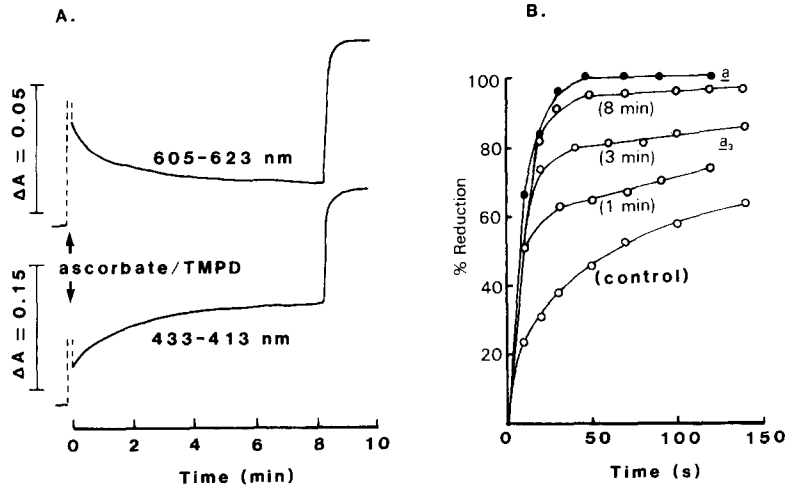

Fig. 2. Spectral changes of cytochrome oxidase following initiation of turnover with ascorbate/TMPD. Sodium ascorbate (30 mM) and TMPD (0.5 mM) was added to a sample of resting cytochrome oxidase (2.8 μM) in potassium phosphate (0.1 M, pH 7.0) at 22° C. (A) Spectral changes at 605–625 nm (upper trace) and 433–413 nm (lower trace) were monitored with time as a measure of steady-state cytochrome *a* reduction and cytochrome a_3 spin state respectively. (B) In separate experiments, sodium dithionite (28 mM) was added at various times following initiation of turnover with ascorbate/TMPD and the spectral changes at 444 nm and 605 nm monitored. The rates of reduction of cytochromes *a* (●———●) and a_3 (○———○) were then calculated as indicated in the methods section and presented as shown. The rate for cytochrome *a* reduction was found to be the same for all samples in the time-scale used and is shown as a single plot.

molecules capable of fast intramolecular electron transfer becomes greater the longer turnover proceeds. Addition of dithionite 8 min after turnover results in a fast reduction of both haems (Fig. 2B). The process of conversion of the enzyme population to a population showing fast intramolecular transfer can also be seen to be accompanied by a shift to the red in the position of the Soret maximum (Fig. 2A), previously noted by Nicholls and Hildebrandt [9], and a fall in the steady-state reduction level of cytochrome *a*. A simple explanation for these findings is that under turnover conditions the population of oxidase molecules converts from a resting to a more catalytically active state. When all the molecules have undergone at least one catalytic cycle, then a simultaneous reduction of both haems occurs on the addition of dithionite. Bickar et al. [23] report a faster rate of cytochrome a_3 reduction on anaerobiosis following a short period of turnover but note that the rate seen was still less than the rate of electron transfer to cytochrome a_3 in the presence of oxygen. On the basis of the present results, it might be suggested that for those experi-

