Spectroscopic evidence for the participation of compound A ($\text{Fe}_{a_3}^{2+}-\text{O}_2$) in the reaction of mixed-valence cytochrome *c* oxidase with oxygen at room temperature*

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1. The reaction of the partially reduced mixed-valence state of cytochrome *c* oxidase ($a^3+\text{Cu}_A^{2+}a_3^{2+}\text{COCu}_B^+$) with O_2 was studied by the rapid-reaction technique of flow-flash spectrophotometry at room temperature. Biphasic absorption records are observed in the time range up to 2 ms in both the Soret and visible spectral regions. The fast-phase rate is O_2 -concentration-dependent and reaches a pseudo-first-order value of $4.5 \times 10^4 \text{ s}^{-1}$ at $680 \mu\text{M}-\text{O}_2$ at 20°C . Under the same conditions the second-phase rate is limited at $6.0 \times 10^3 \text{ s}^{-1}$. Kinetic difference spectra of the two species in the Soret region are not markedly different in form, whereas in the visible region two spectroscopically different species are clearly distinguished. 4. The first intermediate has a peak at 595 nm and a trough at 605 nm. The form of this spectrum resembles that seen in low-temperature studies and assigned to an O_2 -bound form of ferrocycytochrome a_3 . This evidence supports a structure for oxycytochrome *c* oxidase with O_2 bound only to cytochrome a_3 and not bridged between cytochrome a_3 and Cu_B . The second intermediate has a difference spectrum with a trough at 592 nm and a peak at 610 nm. Again, the form of this spectrum is similar to that observed during the O_2 reaction at low temperature and is thought to be a result of electron transfer from the oxidase to bound O_2 . 5. The oxygen profile of the fast phase suggests that a spectroscopically silent species may precede the formation of compound A. These data represent the first spectroscopic distinction, in the physiological temperature range, between O_2 binding and electron transfer during the O_2 reaction of mammalian cytochrome *c* oxidase. 6. A mechanism is presented for the O_2 reaction of the mixed-valence state of cytochrome *c* oxidase involving four intermediate species. Electron transfer during this reaction is slow, relative to that seen with the fully reduced enzyme, and probably accounts for the detectability of the oxyferro species under these conditions.

Cytochrome *c* oxidase (ferrocycytochrome *c*:oxygen oxidoreductase, EC 1.9.3.1) is the terminal member of the mammalian mitochondrial respiratory chain and as such is responsible for the activation and reduction of molecular O_2 to water. The enzyme possesses four prosthetic groups per functional unit, consisting of two haems A, cytochromes *a* and a_3 , and two copper atoms, Cu_A and Cu_B (Malmström, 1979). The reaction of the reduced enzyme with O_2 at room temperature is extremely fast and beyond the resolution of con-

ventional rapid-mixing techniques. Gibson & Greenwood (1963) applied a method combining rapid mixing and flash photolysis to study this reaction. This flow-flash approach involves using the CO-bound reduced enzyme as the starting material. In the dark CO dissociates very slowly ($t_{1/2} = 30 \text{ s}$), allowing for the introduction of O_2 to the solution. When such a mixture is subjected to a flash of light, the CO is rapidly removed and the O_2 reaction ensues. An analogous technique has been adopted by Chance and his colleagues to study the reaction at low temperature (Chance *et al.*, 1975). At about -100°C the reaction of the oxidase occurs on a time scale of seconds to minutes, allowing the reaction to be quenched at intermediate stages.

