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Kinetic Studies on the Reaction between Cytochrome *c* Oxidase and Ferrocytochrome *c*

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In stopped-flow experiments in which oxidized cytochrome *c* oxidase was mixed with ferrocytochrome *c* in the presence of a range of oxygen concentrations and in the absence and presence of cyanide, a fast phase, reflecting a rapid approach to an equilibrium, was observed. Within this phase, one or two molecules of ferrocytochrome were oxidized per haem group of cytochrome *a*, depending on the concentration of ferrocytochrome *c* used. The reasons for this are discussed in terms of a mechanism in which all electrons enter through cytochrome *a*, which, in turn, is in rapid equilibrium with a second site, identified with 'visible' copper (830 nm-absorbing) Cu_d (Beinert *et al.*, 1971). The value of the bimolecular rate constant for the reaction between cytochromes c^{2+} and a^{3+} was between 10^6 and $10^7\,M^{-1}\cdot s^{-1}$; some variability from preparation to preparation was observed. At high ferrocytochrome *c* concentrations, the initial reaction of cytochrome c^{2+} with cytochrome a^{3+} could be isolated from the reaction involving the 'visible' copper and the stoicheiometry was found to approach one molecule of cytochrome c^{2+} oxidized for each molecule of cytochrome a^{3+} reduced. At low ferrocytochrome *c* concentrations, however, both sites (i.e. cytochrome *a* and Cu_d) were reduced simultaneously and the stoicheiometry of the initial reaction was closer to two molecules of cytochrome c^{2+} oxidized per molecule of cytochrome *a* reduced. The bleaching of the 830 nm band lagged behind or was simultaneous with the formation of the 605 nm band and does not depend on the cytochrome *c* concentration, whereas the extinction at the steady-state does. The time-course of the return of the 830 nm-absorbing species is much faster than the bleaching of the 605 nm-absorbing component, and parallels that of the turnover phase of cytochrome c^{2+} oxidation. Additions of cyanide to the oxidase preparations had no effect on the observed stoicheiometry or kinetics of the reduction of cytochrome *a* and 'visible' copper, but inhibited electron transfer to the other two sites, cytochrome a_3 and the undetectable copper, Cu_u.

The steady-state kinetics of the reaction between solubilized preparations of oxidized cytochrome oxidase (EC 1.9.3.1) and its substrate, reduced cytochrome *c*, have been extensively studied by a number of workers (Yonetani, 1960*b*; Smith, 1955; Minnaert, 1961*a*,*b*). However, the pre-steady-state transient situation has been less fully explored. Gibson *et al.* 1965) have examined the spectral changes occurring on mixing reduced cytochrome *c* with its oxidase in a stopped-flow apparatus. They concluded that the spectral changes observed under anaerobic conditions (approx. $0.2\,\mu M\text{-}O_2$) indicated a fast ($k = 10^6$–$10^7\,M^{-1}\cdot s^{-1}$) reduction of cytochrome *a* and a much slower reduction of cytochrome a_3. They also presented evidence which indicates that the reduction of another electron acceptor in the oxidase molecule is dynamically linked to the reduction of cytochrome *a* and, on the basis of previous investigations (Beinert & Palmer, 1964), proposed that this acceptor may be copper. Andréasson *et al.* (1972) have suggested, however, that the reduction of a second electron acceptor in the oxidase molecule only takes place in the presence of oxygen. The interaction of cytochrome *c* with cytochrome *c* oxidase has also been investigated by Antonini *et al.* (1970), whose results indicated that reoxidation of

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partially reduced enzyme is slow, and they have taken this as evidence for multi-electron steps in the reduction of oxygen by the oxidase.

The present paper seeks to extend our knowledge of the reaction between cytochrome *c* oxidase and its substrate, and in particular to monitor the spectral changes occurring in the near-i.r. region of the spectrum which are thought to reflect valency changes in one of the two copper atoms Cu_a associated with cytochrome *a* and a_3.

Materials and Methods

Cytochrome oxidase was prepared by a modification of the method of Yonetani (1960*a*) in which 1 μM-EDTA was incorporated as a chelating agent into the last fractionation steps to ensure removal of adventitiously bound copper which is inactive in electron transfer (Lemberg, 1968). The concentration of oxidase (in terms of haem *a*) was calculated by using $\varepsilon = 21\,000\,M^{-1}\cdot cm^{-1}$ at 605 nm for the fully reduced enzyme (Yonetani, 1960*a*).

Sigma type III cytochrome *c* [Sigma (London) Chemical Co., Kingston-on-Thames, Surrey, U.K.] was used without further purification and the reduced protein was prepared by passing the dithionite-reduced material through a Sephadex G-25 column to remove excess of dithionite and its oxidation products. The reduced cytochrome *c* prepared by this method contained approx. 5% of oxidized protein on leaving the column, but autoxidation increased this to approx. 15% during the course of 1 h. The concentration of cytochrome *c* was calculated by using $\varepsilon = 27\,600\,M^{-1}\cdot cm^{-1}$ at 550 nm for the reduced protein (Schejter *et al.*, 1963).

The buffer used throughout was 0.1 M-potassium phosphate, pH 7.4, containing 1% Emasol 4130 (Kao Soap Co., Tokyo, Japan).

Stopped-flow experiments were carried out in a Durrum–Gibson instrument with a 2 cm light-path and a dead-time of 4 ms.