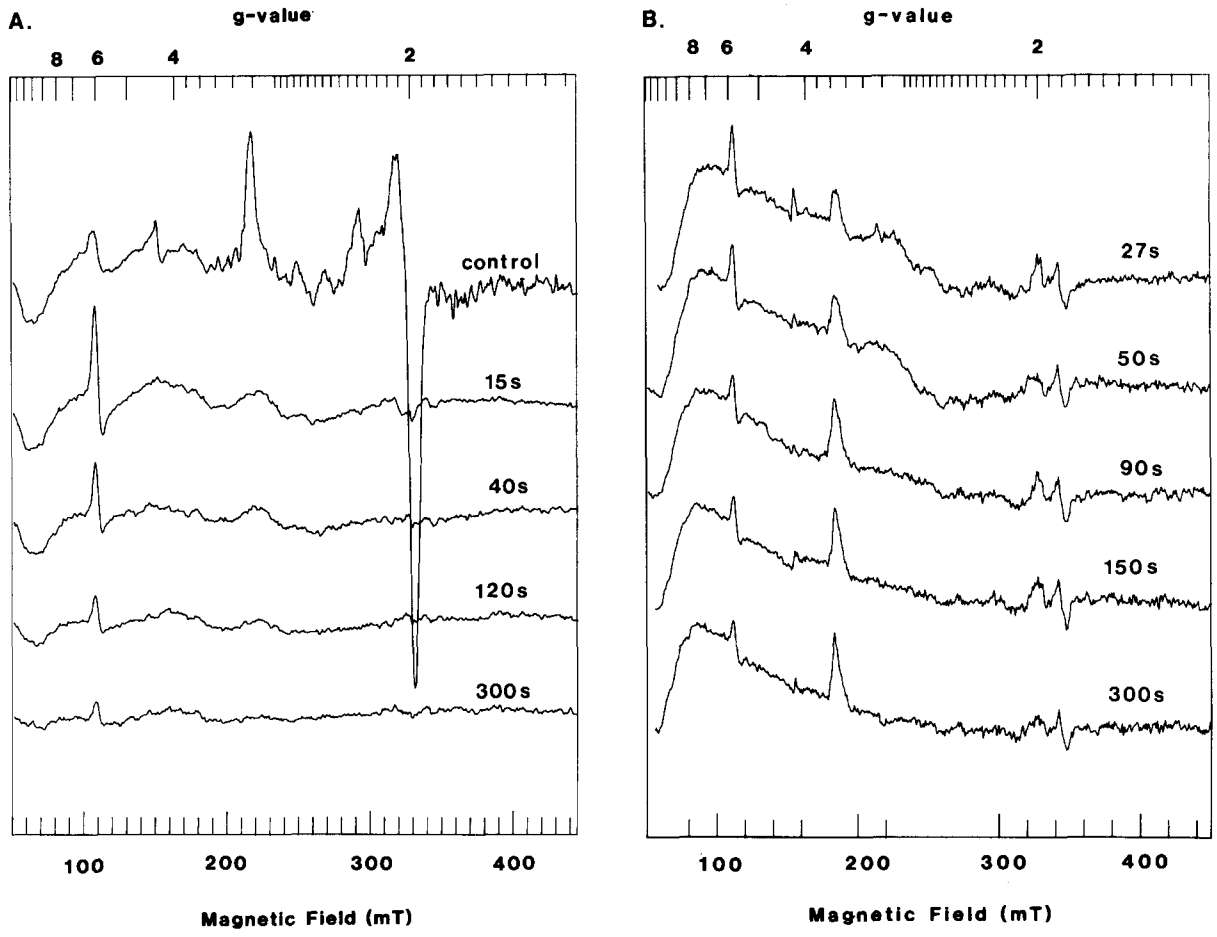

Fig. 3. EPR spectra of cytochrome oxidase following the addition of dithionite to the resting enzyme, (A) in the absence and (B) in the presence of cyanide. (A) Sodium dithionite (22 mM) was added to a solution of cytochrome oxidase (178 μM) in potassium phosphate (0.1 M, pH 7.0). Samples of the mixture were taken at the indicated times and frozen in liquid N_2 for EPR examination. (B) As for (A), but in the presence of potassium cyanide (5 mM). EPR conditions as described in the Materials and Methods section, with the gain in (A) at 2000 and the gain in (B) at 4000.

ments there was an incomplete conversion of the enzyme population to the catalytically active state. A longer period of turnover would have allowed for a full conversion and a simultaneous reduction of both haems on anaerobiosis.