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Two types of CO-bound derivative of cytochrome *c* oxidase may be made: fully reduced and mixed-valence species. The fully reduced species has both haems ferrous and both coppers cuprous, whereas in the mixed-valence enzyme cytochrome *a* and Cu_A are oxidized and cytochrome *a*₃ and Cu_B are reduced and bound by CO (Wilson & Miyata, 1977; Clore *et al.*, 1980). Room-temperature measurements of the reaction of the fully reduced species with O_2 has led to the proposal of a sequential mechanism involving oxidation of cytochrome *a*₃, as the first spectroscopically detectable event, followed by copper oxidation and finally cytochrome *a* oxidation (Greenwood & Gibson, 1967). Experiments with the mixed-valence species at room temperature demonstrated the rapid reactivity of this partially reduced state with O_2 . A single phase of absorbance change was seen and had an O_2 -concentration-dependence similar to that assigned to cytochrome *a*₃ oxidation with the fully reduced enzyme (Greenwood *et al.*, 1974). No spectroscopic evidence of an O_2 -bound state before the electron transfer was observed in either study. However, the low-temperature experiments have revealed new intermediates in the reactions of both the fully reduced and the mixed-valence oxidase with O_2 (Chance *et al.*, 1975; Clore *et al.*, 1980). Most notable was the resolution of an oxyferrocycytochrome *a*₃ species, termed 'compound A' by Chance *et al.* (1975), as the primary intermediate. In the mixed-valence species the formation of compound A is followed by electron transfer, resulting in complexes of partially reduced O_2 and the enzyme. These were designated 'compounds C' by Chance *et al.* (1975) and Denis (1981). However, the participation of these intermediates in the cytochrome *c* oxidase- O_2 reaction at temperatures near the physiological range remains uncertain, as extrapolation over such a wide temperature difference is difficult. The intermediates at low temperature have largely been resolved on the basis of their visible-region absorption spectra. Accordingly, Denis (1981) has demonstrated that the Soret-region changes are nearly featureless during the O_2 reaction of mixed-valence cytochrome *c* oxidase relative to that seen in the visible region. In the previous room-temperature measurements made by Greenwood *et al.* (1974), detailed experiments of the reaction of mixed-valence cytochrome *c* oxidase with O_2 were restricted to the Soret region.

In the present investigation we have made kinetic measurements of absorbance changes during the O_2 reaction of the mixed-valence species of cytochrome *c* oxidase measured at room temperature by the flow-flash technique. The absorbance changes are biphasic in both the Soret and visible regions. Visible-region records resemble those seen at low temperature and support the proposition that compound

A is a functional intermediate during the reaction of cytochrome *c* oxidase with O_2 . In addition, these results describe a clear difference in the reactivity of the mixed-valence and the fully reduced species of cytochrome *c* oxidase.

Materials and methods

NaH_2PO_4 and Na_2HPO_4 were from BDH Chemicals, Poole, Dorset, U.K. Tween-80 (polyoxyethylene sorbitan mono-oleate) was obtained from Sigma Chemical Co., Poole, Dorset, U.K. O_2 , N_2 and CO gases were from British Oxygen Co., Wimbledon, London SW19 3UF, U.K.

Cytochrome *c* oxidase from bovine heart muscle was prepared by the method of Yonetani (1960) as modified in this laboratory (Thomson *et al.*, 1981). The mixed-valence CO-bound derivative was formed by incubation of the oxidized resting enzyme under an anaerobic atmosphere of CO at 20°C for 14 h (Greenwood *et al.*, 1974). This sample was prepared in a large-volume Thunberg cell fitted with a cuvette in order that its condition could be monitored by absorption spectroscopy. The mixed-valence species was transferred from this cell to a storage syringe of the flow assembly under a positive pressure of either CO or N_2 gas.

The flow-flash assembly employed a Durrum-Gibson-type stopped-flow mixing device, which delivered into a 4 cm × 1 cm-diam. observation cell housed in an Applied Photophysics flash-photolysis unit. Maximum flash powers of either 0.2 or 1 kJ were available in less than 2 μs in either case, and the flash tail disappeared with a half-time of about 20 μs. Complementary filtration was used between the observation beam and the photolysis light to minimize interference from the flash decay. The change in intensity of light was stored as a change in voltage with a storage oscilloscope or on a Datalab 905 transient recorder. In either case data collection was triggered by a photodiode mounted in the flash cavity. The oscilloscope screen could be photographed and analysed by semi-logarithmic plots of absorbance change against time. Data from the transient recorder were fed directly into a PDP-8A minicomputer and analysed by using fitting programs previously described by Barber *et al.* (1978).

In all experiments the flash power used was first checked to establish that it was sufficient to obtain complete photolysis of the CO-bound species. In the visible region a power of 90 J was enough, and in the Soret region 450 J was necessary.

