An investigation by e.p.r. and optical spectroscopy of cytochrome oxidase during turnover

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Cytochrome oxidase (EC 1.9.3.1; ferrocycytochrome *c*:oxygen oxidoreductase) was studied during steady-state by optical and e.p.r. methods. Starting with either the 'resting' or the 'pulsed' enzyme, oxidase, cytochrome *c*, ascorbate and O₂ were mixed and the reaction monitored optically. Tetramethylphenylenediamine was used as mediator to poise the steady-state to the desired reduction level. After mixing, the reaction was quenched by the use of rapid-freeze techniques. The e.p.r. spectra of samples captured at increasing tetramethylphenylenediamine concentrations (i.e. higher electron flux) show decreasing $g = 2$ (Cu_A) and $g = 3$ (cytochrome *a*) signals. No Cu_B or $g = 6$ signals (high-spin cytochrome *a*₃) could be found during the reaction. Also, the signal with peaks at $g = 1.69$, 1.78 and 5 as well as the $g = 12$ signal was hardly detectable at higher turnover rates. The only new signal appearing during turnover is a radical signal, which is discussed in terms of a protein radical. Finally, a scheme is presented, proposing a catalytic cycle for cytochrome oxidase with respect to the O₂ binding Cu_B–cytochrome *a*₃ unit.

Cytochrome oxidase has been extensively studied by e.p.r. spectroscopy for many years (Malmström, 1979; Brunori *et al.*, 1981). Although cytochrome oxidase contains two haem *a* groups and two copper ions in its fully oxidized resting condition, only two of the metal sites, cytochrome *a* and Cu_A, are considered to contribute significantly to the e.p.r. spectrum. The enzyme may, nevertheless, exhibit a wealth of e.p.r. signals depending on the valence state of its other metal centres and on its past history. The low-spin Fe(III) signal of cytochrome *a* at $g = 3$ and the Cu(II) signal of the magnetically isolated Cu_A atom at $g = 2$ are always present in the oxidized enzyme, whereas the high-spin Fe(III) signal of cytochrome *a*₃ and the Cu_B signal are generally not observed, owing to antiferromagnetic coupling between these sites. This coupling may, however, be broken by reduction of one of the metals in the binuclear Fe/Cu centre or by binding ligands to one of the pair. Hartzell & Beinert (1974, 1976) have reported the transient appearance of a high-spin ferric $g = 6$ signal during the course of reductive redox titrations of cytochrome oxidase, a feature of the partially reduced enzyme. At higher pH values this signal is converted into a low-spin

ferric signal at $g = 2.6$ (Hartzell & Beinert, 1974; Lanne *et al.*, 1979). A high-spin signal at $g = 6$ has been reported by Brudvig *et al.* (1980) on binding NO to the oxidized enzyme. This signal is thought to arise from breaking the coupling by preferential ligation to and reduction of the Cu_B site. Recently Reinhammar *et al.* (1980) and Karlsson & Andréasson (1981) have observed, for the first time, a Cu_B signal around $g = 2$ by preferentially reducing the iron of cytochrome *a*₃ and (possibly) holding it in the ferrous state as an oxygen adduct.

The fully oxidized enzyme also displays a number of other weaker signals (e.g., the $g = 12$ signal) depending on how it has been prepared and whether it has recently passed through a cycle of reduction and oxidation, e.g., the $g = 1.69$, 1.78 and 5 signals reported by Shaw *et al.* (1979).

What, however, has yet to be established is which of these signals, and thus which enzymic species, are present during turnover. In other words, what is the e.p.r. spectrum of cytochrome oxidase while it is rapidly catalysing the oxidation of cytochrome *c* by molecular oxygen under steady-state conditions? The answer to this question is difficult to obtain for technical reasons. In order to produce adequate e.p.r. spectra one must use moderately high enzyme concentrations (high at least for steady-state assays, i.e. approx. 30 μ M). Under such conditions, however, the steady-state lies totally towards the oxidized

Abbreviation used: TMPD, *NNN'*N'-tetramethyl-phenylenediamine dihydrochloride.

