The reaction of fully reduced cytochrome *c* oxidase with oxygen studied by flow-flash spectrophotometry at room temperature

Evidence for new pathways of electron transfer

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1. Absorption changes during the O₂ reaction of reduced bovine cytochrome *c* oxidase were investigated by the rapid-reaction technique of flow-flash spectrophotometry in the Soret, visible and near-i.r. spectral regions. 2. New features in the time courses of absorption change were observed relative to the earlier findings reported by Greenwood & Gibson [(1967) *J. Biol. Chem.* **242**, 1782–1787]. These new features arise in the Soret and near-i.r. regions and allow the reaction to be described at all wavelengths as a composite of three exponential processes. 3. There is a rapid O₂-sensitive phase detectable in the Soret and visible region. The second phase has a rate that is somewhat less dependent on O₂ concentration than is the fastest phase rate and is detectable in all three spectral regions. The rate of the third phase is almost independent of the O₂ concentration and is also detectable in all spectral regions. 4. Analysis of the three phases gives their rates and absorption amplitudes. The fast phase reaches a rate of $2.5 \times 10^4 \text{ s}^{-1}$ at the highest O₂ concentration available at 20°C, whereas the phase of intermediate rate is limited at a value of $7 \times 10^3 \text{ s}^{-1}$ and the slow phase rate is limited at 700 s^{-1} . 5. The ratios of the kinetic difference spectra for the fast phase and the slow phase do not correspond to the spectra of the individual haem centres. 6. A branched mechanism is advanced that is able to reconcile the kinetic and static difference spectra. This mechanism suggests that some of the cytochrome *a* is oxidized along with cytochrome *a*₃ in the initial O₂-sensitive phase. In addition, the model requires that Cu_A is oxidized heterogeneously. This fits with the complex time course of oxidation observed at 830 nm while retaining Cu_A as virtually the sole contributor to absorbance at this wavelength.

Cytochrome *c* oxidase (ferrocytochrome *c*:oxygen oxidoreductase, EC 1.9.3.1) is the terminal member of the mammalian mitochondrial respiratory chain and functions to deliver electrons from cytochrome *c* to O₂ (for reviews see Nicholls & Chance, 1974; Malmström, 1979). Rapid-reaction techniques have been used to study the two half-reactions of the enzyme with its two substrates, ferrocytochrome *c* and O₂. Such studies have led to the view that two of the four metal centres, cytochrome *a* and Cu_A, are responsible for cytochrome *c* oxidation, whereas cytochrome *a*₃ and Cu_B are more closely involved with O₂ activation and reduction (Chance, 1981).

The reaction of cytochrome *c* oxidase with O₂ may be studied at room temperature by a technique combining rapid mixing and flash photolysis

(Gibson & Greenwood, 1963). Greenwood & Gibson (1967) described the absorption changes during the reaction with the fully reduced enzyme with O₂ as being composed of three exponential processes discriminated on the basis of their different rates, O₂-dependencies and spectral contributions. They proposed a sequential mechanism involving O₂ binding, followed by cytochrome *a*₃ oxidation, Cu oxidation and lastly the oxidation of cytochrome *a*. More recently Chance *et al.* (1975) adopted a similar technique for use at low temperature and have been able to resolve intermediate stages of the reaction not observed previously at room temperature. Most notably the studies at low temperature have provided direct evidence for the formation of an O₂-bound state of the reduced oxidase that is spectroscopically, and

presumably structurally, analogous to oxyhaemoglobin. This oxyferro species has been termed Compound A by Chance *et al.* (1975). Previously the existence of this species had been one suggestion among others proposed by Greenwood & Gibson (1967) to account for the limit at high O_2 concentrations on the rate of their fastest spectrophotometric event assigned to the oxidation of cytochrome a_3 . Clore *et al.* (1980) used e.p.r. spectroscopy to define more completely the oxidation state of the individual metal centres in the intermediates trapped at low temperature. The results of this study confirmed the Chance *et al.* (1975) definition of Compound A, but introduced a branch into the linear mechanisms advanced previously. The branch in the mechanism proposed by Clore *et al.* (1980) allows for the oxidation of cytochrome a in the initial electron-transfer step to bound O_2 . Such an interpretation is at variance with the sequential mechanism proposed by Greenwood & Gibson (1967).