The monitoring wavelengths chosen depended on the concentration regimes under which the experiments were conducted. Where the concentrations were low, the wavelengths chosen were 550 nm for the cytochrome *c*, and 605 nm, which is thought to represent mostly cytochrome *a*. At high concentrations, however, the absorbance of the peak at 550 nm was too high and the oxidation of cytochrome *c* was followed either at 560 or 563 nm, the latter wavelength being isosbestic for fully reduced versus fully oxidized oxidase. The changes in the near-i.r. region of the spectrum (at 830 nm say) were corrected for the contribution made by cytochrome *c* in this region, or measurements were made at 740 nm, a wavelength isosbestic for reduced and oxidized cytochrome *c*. Spectra were obtained by using a Cary 14 instrument.

Results

It is convenient to consider the experiments in two categories: those in which the reaction between reduced cytochrome *c* and its oxidase was carried out at low reagent concentrations to examine the fast initial phases of the reaction, and those at high reagent concentration, where it is possible to monitor changes in the weak absorption band of cytochrome oxidase centred at 830 nm.

Low-concentration experiments

On mixing reduced cytochrome *c* with oxidized oxidase in the presence of oxygen a fast reaction is observed at the level of both cytochrome *c* (monitored at 550 nm) and cytochrome *a* (monitored at 605 nm) see Figs. 1 and 2. The rate of this reaction depended, for a given preparation, on the reagent concentration and was found to vary, from preparation to preparation. The second-order rate constants, calculated assuming a simple bimolecular process varied between approx. $4\times10^6\,M^{-1}\cdot s^{-1}$, and $1\times10^7\,M^{-1}\cdot s^{-1}$. The first of these values is in close agreement with that of Gibson *et al.* (1965), for the Yonetani (1960*a*) preparation, but the latter is high, being closer to the value found by these authors for the Griffiths & Wharton (1961*a*) preparation.

Figs. 1 and 2 compare the extinction changes at 550 nm with those at 605 nm. The fast phase at 550 nm gives way to a slower process in which all the available cytochrome *c* is oxidized. However, at 605 nm there is a single process, coincident with the fast

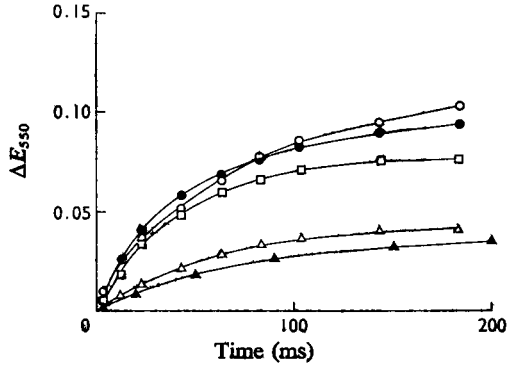

Fig. 1. *Aerobic oxidation of ferrocytochrome c by cytochrome oxidase*

Cytochrome oxidase (4.5 μM) in the presence of 130 μM-O_2 was mixed with the following concentrations of ferrocytochrome *c*: ▲, 1.55 μM; △, 2.85 μM; □, 5.1 μM; ●, 9.8 μM; ○, 18.5 μM. Observations were made at 550 nm (2 cm light-path). Experiments were performed at 21°C, in 0.1 M-potassium phosphate buffer, pH 7.4, containing 1% Emasol 4130.

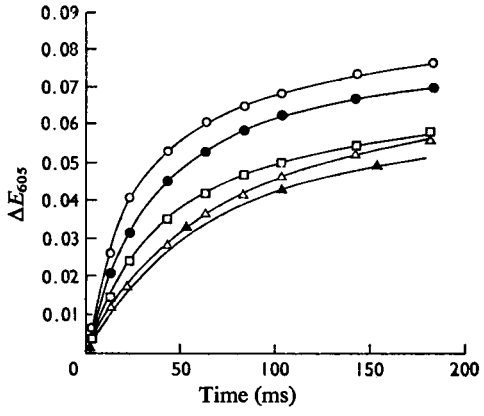

Fig. 2. *Progress curves for the reduction of cytochrome oxidase by ferrocytochrome c in the presence of oxygen* (130 μM)

The formation of reduced cytochrome oxidase was followed at 605 nm. The concentration of cytochrome oxidase was 4.5 μM and the following concentrations of ferrocytochrome *c* were used: ▲, 1.55 μM; △, 2.85 μM; □, 5.1 μM; ●, 9.8 μM; ○, 18.5 μM. All other conditions as in Fig. 1.

oxidation of cytochrome *c*, representing the approach to a steady state, the position of which depends on the concentration of cytochrome *c* (see Fig. 2). The return from the steady-state level of reduction to the fully oxidized enzyme is discussed below. Where the reduced cytochrome *c* is in excess over the oxidase (defined as *a*, a_3, 2Cu) the rapid extinction change at 605 nm corresponds to 80% of the total change that might be expected on going from the fully oxidized to the fully reduced enzyme.

Plots of extinction changes at 605 nm versus those at 550 nm at identical times are shown in Fig. 3. Since time is eliminated, the slope of the graph at any point is directly related to the stoicheiometry of the reaction. Where the cytochrome *c* concentration is low, the plots yield straight lines of slope 1.4, in close agreement with earlier work (Gibson *et al.*, 1965). At higher concentrations of cytochrome *c*, however, a deviation from this linear behaviour is seen. Such behaviour may reflect a change in the stoicheiometry of electron transfer from cytochrome *c* to the oxidase molecule as the concentration of cytochrome c^{2+} changes.