Results

Fig. 1 describes the photolysis and recombination of CO from the mixed-valence species of cytochrome *c* oxidase. Fig. 1(a) shows an oscilloscope

Fig. 1. (a) Photolysis and recombination of CO from mixed-valence cytochrome *c* oxidase and (b) kinetic difference spectrum of mixed-valence CO-bound cytochrome *c* oxidase minus the mixed-valence unbound enzyme

(a) The upper set of curves is a reproduction of the dual-sweep oscilloscope screen. The enzyme concentration was $7.25 \mu\text{M}$ in 100 mM-sodium phosphate buffer, pH 7.4. The CO concentration was 0.5 mM, the temperature 20°C and the observation wavelength 590 nm. The semilogarithmic plot of absorbance change against time is of the recombination of CO with the oxidase. (b) The conditions were identical with those described in (a).

2–5 μs) decrease in absorption due to the photodissociation of CO. The absorption level reached is stable on the time scale of hundreds of microseconds. There is a small disturbance from the flash tail, but no absorption changes, such as those reported by Boelens *et al.* (1982) and assigned to electron redistribution, were seen. Either this redistribution event does not occur with our preparation of enzyme or it occurs so rapidly that equilibrium is established on the same time scale as the photolytic event. After the photolysis of CO, recombination occurs in a monoexponential reaction at a rate that is proportional to CO concentration, with a second-order rate constant of $7.7 \times 10^4 \text{ M}^{-1} \cdot \text{s}^{-1}$. This is close to the value reported by Greenwood *et al.* (1974) for CO recombination to the fully reduced enzyme. In Fig. 1(b) the amplitude of the absorption change between the CO-bound and CO-free states is plotted in a kinetic difference spectrum over the visible region. There are peaks at 550 and 590 nm, with a trough at 610 nm, typical for photolysis of CO from reduced cytochrome *a*₃ (Chance, 1953; Greenwood *et al.*, 1974). In the Soret region results essentially identical with those reported by Boelens *et al.* (1982) were obtained (not shown).

Soret region oxygen kinetics

When the mixed-valence CO-bound oxidase is photolysed in the presence of O₂, absorption changes occur on the microsecond time scale. In Fig. 2 kinetic records in the Soret region at two different O₂ concentrations are presented as semilogarithmic plots of absorption change versus time. At low O₂ concentration (13.5 μM) the absorption change is essentially a single exponential with a half-time of about 500 μs . This is similar in form and rate to that reported previously by Greenwood *et al.* (1974). However, when the O₂ concentration is increased a second phase becomes apparent. In Fig. 2(b), at 345 μM -O₂, the absorption change is biphasic. The second phase has a half-life of about 100 μs . The fast phase may be extracted from the biphasic curve, and is shown in Fig. 2(c) (iii). It has the form of a single exponential with a half-time of about 30 μs .

In Fig. 3 the O₂-concentration-dependence of the rates of the two phases is shown. The fast phase shows a linear dependence on O₂ concentration at low O₂ concentration, with a bimolecular rate of $8 \times 10^7 \text{ M}^{-1} \cdot \text{s}^{-1}$, but becomes non-linear as the O₂ concentration is raised above about 400 μM . The highest observed rate for this phase at 680 μM -O₂ was $3.2 \times 10^4 \text{ s}^{-1}$. The second-phase rate is less O₂-concentration-dependent and reaches a limit of $7 \times 10^3 \text{ s}^{-1}$.

At an O₂ concentration where both phases could be easily discerned the extents of the phases were determined at a number of wavelengths. The extents

record obtained with a dual-trace instrument with the sweep rates set as indicated on the Figure. At 590 nm the flash induces a very rapid ($t_{\frac{1}{2}} =$

Fig. 2. Soret-region absorption changes when mixed-valence CO-bound cytochrome *c* oxidase is photolysed in the presence of O_2 .

In (a) the enzyme concentration was $3.85 \mu\text{M}$ in 100 mM-sodium phosphate buffer, pH 7.4. The CO concentration was 0.5 mM , the temperature 20°C , the monitoring wavelength 445 nm and the O_2 concentration $13.5 \mu\text{M}$. The oscilloscope trace and the corresponding semilogarithmic plot are shown. In (b) the conditions were identical with those described in (a) except that the O_2 concentration was $345 \mu\text{M}$. (i) The oscilloscope trace; (ii) the semilogarithmic plot of the overall absorbance change; (iii) the semilogarithmic plot of the fast phase.