In response to the results from the low-temperature work outlined above, we have used an improved flow-flash device to re-investigate the reaction of fully reduced cytochrome c oxidase with O_2 at room temperature. We report new features in the time courses of absorption change in both the Soret and near-i.r. regions. A branched mechanism is proposed that accounts for the absorption ratios in the Soret and visible regions as well as the complex time course observed at 830 nm while retaining independent spectral properties for cytochromes a and a_3 and Cu_A . This mechanism has some similarities to that proposed by Clore *et al.* (1980), but suggests that both cytochrome a and Cu_A are oxidized heterogeneously. Such a model implies that two different conformers of the reduced enzyme may exist, as required by the two-state model advanced by Brunori and his co-workers (Wilson *et al.*, 1981; Brunori *et al.*, 1983).

Experimental

Cytochrome c , sodium ascorbate and Tween-80 (polyoxyethylene sorbitan mono-oleate) were obtained from Sigma Chemical Co., Poole, Dorset, U.K. NaH_2PO_4 and Na_2HPO_4 were from BDH Chemicals, Poole, Dorset, U.K. O_2 , N_2 and CO were supplied by British Oxygen Corp., Wimbledon, London, 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 fully reduced CO-bound species of cytochrome c oxidase was prepared as follows. A solution of the oxidized resting enzyme was placed under an O_2 -free N_2 atmosphere by five rounds of evacuation and flushing. Ascorbate (2 mM) and

cytochrome c (1:50, c/aa_3) were added to the anaerobic oxidase solution and the mixture was left to incubate at 4°C for 12–20 h until complete reduction was obtained. At this stage the N_2 atmosphere was replaced with CO. This solution could be transferred to a storage syringe of the flow system under a positive pressure of N_2 gas.

The flow-flash assembly and data-collection system employed have been described in recent papers from this laboratory (Brittain & Greenwood, 1982; Hill & Greenwood, 1983).

Results

Fig. 1 shows the change in absorption seen in the Soret region at 445 nm at three different concentrations of O_2 . At the lowest O_2 concentration the time course is a single exponential process. When the O_2 concentration is raised to 274 μM two phases of absorption change become apparent. There is an initial fast phase followed by a slower second phase, in agreement with the data reported earlier by Greenwood & Gibson (1967). However, at the highest O_2 concentration available at ambient pressure a new feature of intermediate rate is observed in the time course of absorption change at 445 nm. A plateau appears in the record of absorption change at 445 nm after the initial fast phase and before the slow phase.

In Fig. 2 time courses at other wavelengths in the Soret region are presented under conditions where the new feature of intermediate rate is observed. At 455 nm the time course of absorption change is

Fig. 1. Absorption changes in the Soret region at different O_2 concentrations during the reaction of fully reduced cytochrome c oxidase with O_2 .

The enzyme concentration was 1.87 μM and it was reduced with 2 mM-sodium ascorbate with 20 nM-cytochrome c as described in the Experimental section. The buffer used was 0.1 M-sodium phosphate, pH 7.4, containing 0.25% Tween-80. The CO concentration was 0.580 mM, the temperature 13°C and the O_2 concentration as indicated on the Figure. All concentrations are those obtained after mixing.

Fig. 2. Absorption changes in the Soret region at high O_2 concentration during the reaction of fully reduced cytochrome *c* oxidase with O_2

The time courses are traces of a dual-sweep oscilloscope screen. Traces (i) and (iv) were recorded on the slower sweep time, whereas traces (ii) and (iii) were recorded on the faster time scale. The enzyme concentration was $1 \mu M$, the CO concentration $500 \mu M$, the O_2 concentration $680 \mu M$ and the temperature $20^\circ C$. The observation wavelengths are indicated on the Figure. All other conditions are the same as those indicated in Fig. 1 legend.

close to that seen at $445 nm$, with a fast phase followed by a plateau and then a slow phase of decreasing absorption. At $430 nm$, where the overall direction of absorption change is now reversed, the phase of intermediate rate is clearly seen to lead to an absorption change in opposition to that of the fast and the slow phases. However, the intermediate phase was too small to permit estimation of an accurate value for its contribution to this spectral region.