High-concentration experiments

On mixing a high concentration of oxidase (approx. 20 μM-haem *a* after mixing) with reduced cytochrome *c* (approx. 100 μM after mixing) in the presence of 40 μM-oxygen, rapid spectroscopic changes were observed at 605 nm, 560 nm, and in the near-i.r. region of the spectrum; these were followed by slower processes taking several seconds to complete. Figs. 4(*a*) and 4(*b*) show the results of a typical experiment. The rapid oxidation of cytochrome *c* in a 'burst' phase is accompanied by the formation of the 605 nm band and the bleaching of the 830 nm band. Where the oxygen concentration is greater than that of the oxidase, these fast processes are followed by turnover in cytochrome *c* oxidation, reappearance of the long-wavelength absorption, and bleaching of the 605 nm band (Fig. 4*b*). The bleaching of the 605 nm band is the slowest process observed (cf. Antonini *et al.*, 1970).

The rapid bleaching at 830 nm [a wavelength at which both haem and copper are thought to make contributions (Greenwood *et al.*, 1974) (Fig. 4*a*)] was found to either parallel or lag slightly behind the formation of the 605 nm band, but was never found to precede it. The contribution of cytochrome *c* to the changes in this long-wavelength region may be discounted, as the traces were either corrected for such contribution or the experiments were repeated at 740 nm, a wavelength isosbestic for ferri- and ferrocytochrome *c*.

The total ΔE observed at 605 nm in the experiment shown in Fig. 4*a* is 0.18: when corrected for the

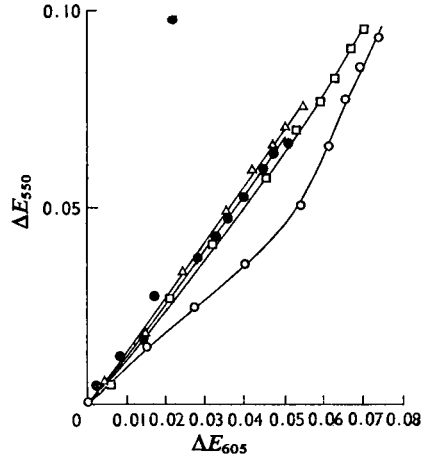

Fig. 3. *Relationship between the changes in absorbance at 550 and 605 nm during the reaction of oxidized cytochrome oxidase* (4.5 μM) *with several concentrations of ferrocytochrome c*

The following ferrocytochrome *c* concentrations were used: ●, 2.85 μM; △, 5.1 μM; □, 9.85 μM; ○, 18.5 μM. The points were obtained by correlating changes in absorbance at the two wavelengths during the first 100 ms of the reaction. Oxygen was present in all experiments (130 μM). All other conditions as in Fig. 1.

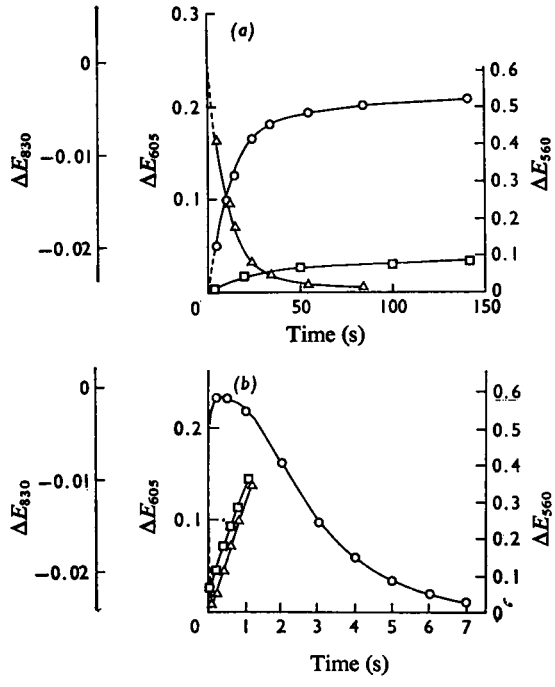

Fig. 4. *Progress curves for the aerobic oxidation of ferrocytochrome c by cytochrome oxidase*

Cytochrome oxidase (22 μM) was mixed with 82 μM-ferrocytochrome *c* in the presence of 40 μM-O_2 and the oxidation process followed at 605 nm (○), 830 nm (△) and 560 nm (□). All other conditions as in Fig. 1.

loss of absorbance in the dead-time of the instrument (i.e. that time which elapses between mixing and observation) the ΔE at 605 nm is increased to 0.23. This accounts for 48% of the expected ΔE at this wavelength if the oxidase was fully reduced. Taking the extinction coefficients for haem *a* proposed by Yonetani (1960*a*), the ΔE would account for 68% of the expected contribution of cytochrome *a* on full reduction. It seems likely in the light of the known slow reduction of cytochrome a_3 (Gibson *et al.*, 1965) that over this time-range only cytochrome *a* is reduced, and the low value reflects a steady-state situation in the presence of oxygen in which only a portion of the cytochrome *a* is reduced. An alternative explanation might be that we lose much more in the dead-time, i.e. that the extrapolation back to zero time is incorrect. However, this can be ruled out, as the slow bleaching of the 605 nm absorption (Fig. 4*b*) from the steady-state value to the fully oxidized condition gives the same change in absorbance.