The species of oxidase formed a few seconds after the addition of dithionite to the resting enzyme would appear to be a half-reduced high-spin a_3 form of the enzyme. Cytochrome a becomes fully reduced as also does Cu_A, as assessed by the disappearance of the 830 nm band in the optical spectrum (results not shown) and the loss of the $g = 2$ signal in EPR (Fig. 3). Ferric haem a_3 remains high spin throughout the reaction, as assessed by its Soret absorption maximum at 418 nm (Fig. 1), and also from the presence of the weak absorption band around 655 nm (Fig. 4), attributed to high-spin haem a_3 [30]. In the EPR spectrum, the $g = 3$ signal, attributed to cytochrome a^{3+} [31], largely disappears, although, as noted by Johnson et al. [32], this does not exactly parallel the reduction of cytochrome a as monitored by optical absorbance. A high-spin haem signal at $g = 6$ appears a few seconds after the addition of dithionite (Fig. 3a) then also slowly

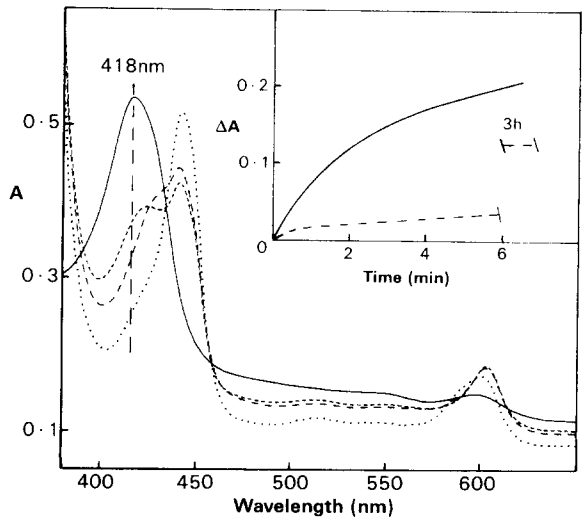

Fig. 5. Dithionite-induced spectral changes in cytochrome oxidase in the presence of cyanide. Sodium dithionite (28 mM) was added to cytochrome oxidase (3.6 μM) in potassium phosphate (0.1 M, pH 7.0) in the presence of potassium cyanide (5 mM). The spectrum was recorded before (continuous line), and 1 min (short-dashed line), 10 min (long-dashed line) and 24 h (dotted line) after dithionite addition. Inset: Rate of appearance of the spectroscopically-visible (433–413 nm) cyanide complex following dithionite addition. Dashed line shows the rate of appearance of the cyanide complex in the absence of dithionite.

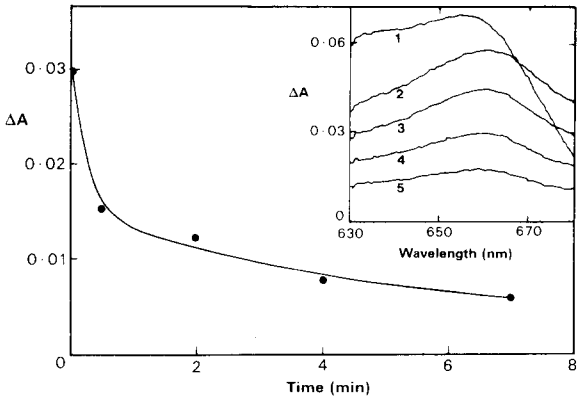

Fig. 4. Rate of change of the 655 nm band in resting cytochrome oxidase following the addition of dithionite. Sodium dithionite (28 mM) was added to a solution of cytochrome oxidase (35 μM) in potassium phosphate (0.1 M, pH 7.0) and the rate of disappearance of the absorbance at 655 nm monitored with time. Inset: Spectral scans taken before (1), and 15 s (2), 90 s (3), 220 s (4), and 420 s (5) after dithionite addition. The large change in the α-band region of haem a contributes to the the apparent initial drop in the 655 nm band absorbance seen in the main plot.

decays at approximately the same rate as the rate of reduction of haem a_3. The $g = 12$ signal, used by Baker et al. [40] as an indicator of the species of enzyme that shows only slow reactivity with cyanide, also decays at the same rate (Fig. 3a). In the presence of cyanide (Fig. 3b), the $g = 12$ signal remains largely unaffected by dithionite whereas the decay of the $g = 6$ signal is associated with the growth of a $g = 3.6$ signal (Fig. 3b) indicative of the formation of a low-spin a_3/CN complex [31,32]. The rate of formation of the $g = 3.6$ signal was found to parallel the rate of formation of the spectroscopically visible cyanide complex (Fig. 5). No EPR signals which could be attributed to copper in any significant amounts could be detected after dithionite addition, either in the presence or absence of cyanide (Fig. 3).