By extrapolating the slow-phase time course back to zero time it was possible to determine the contribution of both the fast-phase and the slow-phase components to the overall difference spectrum. Fig. 3 shows such kinetic difference spectra of the fast and the slow phases in the Soret region during the oxidation of fully reduced cytochrome *c*

Fig. 3. Kinetic difference spectra in the Soret region obtained during the reaction of cytochrome *c* oxidase with O_2

The enzyme concentration was $1.38 \mu M$ at pH 7.4 at $20^\circ C$. The CO concentration was $500 \mu M$ and the O_2 concentration $135 \mu M$. The amplitudes for each phase were determined by analysis of the time courses on semi-logarithmic plots of absorbance change against time. ●, Fast phase; ○, slow phase.

oxidase by O_2 . Both spectra peak at $445 nm$, with the slow phase having a trough at $420 nm$ and the fast phase a trough at $425 nm$. With regard to the relative contribution of the fast and the slow phases at $445 nm$, the peak of the static reduced-minus-oxidized difference spectrum is 2.6:1 in favour of the fast phase. This value and the form of the difference spectra agree well with the data reported by Reichardt & Gibson (1982) with the bovine heart enzyme, obtained with a stopped-flow laser-flash system.

Fig. 4(a) reports the O_2 -dependence of the pseudo-first-order rate constants for the absorption change of the fast and the slow phases as measured in the Soret region. The fast-phase rate increases proportionately with O_2 concentration up to about $200 \mu M$, after which the rate increases in a non-proportional manner. At low O_2 concentration, where the rate is proportional to O_2 concentration, a bimolecular combination rate of $8.5 \times 10^7 M^{-1} \cdot s^{-1}$ may be calculated. A double-reciprocal plot of the fast-phase data shown in Fig.

Fig. 4. Dependence on O_2 concentration of the rates of the fast phase and slow phase observed in the Soret region. The conditions were identical with those described in Fig. 1 legend. (a) Observed rates plotted against O_2 concentration for the fast phase (●) plotted on the left-hand scale and the slow phase (○) plotted on the right-hand scale. (b) Double-reciprocal plot of the fast-phase data.

4(b) is linear and allows the determination of the maximum pseudo-first-order rate at infinite O_2 concentration of $3.5 \times 10^4 s^{-1}$ and an apparent K_m for oxygen of $285 \mu M$. The unusually high value for the K_m for O_2 is in agreement with that found at low temperature by Chance *et al.* (1975). In contrast, the rate of the slow phase is virtually independent of O_2 concentration and reaches a limit of about $800 s^{-1}$ at $680 \mu M$ O_2 at $20^\circ C$. These values are in good agreement with those reported by Greenwood & Gibson (1967).

As previously reported by Greenwood & Gibson (1967), three phases of absorption change are also seen in the visible region and become better defined in time as the O_2 concentration is increased. In Fig. 5 time courses at three wavelengths in the visible region are shown at an O_2

Fig. 5. Visible-region absorption changes during the reaction of fully reduced cytochrome *c* oxidase with O_2 . The enzyme concentration was $2.75 \mu M$, the temperature $20^\circ C$ and the pH 7.4. The enzyme was reduced with 3.15 mM-sodium ascorbate plus 40 nM-cytochrome *c*. The CO concentration was $500 \mu M$ and the O_2 concentration $135 \mu M$. The observation wavelengths are indicated on the Figure. The time courses are all recorded on the same time scale, but are shifted along the absorption axis for ease of comparison.

concentration of $135 \mu M$, where all three phases may be discriminated. At 607.5 nm a fast phase of decreasing absorption is followed by a phase of intermediate rate increasing in absorption and finally a slow phase of decreasing absorption. At 570 nm the trace begins with a lag phase followed by a phase of increasing absorption and finally a slow phase of decreasing absorption. At 645 nm all three phases proceed in the direction of increasing absorption. For this reason what must be a small contribution of the intermediate-phase component is difficult to discern, but the fast-phase and slow-phase components are easily seen. Therefore, as in the Soret region, the time courses in the visible

region may be described as being composed of three consecutive exponential processes.

By analysis of the time courses on semi-logarithmic plots of absorbance change against time, the amplitudes for the three phases were determined, and these are presented as kinetic difference spectra in Fig. 6 over the range 570–690 nm. All three components are most intense at 605 nm, the α -band maximum of the spectrum of reduced cytochrome *c* oxidase. The slow phase is the dominant phase and is seen at all wavelengths. The amplitudes of the fast and intermediate rate phases are of equal intensity but of opposite sign at 605 nm. The intermediate rate phase has significant contribution at wavelengths around 580 nm, whereas the fast phase contributes from 585 to 650 nm. The O_2 -concentration-dependences of the fast-phase and slow-phase rates are the same as that reported for the corresponding phases in the Soret region (not shown). The O_2 -concentration-

Fig. 6. Kinetic difference spectra in the visible region for the three phases observed during the reaction of cytochrome *c* oxidase with O_2 .