Fig. 3. O_2 -concentration-dependence of the pseudo-first-order rate constants for the fast phase and the slow phase of absorption change during the O_2 reaction of mixed-valence cytochrome *c* oxidase

The conditions were identical with those outlined in the legend to Fig. 2. $\bullet$, Fast phase (plotted on the left-hand scale); $\circ$, slow phase (plotted on the right-hand scale).

of these experiments are shown in kinetic difference spectra for the fast-phase and slow-phase components in Fig. 4. The absorption differences are plotted such that a decrease in absorption appears as a trough in the difference spectrum. At all the wavelengths studied in the Soret region the fast phase contributed most of the observed absorbance change and both phases proceeded in the same direction. The fast-phase component has a peak at 430 nm and a trough at 445 nm . The form of this difference spectrum is similar to that for the single fast phase reported by Greenwood *et al.* (1974). The second phase has a peak at 430 nm with a trough at 440 nm . The difference in the form of these spectra, though small, suggests that two unique intermediates are being observed rather than merely a kinetically heterogeneous population of enzyme molecules.

In the earlier study by Greenwood *et al.* (1974) of the mixed-valence CO-bound species reacting with O_2 , only a single rapid phase of absorption change in the Soret region was observed. The results shown in Figs. 3 and 4 may explain why the second phase reported in the present paper was not observed previously. The maximum rate recorded for the fast phase by Greenwood *et al.* (1974) was about $1.7 \times 10^4 \text{ s}^{-1}$ compared with about $3.2 \times 10^4 \text{ s}^{-1}$ in the present paper. Thus the fast-phase rate would have only been a little more than twice that of the slow phase. Since at most wavelengths in this region

of the two kinetic phases were determined by the extrapolation of the two linear portions of the semilogarithmic plots back to zero time. The results

Fig. 4. Soret-region difference spectra of mixed-valence cytochrome *c* oxidase reacting with O₂

The enzyme concentration was 1.96 μM and the O₂ concentration 135 μM . O, Fast phase; ●, slow phase. The other conditions were the same as those given in the legend to Fig. 2.

the fast phase makes up most of the change in absorption and both phases proceed in the same direction, the slower phases could have easily been overlooked.

A phase of absorption change on the time scale of seconds was reported to occur in the Soret region in the earlier study (Greenwood *et al.*, 1974). We have not made measurements over this time period because of interference from non-specific light-scattering changes in our samples occurring on the same time scale. The present measurements are therefore restricted to the first 10 ms after CO photolysis.

Visible-region O₂ kinetics

Two phases of absorption change are also observed in the visible region when the mixed-

Fig. 5. Visible-region absorption changes during the reaction of mixed-valence cytochrome *c* oxidase with O₂

The enzyme concentration was 4.05 μM and the O₂ concentration 135 μM . The two sets of traces were recorded on a dual-sweep oscilloscope. Traces (a) and (d) were recorded at the faster sweep rate, whereas traces (b) and (c) were recorded at the slower sweep rate. The measuring wavelengths are indicated on the Figure.

valence CO-bound species of cytochrome *c* oxidase is photolysed in the presence of O₂. In Fig. 5 the records of absorption change at 590 and 605 nm were recorded with a dual-sweep oscilloscope. The fast sweep was set to register the rapid absorption changes, while the slow-sweep rate was sufficient to monitor the overall absorption change through both the fast phase and slow phase. At 590 nm, after the flash and CO photodissociation (not shown), there is a phase of increasing absorption followed by a slower phase of decreasing absorption. At 605 nm the form of the kinetic record is reversed, with the fast phase being a decrease in absorption followed by a second phase of increasing absorption. In Fig. 6 records at four different O₂ concentrations are shown, as measured at 597 nm, which is the isosbestic wavelength for the $a_3^{2+}\text{-CO} \rightarrow a_3^{2+}$ reaction. As the O₂ concentration is increased, both the overall extent of the absorption change and the rate of both phases