Fig. 6 shows the results of anaerobic reduction of the resting enzyme using TMPD (Fig. 6a) and ferrocytochrome c (Fig. 6b), in the presence of cyanide. It can be seen that following an initial

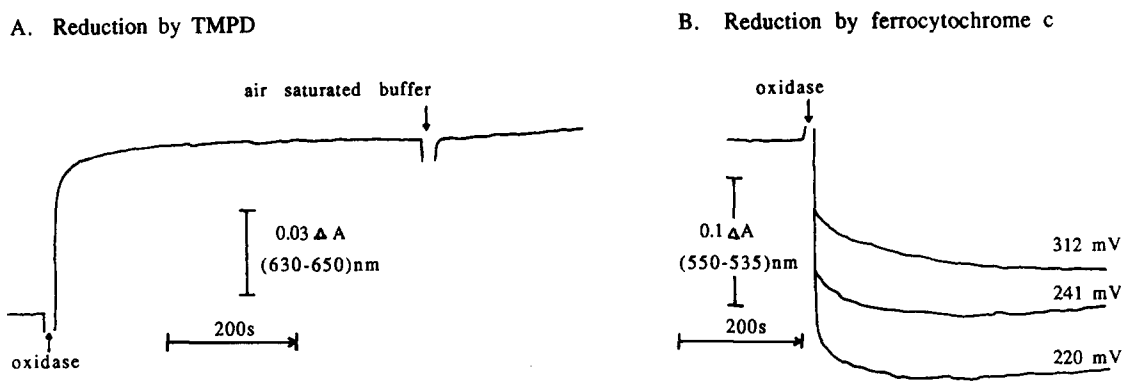

Fig. 6. Oxidation of (A) TMPD and (B) ferrocytochrome *c* on the addition of resting cytochrome oxidase under anaerobic conditions in the presence of cyanide. (A) Anaerobic cytochrome oxidase (see methods section) (2.9 μM) was added to an anaerobic solution of TMPD (500 mM) in Hepes buffer (50 mM, pH 7.0) containing potassium cyanide (5 mM). The absorbance change at 630–650 nm, was monitored with time. At the point indicated, 30 ml of air-saturated HEPES buffer was added to the total volume of 3 ml. (B) Anaerobic cytochrome oxidase (2.9 μM) was added to anaerobic mixtures of ferro- and ferricytochrome *c* (total cytochrome *c* concentration 73 μM) in Hepes buffer (50 mM, pH 7.0) containing potassium cyanide (5 mM). The absorbance changes at 550–535 nm were monitored with time. The redox potential of the mixture for each experiment was calculated from the final ratio of oxidised to reduced cytochrome *c*.

rapid oxidation of either reductant, a slow oxidation phase occurs over a period of 1–2 min. With TMPD as reductant over the redox range 100–200 mV (Fig. 7), an average value of 2.3 ± 0.2 electrons per oxidase molecule was found for the fast initial entry leading to a final total value of 3.0 ± 0.2 electrons per oxidase molecule. Variations in the value for electron entry in the initial fast phase corresponded closely to variations between samples in the proportion of oxidase molecules showing a fast intramolecular electron transfer. However, the final total of electrons per oxidase, in the presence of cyanide, always approximated to a value of 3 at redox values below +200 mV. Above +200 mV (Fig. 7), the number of electrons per oxidase/cyanide complex falls to lower values suggestive of the presence of a single-electron redox center with an apparent mid-point potential of 240 mV, probably Cu_A. Interestingly, the slow phase of electron entry is still present at the more positive redox values, although the number of electron equivalents corresponding to the slow phase in the presence of cytochrome *c* fell from 0.7 to 0.5. It would appear that this phase of electron transfer corresponds to the reduction of a more positive centre (Cu_B?) at a rate still limited in the resting enzyme by a slow kinetic transition.