Fig. 4(*a*) shows that the changes at 830 nm are closely related in time to those at 605 and 560 nm. The possibility that the changes in the long-wavelength absorption belong entirely to the oxidase haem may be excluded on the basis of the following evidence. First, the re-formation of the 830 nm band is much faster than the bleaching of the haem band at 605 nm and partially occurs while the 605 nm band is in a steady state of reduction. Secondly, the observed ΔE in the burst phase of cytochrome *c* (from 5 to 50 ms) corresponds to approx. 1.1 electron equivalents (1 electron equiv. is equivalent to concentration of cytochrome *a*) whereas the ΔE at 605 nm during this same time-range accounts for only about 0.5 equivalent. It follows therefore that some other redox site is accepting electrons during this time, and that valence changes in this site are reported by the changes in the 830 nm band. The changes we observe at 830 nm are approximately half those expected on going from fully oxidized to fully reduced enzyme.

A similar situation was found for those experiments performed at low oxygen concentration (approx. 5 μM) and at high reagent concentrations. In the first 50 ms of observation 1 equivalent of cytochrome c^{2+} was oxidized and in the same time-period we observed changes at 605 nm which corresponded to about 0.5 equivalent of cytochrome *a*, and changes at 830 nm which accounted for 0.4 equivalent (see Fig. 5).

In all experiments we see the same basic pattern, i.e. more cytochrome *c* oxidized in the burst phase than cytochrome *a* reduced, and we are faced with the following alternatives to explain this discrepancy. (1) We have used the wrong Δ extinction coefficient at 605 nm and we should use a much lower value, say 11 000 $M^{-1} \cdot cm^{-1}$. This implies approximately equal contributions for cytochromes *a* and a_3 at this wavelength, as suggested for the oxidase in the presence of cytochrome *c* (Muijsers *et al.*, 1972). In the experiment depicted in Fig. 5 this would

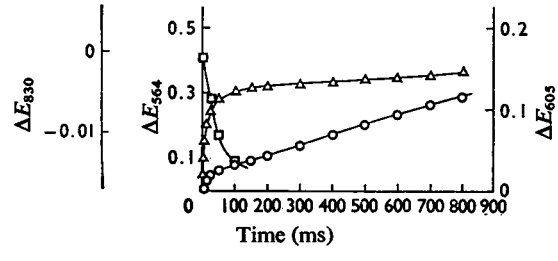

Fig. 5. *Oxidation of ferrocytochrome c by cytochrome c oxidase in the presence of a low oxygen concentration*

Cytochrome oxidase (21 μM) was mixed in the stopped-flow apparatus with 125 μM-ferrocytochrome *c* in the presence of approx. 5 μM-O_2. The progress curves were followed at 605 nm (△), 563 nm (○) and 830 nm (□). All other conditions as in Fig. 1.

increase the amount of cytochrome *a* reduced from 0.5 to 0.7 equivalent. However, if this were so we would expect a large slower change at 605 nm to account for the cytochrome a_3 reduction, which is known to occur under these conditions (Gibson *et al.*, 1965) and which should now be substantial at this wavelength. As we see only small slow changes at 605 nm and since the enzyme is fully reduced at the end of the experiment we feel that our results are not consistent with cytochromes *a* and a_3 making an equal contribution at this wavelength. (2) The alternative is that we lose a large fraction of the ΔE at 605 nm in the dead-time under these conditions. If so, then we must also lose ΔE at 563 nm and thus if we lose approx. one-half of the cytochrome *a* contribution in the dead-time we must lose at least 0.5 equivalent of cytochrome *c*. Thus over the first, say, 100 ms, approx. 2 equivalents of cytochrome *c* are used, indicating that not only cytochrome *a* but also another site is accepting electrons. The non-synchronous changes at 830 nm may well reflect this site and thereby implicate copper as an early electron acceptor in the oxidase molecule (cf. Beinert & Palmer, 1964).

No experiments were conducted which could be considered truly anaerobic, the lowest oxygen concentration being estimated at approx. 5 μM. Increasing the oxygen concentration from this value up to the fully oxygenated condition did not affect the rate of the reduction of cytochrome *a* monitored at 605 nm but did lower the steady-state level of reduction. Also the rate of bleaching at 830 nm was independent of oxygen concentration.

Where the reducing equivalents, present in the form of cytochrome *c*, are only slightly in excess over the oxidizing equivalents of oxygen in solution, complex kinetic patterns are observed, as for example in Fig. 6. A steady-state level of reduction of the cytochrome *a* observed at 605 nm and a steady-state turnover of cytochrome *c* occupy the first second of the reaction time. Thereafter cytochrome *a* begins to oxidize until a sharp transition point is reached (presumably when the oxygen is exhausted), after which the cytochrome *a* is once more reduced, but at a very low rate.

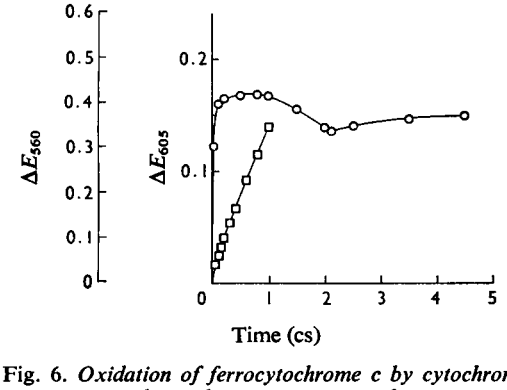

Fig. 6. *Oxidation of ferrocytochrome c by cytochrome c oxidase in limiting oxygen conditions*

Progress curves at 605 and 560 nm were obtained on mixing 22 μM-cytochrome oxidase with 114 μM-ferrocytochrome *c* in the presence of approx. 5 μM-O_2. The reaction was followed at 605 nm (○) and 560 nm (□). All other conditions as in Fig. 1.