The conditions were identical with those indicated in Fig. 5 legend. The phase amplitudes were determined from semi-logarithmic plots of absorbance change against time. ●, Fast phase; □, intermediate phase; ○, slow phase.

dependence of the intermediate rate is identical with that of the corresponding phase measured at 830 nm (see Fig. 8).

In Fig. 7 two time courses of absorption change are compared. At 830 nm the absorption is increasing with time. At this wavelength the time course begins with a lag phase followed by a phase of increasing absorption equivalent to the intermediate phase measured in the visible region. The time course at 830 nm finishes with a slow phase of increasing absorption. In Fig. 7 the time course at 830 nm is compared with a kinetic record obtained under identical conditions in the visible region at 610 nm, a wavelength within the α -band of the spectrum of the reduced enzyme. Since this visible-region trace at 610 nm is identical in form with that obtained at the much lower protein concentration used in the visible-region work reported above (see Fig. 5) and with that reported earlier by Greenwood & Gibson (1967), we conclude that the complex time course observed at 830 nm is not a consequence of the much higher enzyme concentration required for work in this spectral region. Kinetic records were also collected in the near-i.r. region at 730, 790 and 880 nm and were found to be identical in their form with that at 830 nm (not shown), indicating that the spectral contribution of the kinetic components is homogeneous throughout the broad absorption band centred at 830 nm.

Fig. 8 shows the O_2 -concentration-dependence of the two phases of absorption change seen at 830 nm. The rate of the faster phase at 830 nm, which corresponds in time to the intermediate phase seen in the visible region, increases in a non-proportional manner with O_2 concentration above $100 \mu M$ and reaches a rate limit of $7000 s^{-1}$. This behaviour was identical with that of the

Fig. 7. Time courses of absorption change at 830 nm and 610 nm during the reaction of cytochrome *c* oxidase with O_2 . The enzyme concentration was $8.90 \mu M$, the temperature $20^\circ C$ and the pH 7.4. The enzyme was reduced with 5 mM-sodium ascorbate plus 150 nM-cytochrome *c*. The CO concentration was $500 \mu M$ and the O_2 concentration $345 \mu M$.

intermediate phase observed in the visible region. The slow-phase rate at 830nm was virtually independent of O₂ concentration over the range studied and reached a rate limit of 800s⁻¹.

Fig. 8. Dependence of the observed rates of absorption change at 830nm on O₂ concentration. The conditions were identical with those indicated in Fig. 7 legend. ○, Fast phase, plotted on the left-hand scale; ●, slow phase, plotted on the right-hand scale.

Table 1 summarizes the values of the rate constants obtained in the different spectral regions at two different concentrations of O₂.

Discussion

In Table 2 values for the ratios of the amplitudes of the fast and the slow phases obtained during the oxidation of fully reduced cytochrome *c* oxidase at the Soret and α-band maxima are compared with values obtained in kinetic reductive experiments with both ferrocytochrome *c* and artificial reductants and with ratios assigned for cytochromes *a* and *a*₃ as a result of the use of static spectrophotometry of various liganded forms of the enzyme. The values from static experiments suggest that cytochromes *a* and *a*₃ make an equal contribution to the reduced-minus-oxidized difference spectrum, whereas in the visible region at 605nm the absorbance of cytochrome *a* is 4 times that of cytochrome *a*₃. The reductive-kinetic technique generates half-reduced forms of the oxidase with cytochrome *a* reduced and cytochrome *a*₃ oxidized, and the data obtained may be used to calculate the relative contributions of the two haem species to the spectrum of the oxidase. These values are shown in Table 2 and are in good agreement with those determined in the static spectrophotometric studies. Similarly the oxidative-kinetic experiments have been proposed to generate half-

Table 1. Observed rates of absorption change in the Soret, visible and near-i.r. regions during the O₂ reaction of fully reduced cytochrome *c* oxidase

All measurements were made at 20°C in 0.1 M-sodium phosphate buffer, pH 7.4, with 0.25% Tween-80. The enzyme concentrations used were 1.3 μM in the Soret region, 2.75 μM in the visible region 15.7 μM in the near-i.r. region. Values in the Soret and visible regions are the means ± s.d. for four determinations. In the near-i.r. region the numbers are from a single experiment.