Fig. 6. Visible-region absorption changes measured during the reaction of mixed-valence cytochrome *c* oxidase at a number of different O_2 concentrations. The enzyme concentration was $7.25 \mu M$ and the O_2 concentrations were $27 \mu M$ (—), $67.5 \mu M$ (---), $135 \mu M$ (-·-·-) and $340 \mu M$ (- - - -). The measuring wavelength was 597 nm .

increase. The form of these records is very different from that reported previously by Greenwood *et al.* (1974). However, in that work observation in the visible region was restricted to a single O_2 concentration ($13 \mu M O_2$). At such an O_2 concentration the amplitude would have been small and completely obscured by interference from the photolysis light with the instrument in use at that time. The improved apparatus used in the present work allows observation of absorption changes without interference from the photolysis flash.

In Fig. 7 the observed rates of the fast phase and the slow phase are shown as a function of oxygen concentration. The form of these is similar to their counterparts observed in the Soret region. However, the maximum rate observed for the fast phase is higher than that observed in the Soret region, whereas the slow-phase rate is somewhat lower. The two sets of results were, however, obtained with different preparations on different days, and we do not consider these relatively small differences significant at this stage. Thus the O_2 reaction of the mixed-valence species of cytochrome *c* oxidase may be described as consisting of two spectrophotometrically visible intermediate species occurring over the first 2 ms. Spectroscopic evidence of a third species on the millisecond time scale was also seen at wavelengths in the range of $605\text{--}620 \text{ nm}$ (see Fig. 5). Such changes do not occur at other wavelengths in either the visible or the Soret region. This third phase may correspond to the shift in the α -band maximum from 610 to 606 nm detected by Denis (1981) at low temperature.

In Fig. 8 the kinetic difference spectra of the two components are shown in the visible region from 540 to 630 nm . The fast phase had a peak at 595 nm with a trough at 605 nm . The second phase has a trough at 592 nm with a peak at 610 nm . Neither of these spectra resembles the single one reported previously and assigned to cytochrome a_3^{2+} oxidation.

Fig. 7. O_2 -concentration-dependence of the fast-phase and slow-phase rates of the reaction of mixed-valence cytochrome *c* oxidase with O_2 measured at 590 nm . The enzyme concentration was $3.87 \mu M$ and the other conditions were the same as those given in the legend to Fig. 2. ●, Fast phase (plotted on the left-hand scale); ○, slow phase (plotted on the right-hand scale).

Fig. 8. Kinetic difference spectra in the visible region during the O_2 reaction of mixed-valence cytochrome *c* oxidase.

The enzyme concentration was $3.69 \mu M$ and the O_2 concentration $680 \mu M$. The other conditions were identical with those described in the legend to Fig. 2. ●, Fast phase; ○, slow phase.

Table 1. *Pseudo-first-order rate constants for the two phases of absorption change observed in the Soret and visible spectral regions*

The values were determined at 20°C and 680 μM-O₂. Each value is the mean and standard deviation of four separate determinations.

	$k_{\text{obs.}} \text{ (s}^{-1}\text{)}$	
	Fast	Slow
Soret region	$3.5 (\pm 0.5) \times 10^4$	$7.0 (\pm 1.0) \times 10^3$
Visible region	$4.5 (\pm 0.6) \times 10^4$	$5.0 (\pm 0.7) \times 10^3$

In Table 1 the pseudo-first-order rate constants for the two phases of absorption change as observed in the Soret and visible regions are compared. The observed values for both phases are somewhat different as recorded in the two regions. But, in view of the fact that the experiments were performed with different samples of enzyme on different days, we consider the relatively small differences to be insignificant. Therefore we conclude that the two phases in the spectral regions reflect the same molecular events.