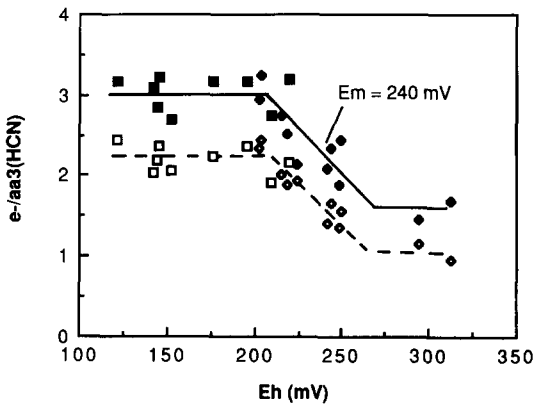

Fig. 7. Electron uptake by resting cytochrome oxidase, in the presence of cyanide, as a function of redox potential. Conditions of measurement were as indicated in Fig. 5. Reductions using TMPD, at various $TMPD^+$/TMPD ratios are indicated by the square symbols. Reductions using cytochrome *c* at various c^{3+}/c^{2+} ratios are indicated by the diamond symbols. Both the initial fast phase of electron uptake (open symbols, dashed line) and the final total change (solid symbols, solid line) are shown.

Also shown in Fig. 6A is the effect of oxygen addition (air-saturated buffer) to the anaerobic system of the TMPD-reduced oxidase/cyanide complex. According to the O-loop mechanism [37], addition of oxygen to the enzyme, previously reduced under conditions which excluded O_2 or H_2O_2, and in the presence of cyanide to inhibit turnover, should result in the take-up of a further two reducing equivalents as the putative hydrogen

carrier, O_2, is reduced to H_2O_2. No such uptake can be seen (Fig. 6A). The only change subsequent to oxygen addition is a slow production of $TMPD^+$ at a rate commensurate with the measured rate of TMPD autoxidation in an aerobic system.

Discussion

The results in Fig. 1 confirm earlier reports [4,22,28,33–35] of the biphasic reduction of ferric cytochrome oxidase in the resting form. Analysis of the spectral changes at 444 nm and 605 nm now clearly permits us to attribute the second phase of reduction to a slow intramolecular electron transfer to cytochrome a_3. The long half-time of this phase (around 50 s) agrees with the slow process of reduction reported for several oxidase preparations when reductants other than cytochrome *c* are used [22,28,34]. A small proportion of molecules in the present preparations showed a faster cytochrome a_3 reduction rate and this proportion increased during turnover. Addition of dithionite to the sample at different times after initiation of turnover reveals the changes in the population of oxidase molecules between slow and fast species.

The rate constants for electron transfer within the enzyme have been reported to be oxygen-sensitive [24,36], and it has been suggested by Mitchell et al. [37] that molecular oxygen is required for fast intramolecular electron transfer. The present results do not support this conclusion. The rate of removal of oxygen from the suspending medium by dithionite under the present conditions would be largely independent of whether the dithionite was added before or during turnover, and yet the rate of cytochrome a_3 reduction increases greatly following turnover. We therefore conclude that turnover initiates a conversion of the resting oxidase to a form which can exhibit fast intramolecular electron transfer even in the absence of oxygen.

The results shown in Figs. 2B and 6A provide a direct falsification of a simple type of O-loop mechanism [37]. If such a mechanism were correct, then from a consideration of the results shown in Fig. 2B it would have to be assumed that the resting and activated (pulsed) forms of the enzyme differed by the fact that H_2O_2 is absent in the resting form of the enzyme but present (presumably trapped internally) in the pulsed form. Addition of dithionite or other reductant to the resting enzyme in the absence of O_2 would therefore only result in the reduction of *a* and Cu_A. However, a similar addition of reductant to the pulsed enzyme would result in full reduction as reducing equivalents were transferred to the a_3/Cu_B centre by the internal H_2O_2. Fig. 6A shows the assumption to be false. Reduction of the oxidised resting enzyme under strictly anaerobic conditions in the presence of cyanide and using reductants (TMPD and c^{2+}) that avoid any direct production of H_2O_2, results in the uptake of three electron equivalents (to *a*, Cu_A and Cu_B?). Reintroduction of O_2 does not result in any further electron uptake. There is no evidence for the formation of H_2O_2 from the reintroduced O_2.