[O ₂] (μM)	Phase	<i>k</i> _{obs.} (s ⁻¹)		
		Soret region (445 nm)	Visible region (605 nm)	Near-i.r. region (830 nm)
135	1	1.0 × 10 ⁴ ± 0.31 × 10 ⁴	1.3 × 10 ⁴ ± 0.22 × 10 ⁴	—
	2	—	5.5 × 10 ³ ± 0.62 × 10 ³	5.8 × 10 ³
	3	450 ± 78	780 ± 90	600
680	1	2.6 × 10 ⁴ ± 0.31 × 10 ⁴	2.5 × 10 ⁴ ± 0.35 × 10 ⁴	—
	2	—	7.9 × 10 ³ ± 1.9 × 10 ³	7.1 × 10 ³
	3	800 ± 190	850 ± 100	760

Table 2. Values for the ratio of the absorbances of cytochrome *a* to *a*₃ of the reduced-minus-oxidized difference spectrum of cytochrome *c*

Method	<i>a/a</i> ₃ ratio		Reference
	Soret region (445 nm)	Visible region (605 nm)	
Static	0.9	4.4	Vanneste (1966)
Reductive kinetic	1	3.5	Greenwood <i>et al.</i> (1976)
O ₂ reaction	0.43	1.8	Present work

reduced states of the oxidase with the cytochrome a_3 oxidized and cytochrome a reduced over the initial phase of the O_2 reaction (Greenwood & Gibson, 1967). However, the ratios obtained by this method for the spectral contributions of cytochromes a and a_3 as shown in Table 2 do not agree with those obtained by the other methods. If one assumes that the absorption coefficients of cytochrome a and a_3 are independent of the oxidation or ligation state of one another (see Wikström *et al.*, 1981), then there is a discrepancy in the results obtained by the oxidative-kinetic approach. The model advanced by Greenwood & Gibson (1967) to explain the O_2 reaction of reduced cytochrome *c* oxidase is shown in scheme 1 of Fig. 9. In this model O_2 binding is followed by cytochrome a_3 oxidation, then Cu_A oxidation and finally oxidation of cytochrome a . However, this model is not able to account for the relative absorption changes observed during the fast and

the slow phases. We suggest an alteration of this model that introduces a branch in the mechanism after initial O_2 binding and proposes that the oxidation of cytochrome a_3 and some cytochrome a occurs during the initial rapid, O_2 -sensitive, phase of absorption change. This branched mechanism is shown as scheme 2 in Fig. 9. The values obtained for the ratio of the fast and the slow phases in both the Soret and visible spectral region obtained from the O_2 reaction may be reconciled with those obtained by the other techniques by having 40% of cytochrome a oxidized in the rapid O_2 -sensitive phase.

The branched mechanism shown in scheme 2 of Fig. 9 commences with an O_2 -binding step after the removal of CO by photolysis. This results in the formation of an O_2 complex of reduced cytochrome a_3 , or Compound A as designated by Chance *et al.* (1975). Although this species is not observed spectrophotometrically under the condi-

Fig. 9. Schemes for the reaction of fully reduced cytochrome *c* oxidase with O_2

Scheme 1: the Greenwood-Gibson mechanism (after Greenwood & Gibson, 1967). Scheme 2: a branched mechanism.

tions used in the present work, the limit in the rate of the fast phase seen during the reaction is taken as evidence of the formation of cytochrome a_3^{2+} - O_2 complex. The apparent K_m for O_2 of $285\mu M$ reported in the present paper may be compared with the K_D for O_2 of $340\mu M$ from low-temperature experiments (Chance *et al.*, 1978) and the K_m for O_2 of about $1\mu M$ from steady-state measurements (Petersen *et al.*, 1976). Chance *et al.* (1978) concluded that there was a significant 'off' rate for O_2 in the initial complex, whereas the high affinity reported from steady-state experiments (Petersen *et al.*, 1976) is thought to be due to the relatively low turnover rate of the oxidase as compared with the initial O_2 -binding and electron-transfer steps. The apparent K_m for O_2 reported in the present paper is probably due to the high rate of the initial electron-transfer step as $K_m \approx k_2/k_{on}$, where k_{on} is the bimolecular O_2 -binding step and k_2 is the rate of the electron-transfer step. Computer simulation studies also indicate that O_2 'off' rate of between 1×10^3 and $5 \times 10^3 s^{-1}$ is necessary to account for the negligible accumulation of Compound A seen in this work (D. Barber, C. Greenwood & B. C. Hill, unpublished work). We have recently observed the formation of Compound A directly at room temperature during the reaction of the mixed-valence derivative of cytochrome *c* oxidase with O_2 . We suggested that this was possible because the initial electron-transfer step following O_2 binding is much slower with the mixed-valence species than with the fully reduced enzyme and leads to significant accumulation of Compound A (Hill & Greenwood, 1983).