Effect of cyanide

Some experiments were carried out with cytochrome oxidase which had been incubated with 10 mM-cyanide. Fig. 7 shows the comparison between aerobic experiments in the presence and absence of cyanide monitored at 605, 740 and 563 nm.

The changes at 605, 740 and 563 nm in the absence of cyanide are in accord with the experiment shown in Fig. 4. From Fig. 7 we see the same temporal relationships between the processes monitored at the various wavelengths, as shown in Fig. 4, and also the stoicheiometry remains the same, i.e. during the first 50 ms 1.6 equivalents of cytochrome *c* are oxidized, 0.7 equivalent of cytochrome *a* is reduced and 0.9 equivalent may be accounted for in the long-wavelength region. There are, however, large differences between the experiments in the presence and absence of cyanide during the time-range 50 ms to several seconds. In this time-range a very much slower turnover rate for cytochrome *c* is observed than in the absence of cyanide. It may be emphasized that even under these conditions of very high cyanide concentration the enzyme was not completely inhibited and a very slow turnover could be discerned, continuing for several minutes after mixing. In the presence of this inhibitor there is a small loss of absorbance at 605 nm after a protracted steady state. At 740 nm a short steady-state period is followed by a partial re-formation of absorbance at a rate somewhat lower than that observed for the re-formation of the absorbance in the absence of cyanide.

Discussion

On mixing oxidized cytochrome *c* oxidase with its substrate, reduced cytochrome *c*, we observe extinction changes at 605 and 550 nm which are fast, synchronous and dependent on the cytochrome *c* concentration. This close correlation between the optical events associated with redox changes in cytochromes *a* and *c* clearly suggest direct electron transfer between the two. The alternative is that the first acceptor on the oxidase reacts with cytochrome c^{2+} at the rate measured for the cytochrome c^{2+} oxidation and subsequently passes on the electron

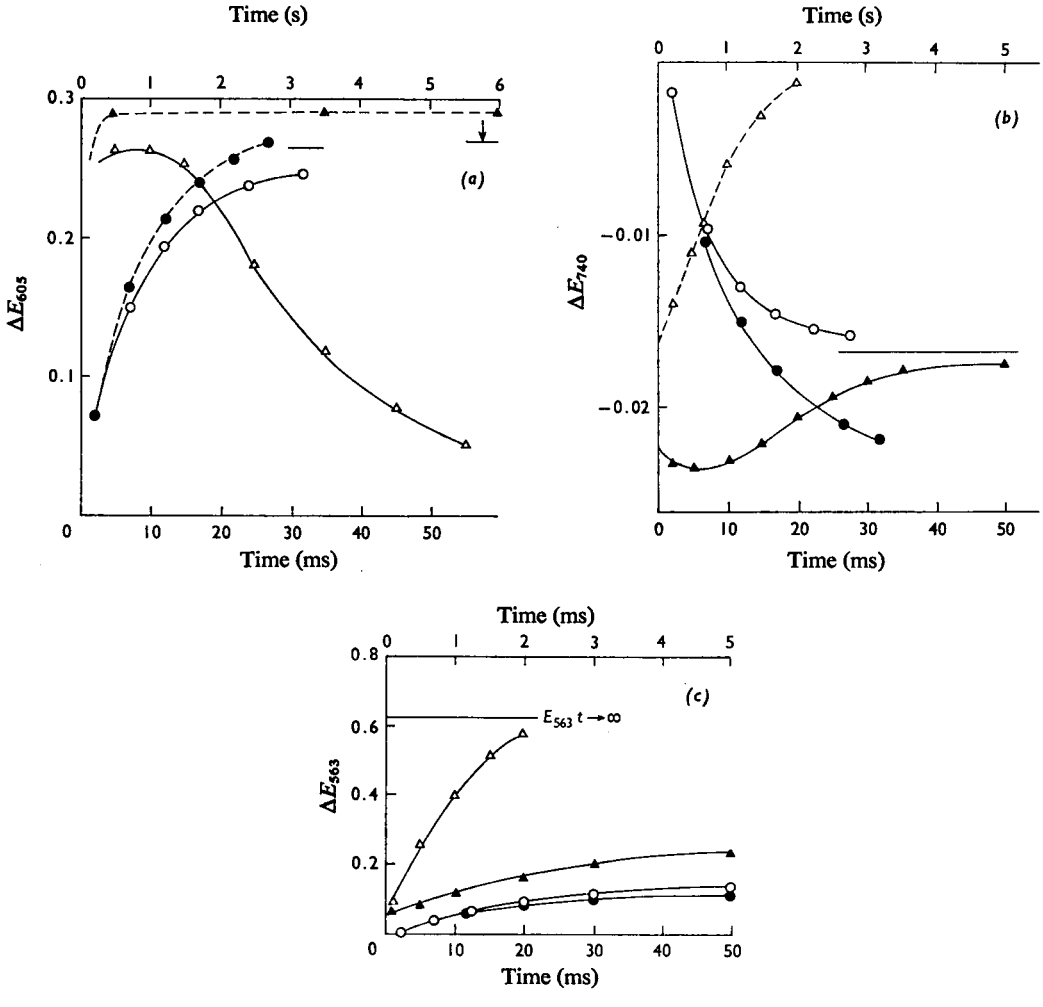

Fig. 7. *Effects of NaCN on the aerobic oxidation of ferrocytochrome c by cytochrome oxidase*