According to the kinetic model of Bickar et al. [23], the slow internal electron transfer observed with resting oxidase results from a thermodynamic barrier produced by a low redox potential of cytochrome a_3, favouring oxidised a_3 and reduced *a*. The two centers are considered to be in redox equilibrium by fast reversible electron transfer. Removal of the species a^{3+}/a_3^{2+} (for example by O_2) or generation of the species a^{2+}/a_3^{3+} (for example by increasing the rate of electron transfer from reductant to cytochrome *a*) should affect the rate of intramolecular transfer. Some indication of these effects have been reported [23]. However, a close analysis of the reduction of enzyme exposed to dithionite after various times of turnover shows that what appears to be changing by exposure to oxygen is the relative proportions of resting and pulsed forms of the enzyme in the mixture. This results in a biphasic reduction of a_3 as the relative proportions of the fast (pulsed) and slow (resting) phases change without any noticeable effect on the individual rate constants themselves. We see a similar effect when cytochrome *c* is present during reduction by dithionite.

Further evidence contrary to the redox-equilibrium model is the finding that the rate of a_3 reduction in the resting enzyme is not affected by changes in reductant (dithionite) concentration over a wide range. The results show that the dithionite concentration can be lowered to a level where cytochrome *a* reduction is slowed to a few seconds (Fig. 1), yet no significant change can be

seen in the intramolecular electron-transfer rate to cytochrome a_3 compared to that measured when very high dithionite concentrations are used. The apparent increase in this rate in the presence of cytochrome *c*, reported in Ref. 23 and discussed above, appears to be a consequence of the effect of cytochrome *c* on the resting/pulsed transition. A final piece of evidence against a possible fast electron equilibration between *a* and a_3 is the finding that the slow rate of intramolecular electron transfer (presumably to Cu_B in this case) remains unaltered in the presence of cyanide where the redox potential of a_3 is very much lowered by the formation of the ferric a_3/cyanide complex. The slow phase of electron entry into the oxidase is also still present over a range of redox values including the more positive ones where electron entry into Cu_A does not occur (Fig. 6). We conclude, contrary to the model of Bickar et al., that there is a kinetic rather than thermodynamic barrier for electron transfer between a/Cu_A and a_3/Cu_B in the resting enzyme.

Scholes and Malmström report that a two electron reduction of cytochrome oxidase is sufficient to trigger a conformational transition of the enzyme to the active (pulsed) state [27]. The basis for their conclusion is that cyanide binding, following an initial 2-electron addition of the enzyme, appears to be sufficiently fast to inhibit turnover [27,38]. Cyanide binding to the resting form of most preparations of cytochrome oxidase is at a low rate (days) [39], although several faster phases of binding can be present depending on the method of oxidase preparation [12,40]. On the other hand, binding to the partially reduced, cyanide-sensitive, form of the enzyme occurs at a rate calculated as in excess of $10^6\ M^{-1} \cdot s^{-1}$ [41]. The question of interest to the present study is how many electron equivalents are required to produce the cyanide-sensitive form from the fully oxidised resting species. Nicholls and Chanady [42] found the first two electrons introduced into the ferric-oxidase/cyanide complex resulted in the reduction of haem *a* with the second equivalent reducing a spectroscopically silent center (presumed to be Cu_B). Reduction of the 830 nm chromophore (EPR-detectable copper) required a further one-electron entry at more negative redox values. Jones et al. [41] in a re-examination of the reactions of cyanide with cytochrome oxidase concluded that the entry of either two or three electrons was necessary to produce the partially reduced, cyanide-sensitive, form. Both these findings are in agreement with the present results (Fig. 7). At redox values more negative than around +200 mV, three electrons titrate into the oxidase in the presence of cyanide. As the redox potential of the system is made more positive, Cu_A becomes non-reducible and two-electron equivalents enter. At redox potentials approaching 300 mV, an effect on cytochrome *a* would also be seen, the redox potential for *a* being around 285 mV under conditions where a_3 remains oxidised and liganded to cyanide [24]. The conclusion of Scholes and Malmström [27] that reduction of both cytochrome *a* and Cu_A is sufficient to trigger a conformational transition to the cyanide-sensitive state was based on measurements of electron entry into the enzyme over the first five seconds of the reaction (Fig. 1a of Ref. 27). The present results show that below +200 mV, a rapid two electron entry is followed by a further slow one-electron entry into the enzyme. The rate for this reaction is the same as the rate of development of the oxidase/cyanide complex and also, in the absence of cyanide, the rate of increasing turnover of the enzyme mixture (Fig. 2). At more positive redox values where the total number of reducing equivalents is lower, the slow electron entry is still present. A simple explanation for the absence of turnover resulting in the presence of cyanide is that both turnover and cyanide binding are limited by the slow rate of conversion of the enzyme from the resting to the active form. Until this occurs, turnover is very low (or is it absent?) and cyanide binding is extremely slow. As soon as the transition takes place (at the same rate as entry of the third electron), cyanide binding occurs to ferric a_3 before the Cu_B/a_3 center can become fully reduced to react with oxygen. From the reaction scheme