Scheme 2 of Fig. 9 suggests that the first event after O_2 binding is the transfer of two electrons to form a peroxo intermediate. A two-electron-transfer step by-passes the thermodynamically unfavourable formation of a superoxide intermediate (George, 1965). It is at this stage that the branch is introduced. The two electrons transferred in this step may originate from either cytochrome a_3 and Cu_B or from cytochrome a_3 and cytochrome *a*. This heterogeneity may simply be a manifestation of an intrinsic difference in the rates of electron transfer from different metal centres in the enzyme to bound O_2 . Alternatively, it may reflect the equilibrium position between two different conformations of the reduced protein, as is required by the two-state model of cytochrome *c* oxidase advanced by Brunori and co-workers (Wilson *et al.*, 1981; Brunori *et al.*, 1983). This could account for the heterogeneous oxidation of cytochrome *a* proposed in the present paper.

The second electron-transfer step in scheme 2 of Fig. 9 involves the oxidation of Cu_A , as evidenced by the change at 830nm. The origin of the absorption band centred at 830nm has been of

some controversy. Powers *et al.* (1981) have attributed a major contribution from Cu_B to this absorption band, whereas Andréasson *et al.* (1972) have assigned contributions from Cu_A and cytochrome *a* to this spectral region. However, there is growing evidence from spectroscopic potentiometric-titration experiments that this band is nearly entirely due to the e.p.r.-detectable Cu or Cu_A (Babcock *et al.*, 1978; Beinert *et al.*, 1980; Greenwood *et al.*, 1983). But, as shown in the present work (see Fig. 8), the time course of the O_2 reaction at 830nm is not simple. The initial lag in the time course of absorption change at 830nm corresponds to the two-electron-transfer step and as specified by scheme 2 of Fig. 9. The simplest explanation of such a lag is that none of the metal centres involved in this step contributes absorption at this wavelength. Alternatively, a lag could arise from a small contribution of a fast-phase component in opposition to the following absorption change. In either case we conclude that only a very small contribution could be attributed to haem absorption changes in this region in the initial kinetic phase. At 830nm two phases of absorption change follow the initial lag. In the Greenwood & Gibson (1967) mechanism, as shown in scheme 1 of Fig. 9, these correspond to the oxidation of Cu_A and cytochrome *a*, whereas in the branching mechanism both phases may be accounted for by the oxidation of Cu_A . The two phases at 830nm represent the proportions of enzyme molecules in the two branches of scheme 2 in Fig. 9. The observed ratio of the fast/slow phases at 830nm is 60:40, as predicted by the proportion in each branch necessary to account for the cytochrome *a* and a_3 absorption contributions in the Soret and visible regions (see above). Therefore the branching mechanism shown in scheme 2 of Fig. 9 is able to account for the complex kinetics at 830nm and retain Cu_A as the sole chromophore contributing at this wavelength. This result also suggests that interconversion between the two branches of scheme 2 must be slow relative to the rates of the individual steps in the branches. The second and third electron-transfer steps in each branch are shown to occur at identical rates. This implies that the rate limit to electron transfer within the complex is imposed by a shared event such as the binding of protons. For this reason the dependence of the form of these kinetics as a function of pH may be interesting to explore.

Greenwood & Gibson (1967) assigned the second phase of absorption change seen in the visible region at the α -band to be due to a contribution by copper at this wavelength. This phase of absorption change is now known to occur in the Soret region as well. Similarly, the new feature in the Soret-region kinetic records could

also be assigned to a spectral contribution from copper. However, this is considered to be unlikely because of the negligible absorption of known copper proteins in this spectral region. Alternatively, this Soret-region feature could be due to a perturbation of the haem absorption spectra caused by the addition of an electron to the putative peroxo intermediate formed after the initial two-electron-transfer step.

The mechanism of scheme 2 in Fig. 9 is an advance on that presented by Greenwood & Gibson (1967). It is not the only mechanism that will account for the observed kinetic profiles, but it has the advantage of conforming with some of the spectroscopic models being proposed for the oxidase (Beinert *et al.*, 1980; Wikström *et al.*, 1981; Greenwood *et al.*, 1983). In addition, it is aligned more closely with the interpretation of the most detailed low-temperature kinetic studies, which also include a branch in the mechanism after O₂ binding (Clore, 1981).

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