Cytochrome oxidase (22 μM) was mixed with 90 μM-ferrocytochrome *c* in the presence of 130 μM-O_2, and the curves (*a*), (*b*) and (*c*) were obtained at 605, 740 and 563 nm respectively. In each figure the upper time-scale, in s, refers to changes described by triangles and the lower time-scale, in ms, to changes represented by circles. Open and closed symbols refer to experiments conducted in the absence and presence of 10 mM-NaCN. All other conditions as in Fig. 1. The Figure was constructed as follows. The change in absorbance between the steady-state level of reduction and the fully oxidized state were calculated from the progress curves occurring in the slow time-range in the absence of cyanide. The fast processes preceding these slower changes could now be fixed, as the position of the steady state being approached in the absence of cyanide is known. It may be seen that positioning the fast processes in this manner is consistent with what would be expected from extrapolating the observed progress curves to zero time, thereby accounting for the dead-time of the apparatus. The fast process in the presence of cyanide was positioned by assuming that approximately the same absorbance was lost in the dead-time as in the experiments in the absence of cyanide, an assumption which is verified by a comparison of the absorbance lost in the dead-time found by extrapolating the observed curves to zero time. Finally, once the extent of reduction approached by the fast process was fixed the slow progress curves occurring in the presence of cyanide could also be positioned.

to cytochrome *a* at a rate much faster than this: thus cytochrome *a* would become reduced with no measureable lag.

The plots of ΔE_{605} versus ΔE_{550} suggest a change in stoicheiometry as the cytochrome c^{2+} concentration changes (Fig. 3). At low concentrations, the slope $\Delta E_{550}/\Delta E_{605}$ is 1.4, close to the value of 1.6 found by Gibson *et al.* (1965). These authors concluded that more than one redox site in the oxidase unit was being titrated by the cytochrome *c*. At higher cytochrome *c* concentrations the first portion of the curve indicates a lower stoicheiometry. It is possible to rationalize this behaviour by the following model:

$$c^{2}+a_{o} \rightleftharpoons c^{3+}+a_{r} \quad \text{(i)}$$

$$a_{r} \rightleftharpoons a'_{o} \quad \text{(ii)}$$

$$c^{2+}+a'_{o} \rightleftharpoons c^{3+}+a'_{r} \quad \text{(iii)}$$

a_o represents the fully oxidized oxidase molecule, a_r the oxidase molecule in which cytochrome *a* is reduced, a'_o the oxidase molecule in which cytochrome *a* is again oxidized but some other site in redox equilibrium with it is reduced, a'_r the oxidase which has both cytochrome *a* and this other site reduced. c^{2+} and c^{3+} represent reduced and oxidized cytochrome *c* respectively. From the known redox potentials of cytochromes *c* and *a* (Muijsers *et al.*, 1972) the forward and back rates in eqns. (i) and (iii) should be comparable. The exact position of the equilibrium for eqn. (ii) is less clear although Tiesjema *et al.* (1973) report a value of 280mV for copper suggesting that this equilibrium lies slightly to the right. It may be appreciated that where the intramolecular electron transfer a_r to a'_0 is comparable with the rate of the reaction between c^{2+} and a_0, cytochrome *a* will not retain all the electrons fed into it. Thus oxidation of cytochrome *c* will only partially be accounted for by reduction of cytochrome *a* during this period, and consequently extinction changes at 550nm would exceed those expected on the basis of the cytochrome *a* changes monitored at 605nm. However, where the rate of electron transfer into cytochrome *a* from cytochrome *c* exceeds the rate of exit into the other site, the reduction of cytochrome *a* will, at least for a period of time, parallel the oxidation of cytochrome c^{2+}. It is possible, in principle, to pass from the first situation to the second by increasing the cytochrome c^{2+} concentration, thereby increasing the rate of electron entry into cytochrome *a*.

Although Fig. 3 may show a change in the stoicheiometry, the quantitative analysis of the results is difficult. The numerical values for the stoicheiometry, i.e. number of cytochrome *c* molecules oxidized/molecule of cytochrome *a* reduced depends wholly on the extinction coefficients used. There is a large range of choice for these extinctions (Muijsers *et al.*, 1972; Yonetani, 1960*a*; Van Gelder & Muijsers, 1964), but from the values used by Gibson *et al.* (1965) or those given in the Materials and Methods section,