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condition, as the rate of oxidation of ferrocytochrome *c* by such high enzyme concentrations competes very efficiently with the re-reduction of cytochrome *c* by ascorbate, the normal reductant used in steady-state assays. If, however, TMPD is added as an electron mediator between ascorbate and cytochrome *c* in order to poise the steady-state at some desired intermediate level of reduction, then the steady-state lasts only approx. 1–10 s, thus necessitating the use of rapid-freeze techniques to trap the steady-state. Rapid-freeze techniques are also demanded by the recent observations by Jensen *et al.* (1981) that slow-freeze methods allow electron redistribution to occur before freezing is complete, thus changing the e.p.r. spectrum of any sample trapped in steady-state.

To overcome these problems we have developed an apparatus that allows us to monitor both the redox level of the steady-state by absorption spectroscopy and to capture the sample in isopentane cooled by liquid N₂ by rapid-freeze methods. By using this method we have been able to examine the e.p.r. spectrum of cytochrome oxidase while it is engaged in rapid turnover, catalysing the oxidation of ferrocytochrome *c* by dioxygen. We have also examined the e.p.r. properties of the steady-state intermediates of cytochrome oxidase starting from either the so called 'resting' or 'pulsed' derivatives (Antonini *et al.*, 1977).

The main conclusions may be summarized as follows. (1) Starting from either 'resting' or 'pulsed' enzyme the $g = 2$ (Cu_A) and $g = 3$ (cytochrome *a*) signals are present in steady-state to an extent that reflects the overall reduction level as estimated from the optical absorption data. Thus in the absence of TMPD they are fully expressed, whereas, on increasing the electron entry rate into cytochrome *c* by addition of TMPD, these e.p.r. signals are decreased. (2) No $g = 6$ or Cu_B signals appeared, indicating that species giving rise to these signals are either outside the catalytic cycle or poorly populated. (3) Although quantification is difficult, it appears that the peaks at $g = 1.69$, 1.78 and 5 (Shaw *et al.*, 1979) as well as at $g = 12$ observed in some species of the fully oxidized enzyme (Brudvig *et al.*, 1981) become smaller during turnover and tend to disappear at the highest turnover rates explored. (4) A radical signal appears during turnover.

These conclusions are discussed in relation to a suggested catalytic scheme.

Materials and methods

Enzyme and chemicals

Beef heart cytochrome oxidase was prepared either by the method of Van Buuren (1972) or (with minor modifications) by the method of Yonetani (1960). The former yielded enzyme containing 12 nmol of haem a/mg of protein and an absorbance

ratio A_{444} (reduced)/ A_{420} (oxidized) of 2, whereas the corresponding values for the latter preparation were 9.7 and 2.4 respectively.

Cytochrome *c* and cytochrome oxidase concentrations were determined as described by Jensen *et al.* (1981).

All experiments were performed in Hepes [4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid] buffer. Chemicals were all of analytical grade.

E.p.r. spectra

E.p.r. spectra at 9 GHz were recorded on a Varian E-9 spectrometer equipped with an Oxford Instruments ESR-9 helium-flow cryostat or on a Varian E-3 spectrometer at 77 K. The E-9 spectrometer was connected on-line to a Nova 3 minicomputer, in which signal averaging and difference spectra were performed. Integration of the signals was by the method of Aasa & Vänngård (1975).

Rapid-freeze experiments

To monitor the redox states of the components of steady-state assays of cytochrome oxidase containing high cytochrome *c* and enzyme concentrations, we have used the apparatus shown in Fig. 1. This consists of a computer (Nova 3 minicomputer) controlled ram (Update Instruments Inc.), which drives solutions from mounted syringes through a simple 'T' mixer and into an optical cell of 160 μ l volume and 2 mm pathlength (see Fig. 1b). The push-length and ram velocity could be selected as could the number of pushes to be delivered and the time interval between pushes. The outflow of the cell passed through a nozzle, 0.2 mm in diameter, suspended just above the surface of isopentane cooled by liquid N₂. The optical cell was also furnished with flexible light guides bringing light of selected wavelengths from a monochromator (Shoefel GM 100-2) and delivering it to an EMI 9592 B photomultiplier. The photomultiplier output was displayed on an oscilloscope (model 564 Tektronix storage scope). The apparatus is therefore a form of multi-push stopped-flow. The first push mixes the solutions and at selected points in the ensuing optically monitored progress curves, a second push dispels the mixture into cold isopentane. The solution exhausted from the cell by the first push was discarded, whereas that from the second push was collected in an e.p.r. tube.