$$\text{resting (closed) form} \xrightarrow[(0.02\ s^{-1})]{ne^-} \text{active (open) form} \xrightarrow[(10^6\ M^{-1} \cdot s^{-1})]{HCN} \text{inhibited form}$$

there is no reason to postulate the initial formation of an inhibitory but spectroscopically silent cyanide complex [27] as long as the turnover rate

of the nonliganded resting form is equal to or less than the resting to active transition. For the preparation used in the present work, which comprises enzyme predominantly in the resting form, there is also no evidence to conclude that the cyanide-sensitive form of the enzyme results from the reduction of cytochrome a and Cu_A alone. The half-reduced species still contains high-spin cytochrome a_3 spin coupled to Cu_B. The formation of the a_3/cyanide complex as monitored by the visible absorbance and EPR spectral changes occurs at the same rate as the slow reduction of Cu_B. These conclusions are in agreement with the magnetic circular dichroism work of Thomson et al. [44] and Hill et al. [45] who conclude that, in the partially reduced enzyme, a cyanide bridge is not present and Cu_B is reduced. Both a visible and an EPR detectable a_3/cyanide complex can be seen. The binding of cyanide to the fully oxidised Cu_B/a_3 center to form a bridging ligand between the metal atoms requires several hours. Even so, this species can still be clearly detected in the Soret region of the visible spectra (Ref. 45; see also Fig. 4) even though cytochrome a_3 and Cu_B remain EPR silent until made EPR detectable by addition of NO.

Fig. 8 summarises the present findings with regard to the activation by reduction of the resting form of cytochrome oxidase. At redox values below approx. +200 mV, an initial fast reduction of cytochrome a and Cu_A takes place with the entry of 2 electron equivalents into the enzyme (stage 1 in Fig. 8). Above +200 mV, the number of electron equivalents associated with this fast phase drops, first with the titration of Cu_A (around +250 mV) and then as cytochrome a titrates (around +320 mV with unliganded oxidised a_3). Cytochrome a_3 remains high spin and spin-coupled to Cu_B with no EPR resonances from either. The slow entry of a third electron into the system (stage 2) is associated with the reduction of Cu_B and the appearance of a $g = 6$ EPR signal from a_3. The amount of $g = 6$ species present at any time in the sample will depend on the rate of electron transfer to Cu_B and the rate of the subsequent reactions of a_3. The reduction of Cu_B and the uncoupling of a_3 then results in a conformational transition (stage 3) to produce the cyanide-sensitive species and also enzyme capable of a