Discussion

Kinetic spectrophotometry shows that there are two intermediate states of cytochrome *c* oxidase detectable over the first few milliseconds when the partially reduced mixed-valence species is allowed to react with O₂ at room temperature. The overall forms of the kinetic records are very similar to those recorded at low temperature and reported by Chance *et al.* (1975) and Clore (1980). The visible-region kinetic difference spectrum suggests that the first spectroscopically detectable intermediate is the O₂-bound form of reduced cytochrome *a*₃ or the compound A described by Chance *et al.* (1975). In the Soret region the formation of compound A is accompanied by a decrease in absorption at 445 nm. In the previous study of the O₂ reaction of mixed-valence cytochrome *c* oxidase made by Greenwood *et al.* (1974), this change had been assigned to cytochrome *a*₃ oxidation, and was the only spectroscopic event reported on the microsecond-to-millisecond time scale. The present results show that the initial spectroscopic event assigned to O₂ binding causes decreased absorption at 445 nm. This is not surprising in view of the model studies made by Babcock & Chang (1979), where various adducts of isolated haem A were compared. The absorption spectrum of the O₂-bound form of reduced haem A was less intense in the Soret region, but more intense in the visible region, than those of the corresponding unbound or CO-bound species. Thus the failure to observe the formation of

compound A previously may be seen as a consequence of the inability of the Soret-band region to distinguish between cytochrome *a*₃²⁺ oxidation or ligation by O₂. Moreover, in low-temperature experiments a similar pattern has been reported by Denis (1981), who recorded changes in the visible and Soret regions and found three spectroscopically distinguishable species in the visible region during the reaction of the mixed-valence species with O₂. However, there were no spectroscopic shifts apparent in the Soret region during the same reaction. In addition, the absorption changes in both spectral regions found by Denis (1981) at low temperature closely resemble those reported in the present paper at room temperature. The difference between the Soret and visible spectral regions in resolving intermediate states during the O₂ reaction of cytochromes is made clear by the results obtained in the present study.

The spectral features described in the present paper for compound A resemble those reported by Babcock & Chang (1979) for the O₂-bound form of isolated haem A, suggesting that in these species O₂ is bound in a similar manner. This conclusion contradicts the suggestion made by Erecinska & Wilson (1978) that compound A was formed appreciably only as a consequence of low temperature and that at room temperature O₂ would be bound as a bridged ligand between cytochrome *a*₃ and Cu_B (I):

The results obtained by Alben *et al.* (1981), showing that at low temperature CO photolysed from cytochrome *a*₃ binds to Cu_B, have led Wikström *et al.* (1981) to propose an alternative structure for compound A containing O₂ bound to cytochrome *a*₃ and CO to Cu_B (II):

As we do not expect there to be any significant barrier to CO migration from the active site of cytochrome *c* oxidase at room temperature, our finding of compound A eliminates this possibility. Therefore we accept the suggestion made by Chance *et al.* (1975) that, in oxycytochrome *c* oxidase, O₂ is bound to the iron of cytochrome *a*₃ alone and is not bridged between cytochrome *a*₃ and Cu_B. Moreover, the type of binding by O₂ in compound A is also revealed by i.r. spectroscopy for CO in its complex with cytochrome *c* oxidase (Yoshikawa & Caughey, 1982).

In Scheme 1 a mechanism is presented for the

Scheme 1. Scheme for the reaction of mixed-valence CO-bound cytochrome *c* oxidase in a flow-flash experiment with O_2

The nomenclature of Chance *et al.* (1975) is used for compounds A and C, and the 607 nm compound is the name given by Nicholls (1979).

reaction of the mixed-valence state of cytochrome *c* oxidase with O_2 . The process begins with the photolysis of CO from cytochrome a_3^{2+} , generating a partially reduced state of cytochrome *c* oxidase. Boelens *et al.* (1982) have argued that, at physiological temperature, after CO removal there is electron redistribution between the cytochrome a_3 - Cu_B and Cu_A . This was reported to occur at a rate of about $7 \times 10^3 \text{ s}^{-1}$ and to involve reduction of about 7% of Cu_A . We do not see this event with our enzyme (see Fig. 1a), and therefore conclude that the product of photolysis in our system is nearly completely the species depicted in Scheme 1, with cytochrome *a* and Cu_B oxidized and cytochrome a_3 and Cu_A reduced and ligand-free. We do, however, observe electron redistribution from the cytochrome a_3 - Cu_B pair to both cytochrome *a* and Cu_A on the time scale of minutes when CO is permanently removed from the mixed-valence species, as was shown by Nicholls & Chanady (1981).