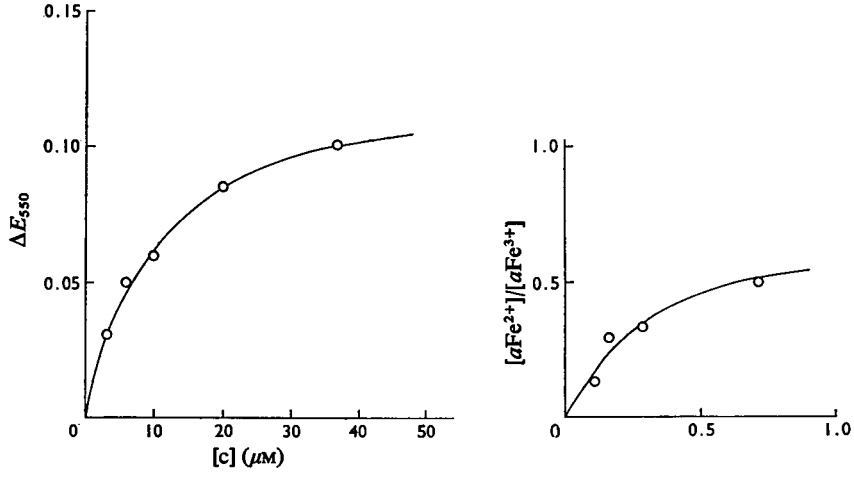

Fig. 8. *'Burst'-phase oxidation of ferrocytochrome by cytochrome oxidase*

(*a*) shows the relationship between the initial 'burst' phase of oxidation of ferrocytochrome *c* measured at 550nm, versus the total ferrocytochrome *c* concentration, when 4.5μM-cytochrome oxidase is mixed with various concentrations of ferrocytochrome *c*. (*b*) represents the ratio of reduced to oxidized cytochrome *c* (ordinate) immediately after the burst phase of the reaction, versus the ratio of reduced to oxidized cytochrome *a* (abscissa). All other conditions as in Fig. 1.

the stoicheiometry for the high-cytochrome *c* case appears to be close to unity (0.9).

The ΔE in the 'burst' phase in cytochrome *c* oxidation is dependent on the total cytochrome c^{2+} concentration, as shown in Figs. 1 and 8. The concentration of oxidized and reduced cytochrome *c* at the end of the 'burst' phase may be calculated from a knowledge of the ΔE in the 'burst' and the initial concentration of cytochrome *c*. A similar calculation can be done for cytochrome *a* by using the comparable changes at 605 nm. If the burst reflects the approach to equilibrium between cytochromes *a* and *c* only, then a plot of (cytochrome *c* reduced/cytochrome *c* oxidized) versus (cytochrome *a* reduced/cytochrome *a* oxidized) at the end of the burst should yield a straight line. Fig. 8 shows such a plot. It appears that the behaviour is not linear and indicates a more complex process than the attainment of equilibrium between cytochromes *a* and *c*. The model in eqns. (i), (ii) and (iii) predicts such behaviour, as only at high cytochrome c^{2+} concentration can reaction (i) be isolated from the subsequent equilibria.

The low-concentration experiments are therefore consistent with the conclusion that electrons enter the oxidase via cytochrome *a* and are rapidly transferred to another site.

The experiments at high concentrations also indicate that electrons are passed rapidly from cytochrome *a* to another site. During the first 50 ms of the observed reaction we invariably see more cytochrome *c* oxidized than can be accounted for by reduction of cytochrome *a*. The second site appears to be associated with absorption in the long-wavelength region of the spectrum. Certainly the changes we observed in this region can be dissociated from those occurring at 605 nm. This is seen most clearly in the kinetics of the slow changes at these wavelengths in the presence of excess of oxygen, where the return of absorbance at 830 (or 740) nm precedes the bleaching of the 605 nm band. It appears therefore that the extinction changes we observe in the high-concentration experiments at 830 nm are not due to changes in the redox state of cytochrome *a*.

We may discount the possibility that electrons are being transferred directly from cytochrome *c* to the chromophore absorbing at 830 nm, as the rate measured at this wavelength was independent of cytochrome *c* concentration at high cytochrome *c* concentrations (t_{50} approx. 8 ms).

In the presence of excess of oxygen the reformation of the absorption at 830 nm parallels the change at 560 nm (Fig. 9). It appears therefore that the steady-state level of reduction of the chromophore absorbing at 830 nm is closely linked to the concentration of reduced cytochrome *c* remaining in the reaction mixture. In the context of the model proposed in eqns. (i), (ii) and (iii) this might mean that the rate of electron transfer from cytochrome c^{2+} to oxygen is limited by the internal transfer of electrons from the 830 nm chromophore to another redox site in the oxidase. From previous studies on

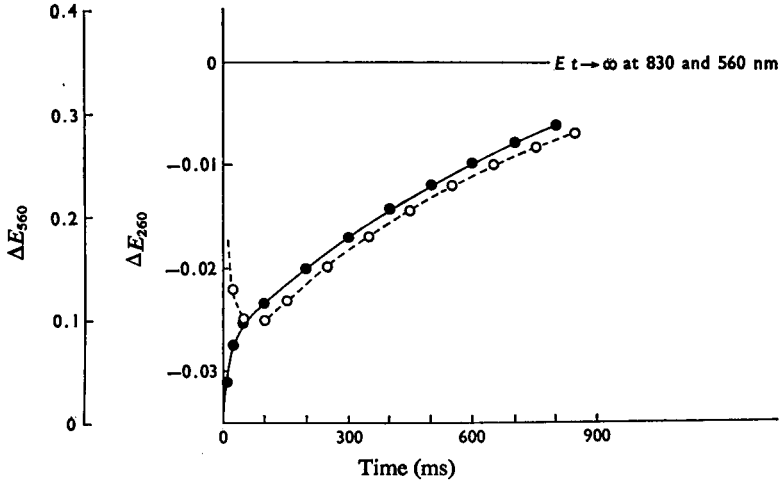

Fig. 9. *Aerobic oxidation of ferrocytochrome c by cytochrome oxidase*

Progress curves obtained on mixing cytochrome oxidase (25.5 μM) with 75 μM-ferrocytochrome *c* in the presence of oxygen (130 μM) are shown. The broken line (○) represents the change in extinction at 830 nm and the solid line (●) the associated change at 560 nm due to oxidation of ferrocytochrome *c*. The upper line on the Figure, which corresponds to zero on the 830 nm absorbance scale, shows the starting and final condition of the 830 nm-absorbing chromophore and also represents the final point at 560 nm. All other conditions as in Fig. 1.