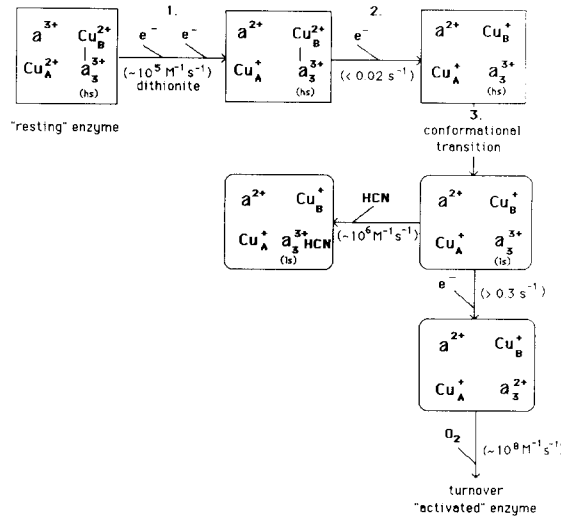

Fig. 8. The electron-transfer sequence for activation by reduction of the resting form of cytochrome oxidase derived from the present experiments. The oxidised resting form of the enzyme is indicated with spin coupling between Cu_b and a_3, with a_3 showing a characteristic high-spin (hs) Soret absorption. Reduction of Cu_B (stage 2) results in the uncoupling of Cu_B from a_3 giving rise to a $g = 6$ EPR signal and inducing a conformational transition (stage 3) to a cyanide reactive form, or in the absence of cyanide, to a form with faster internal electron transfer to the Cu_B/a_3 center. Turnover follows full reduction of Cu_B/a_3 and low-spin (ls) a_3 can be seen as the predominant ferric a_3 species. The value for the rate of cyanide binding to the cyanide sensitive form is taken from Ref. [41]. For further details, see text.

faster intramolecular electron transfer between a/Cu_A and Cu_B/a_3. In the presence of cyanide, the rate of cyanide binding is still faster than electron transfer to the Cu_B/a_3 center, and a partially reduced cyanide complex of the enzyme is formed. Eventually, over a period of several hours (Fig. 5), the cyanide complex of the fully reduced enzyme develops. In the absence of cyanide, intramolecular electron transfer can take place to reduce the Cu_B/a_3 center fully for subsequent reaction with O_2. Low-spin ferric a_3 is present during steady-state turnover of the activated enzyme (Fig. 2a) but its appearance may not be associated with the conformational transition, as indicated in Figure 8, but may be produced at some later stage during the catalytic cycle. On the present evidence it is not possible to clarify this point. The mechanism of how cytochrome c can accelerate the slow intramolecular electron transfer rate also remains unknown. On

the basis of the model shown in Fig. 8, the effect could be explained if cytochrome *c* had some ability to bypass the slow intramolecular electron-transfer pathway and directly reduce Cu_B and hence induce the conformational transition. Such an action would convert some of the enzyme population to the activated form and give rise to the biphasic reduction curve for cytochrome a_3. Previous suggestions have been made for the presence of a more direct pathway from cytochrome *c* to Cu_B [42].

The conversion of the partially reduced species from low to high cyanide reactivity is likely to be associated with some rearrangement of the bridging ligand between Cu_B and a_3 triggered by the reduction of Cu_B. On the other hand, the increase in the intramolecular electron transfer rate to the Cu_B/a_3 center must be associated with a more drastic conformational change to lower or remove the kinetic barrier to Cu_B/a_3 reduction. Alleyne and Wilson [43] report distance changes between cytochrome *c* and cytochrome *a* in the partially reduced cyanide form of the enzyme compared to the resting form. Copeland et al. [47] describe tryptophan fluorescent changes in cytochrome oxidase upon reduction by cytochrome *c*. (In both these reports, the transition was said to be triggered by the reduction of cytochrome *a* and Cu_A but this conclusion was based on the assumption that Cu_B remains oxidised in the partially reduced cyanide complex.) An indicator of a possible link between changes in the Cu_B/a_3 center and protein conformational changes may be provided by the observed change of ferric cytochrome a_3 from high to low spin during activation. Coordination changes in the iron of cytochrome a_3 might mediate a major allosteric transition somewhat analogous to haemoglobin on the binding of oxygen. On the present evidence however, it is not possible to state whether such changes are a cause or a consequence of activation or, indeed, a normal part of the catalytic cycle.

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