A spectroscopically silent step is shown after CO removal and before the formation of compound A. This is invoked to account for the form of the O_2 -concentration-dependence of the fast-phase rate, which becomes limited at high O_2 concentration. Such a result could be due to O_2 migration into a gas pocket thought to surround the ligand-binding site of

the oxidase, as suggested from similar results obtained by De Fonseka & Chance (1980) at low temperature with the mitochondrially bound oxidase. In addition, the formation of compound A follows at such a rapid rate that there would be little accumulation of the putative primary intermediate that would make spectroscopic observation difficult.

The formation of compound A follows the initial spectroscopically silent species in a first-order step with a rate constant of about $4 \times 10^4 \text{ s}^{-1}$. We estimate that compound A accumulates to about 50% of the total enzyme population after about $30 \mu\text{s}$ at the highest O_2 concentration used. It is likely that electron transfer follows O_2 binding, giving rise to the second phase of absorption change and occurring with a rate of $6 \times 10^3 \text{ s}^{-1}$. It is not possible for us to specify with any certainty whether this represents the transfer of one or two electrons. Some workers have suggested that single-electron-transfer steps may occur (Chance & Leigh, 1979; Clore *et al.*, 1980; Nicholls & Chanady, 1981). However, in the case of the low-temperature studies, where the species was trapped and examined by e.p.r. spectroscopy, no new signals were observed, as would be expected if a single-electron transfer event had taken place. In contrast, Reinhammer *et al.* (1980) have observed a new e.p.r. signal from Cu_B in low-temperature trapping experiments when starting from the fully reduced enzyme. In addition, as has been noted in the past, the thermodynamics of O_2 reduction greatly favour an initial two-electron step to the peroxide level (George, 1965; Malmström, 1982). Therefore we present the peroxide-bound enzyme as the product of electron transfer. Evidence of a third rapid phase of kinetic change was apparent in the visible region. The magnitude of the absorption change was small and could be due to a change in the nature of the peroxide-bound state. We suggest that peroxide may become bridged between cytochrome a_3 and Cu_B at this stage. Such a change might be important in turnover to allow a greater throughput of electrons to cytochrome a_3 from the reductive substrate, cytochrome *c*. The ability of Cu_B to expand its ligand-binding shell and accept an exogenous ligand, as it would have to in this instance, has been demonstrated by Brudvig *et al.* (1981).

The events that follow the formation of the peroxide-bound intermediate are not well understood, but it is likely that these states only become populated when starting with the partially reduced material, as their rates of formation do not allow them to participate during turnover. The first of these slow steps has a half-time of 1 s and refers to that phase of absorption change reported by Greenwood *et al.* (1974). We suggest in Scheme 1 that this may be a consequence of peroxide release, which may be why it occurs at such a relatively low

rate. One of the key features of the reaction of cytochrome *c* oxidase with O₂ is that no potentially poisonous partially reduced states of O₂ are released into solution. It should be possible to test this suggestion by assaying for the release of peroxide. The state of the enzyme after the 1 s reaction was identified by Greenwood *et al.* (1974) with an 'oxygenated' species in which cytochrome *a*₃ was ferric and low spin. Nicholls and co-workers (Nicholls, 1979; Nicholls & Chanady, 1981) have studied this species in more detail and prefer a ferrous state for cytochrome *a*₃; however, we favour the suggestion that it is a conformational variant of the oxidized enzyme, since Brittain & Greenwood (1976) showed that it bound cyanide with great facility to give the fully oxidized cyanide-bound species. Further experimentation on the slower-reacting components of the process is required before these questions can be answered. Finally, progress to the resting oxidized enzyme occurs with a half-time of about 30 min.

The reaction sequence shown in Scheme 1 implies a marked difference in the reactivity of the cytochrome *a*₃-Cu_B centre with O₂ between the mixed-valence state as opposed to the fully reduced state of cytochrome *c* oxidase. The combination rate with O₂ appears almost identical with that with either the partially reduced or the fully reduced species, but the subsequent electron-transfer rate to bound O₂ is much lower in the mixed-valence reaction, implying that the O₂-reactive centre is sensitive to the oxidation state of the other metal centres in the enzyme. This difference in reactivity accounts for the fact that the formation of compound A is not observed in the reaction of the fully reduced enzyme with O₂ at room temperature (Greenwood & Gibson, 1967).

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