this absorption band (Griffiths & Wharton, 1961*b*; Wharton & Tzagoloff, 1964) it has been suggested that the identity of the 830nm-absorbing chromophore is copper. It has, however, also been reported that there is a significant haem contribution in this spectral region, although it is still open as to whether this is due simply to cytochrome *a* (Andréasson *et al.*, 1972) or to more complex interactions. Greenwood *et al.* (1974) report the partial bleaching of the 830 nm band in an oxidase species in which both the cytochrome *a* and the Cu_d are thought to be in the oxidized state.

Our findings, which suggest that all electrons enter the oxidase molecule through cytochrome *a*, are in agreement with the results of Andréasson *et al.* (1972). These authors, however, report that under strictly anaerobic conditions only one electron enters the oxidase molecule and that the rate constants for electron transfer within the enzyme are oxygen-sensitive. The results presented here are not necessarily in disagreement with these findings as we have not performed experiments under similar conditions of extremely low oxygen concentration. The suggestion that cytochrome *a* rapidly hands on electrons to copper under the conditions we used is, however, in agreement with the conclusions drawn from rapid-freezing electron-paramagnetic-resonance work (see, e.g., Hartzell *et al.*, 1973).

An important aspect of the results reported above is the fact that cyanide does not affect the kinetics of the fast initial changes. The subsequent changes at 605 and 830nm are, however, greatly affected by the inhibitor: cytochrome *a* remains reduced and the 830nm band returns only partially and at a rate lower than in the absence of cyanide. As the stoicheiometry of cytochrome *c* oxidation in the burst phase remains the same (i.e. two molecules of cytochrome *c* oxidized per oxidase unit) it may be concluded that: (i) cyanide, even at high concentrations, does not react with cytochrome *a* or with the 830nm-absorbing chromophore; (ii) cyanide inhibits electron transfer to the other two sites of the oxidase. The work of Antonini *et al.* (1971) suggests that cytochrome a_3 may not be the cyanide-binding site primarily responsible for inhibition. The remaining site would then be the spectroscopically undetectable copper. The present kinetic data would be consistent with the reaction of cyanide with this site and with the position of this site, in the pathway of electron flow, between the 830nm-absorbing site and cytochrome a_3.

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References

Andréasson, L.-E., Malström, B. G., Strömberg, C. & Vänngard, T. (1972) *FEBS Lett.* **28**, 297–301

Antonini, E., Brunori, M., Greenwood, C. & Malmström, B. G. (1970) *Nature (London)* **228**, 936–937

Antonini, E., Brunori, M., Greenwood, C., Malmström, B. G. & Rotilio, G. C. (1971) *Eur. J. Biochem.* **23**, 396–400

Beinert, H. & Palmer, G. (1964) *J. Biol. Chem.* **239**, 1221–1227

Beinert, H., Hartzell, C. R. & Orme-Johnson, W. H. (1971) in *Probes of Structure and Function of Macromolecules and Membranes* (Chance, B., Yonetani, T. & Mildvan, A. S., eds.), vol. 2, pp. 575–592, Academic Press, New York

Gibson, Q. H., Greenwood, C., Wharton, D. C. & Palmer, G. (1965) *J. Biol. Chem.* **240**, 888–894

Greenwood, C., Wilson, M. T. & Brunori, M. (1974) *Biochem. J.* **137**, 205–217

Griffiths, D. E. & Wharton, D. C. (1961*a*) *J. Biol. Chem.* **236**, 1850–1856

Griffiths, D. E. & Wharton, D. C. (1961*b*) *J. Biol. Chem.* **236**, 1857–1862

Hartzell, C. R., Hansen, R. E. & Beinert, H. (1973) *Proc. Nat. Acad. Sci. U.S.* **70**, 2477–2481

Lemberg, M. R. (1968) *Physiol. Rev.* **49**, 48–121

Minnaert, K. (1961*a*) *Biochim. Biophys. Acta* **50**, 23–34

Minnaert, K. (1961*b*) *Biochim. Biophys. Acta* **54**, 26–41

Muijsers, A. O., Tiesjema, R. H., Henderson, R. W. & Van Gelder, B. F. (1972) *Biochim. Biophys. Acta* **267**, 216–221

Schejter, A., George, P., Glauser, S. C. & Margoliash, E. (1963) *Biochim. Biophys. Acta* **73**, 641–643

Smith, L. (1955) *J. Biol. Chem.* **215**, 833–846

Tiesjema, R. H., Muijsers, A. O. & Van Gelder, B. F. (1973) *Biochim. Biophys. Acta* **305**, 19–28

Van Gelder, B. F. & Muijsers, A. O. (1964) *Biochim. Biophys. Acta* **81**, 405–407

Wharton, D. C. & Tzagoloff, A. (1964) *J. Biol. Chem.* **239**, 2036–2041

Yonetani, T. (1960*a*) *J. Biol. Chem.* **235**, 845–852

Yonetani, T. (1960*b*) *J. Biol. Chem.* **235**, 3138–3144