The ram velocity was chosen to give solution velocities at the exit nozzle that ensured rapid freezing of the samples and samples that could be packed in e.p.r. tubes. The time from the solution leaving the nozzle to the time by which freezing was completed was less than 20 ms.

Results and discussion

Preparations

In a recent paper, Brudvig *et al.* (1981) reported

Fig. 1. Apparatus for freeze-trapping turnover intermediates

(a) Block diagram of the assembled components that combine a stopped-flow optical system with rapid-freeze e.p.r. Abbreviation: PMT, photomultiplier tube. (b) Optical cell.

that cytochrome oxidase, as prepared, is comprised of a mixture of three oxidized forms distinguishable by virtue of their differing e.p.r. spectra and reactivities towards added ligands. These three forms are (a) a form possessing a broad signal of low amplitude at $g = 12$, (b) a form that on exposure to NO exhibits the large $g = 6$ signal of high-spin ferric cytochrome a_3 and (c) the classical 'oxygenated' form that on exposure to fluoride displays a set of e.p.r. signals at $g = 8.5, 6, 4.3$ and 3.2 . Brudvig *et al.* (1981) also report that the proportions of these forms depend on the method of isolation.

We wish to note here that we have confirmed these authors' findings to the extent that we have observed these types of oxidized enzyme by using the above criteria and that oxidase prepared by the Van Buuren (1972) method and by the Yonetani (1960) method seem to contain different proportions of them. Thus the preparation by Van Buuren (1972) appeared similar to that by Hartzell & Beinert (1974), possessing a high proportion of the enzymic form, which gives a $g = 6$ signal with NO, whereas the preparation by Yonetani (1960) was similar to the preparation by Yu *et al.* (1975), being relatively unchanged on addition of NO.

We have also noted, in agreement with the extensive studies by Hartzell & Beinert (1974), that the general shape of the e.p.r. spectrum in the $g = 2.2$ region is preparation-dependent, as are the intensities of the $g = 1.69, 1.78$ and 5 peaks, which we also find as small features of the enzyme as isolated.

The point we wish to make in the present context, however, is that the broad conclusions we draw from the studies of the enzyme in turnover, described below, do not depend on the nature of the preparation with which we started (i.e. by the Van Buuren or Yonetani methods). Thus it may be that all the forms of the oxidized enzyme are capable of

generating those enzymic species that are important as intermediates in the catalytic cycle.

Slow-freeze experiments

Jensen *et al.* (1981) have recently shown that the e.p.r. spectrum of cytochrome oxidase undergoing slow turnover in the presence of ascorbate and low cytochrome c concentrations depended markedly on whether the steady-state sample was frozen slowly or rapidly. Slow freezing caused electron redistribution leading to a greater reduction of cytochrome a and Cu_A than was observed in the rapidly frozen samples. In the course of the present study we have performed some slow-freeze experiments at high cytochrome c concentrations and thus at higher turnover rates than explored by Jensen *et al.* (1981). Fig. 2 shows the results of such an experiment in which $191 \mu\text{M}$ -oxidase and $200 \mu\text{M}$ -cytochrome c were reduced by 10 mM -ascorbate. Fig. 2(b) shows the e.p.r. spectrum after briefly (approx. 10 s) passing a stream of O_2 through the sample in an e.p.r. tube and then freezing it in liquid N_2 (slow freeze). In comparison with the spectrum of the control (Fig. 2a), a strong signal around $g = 6$, a feature of cytochrome a_3 , is well resolved, presumably due to partial reduction of the Cu_B site. Also the cytochrome a signal at $g = 3$ and the Cu_A signal around $g = 2$ are attenuated, indicating partial reduction of these components. The spectrum shown in Fig. 2(b) is typical of those obtained from partially reduced oxidase when O_2 is depleted (Aasa *et al.*, 1976; Hartzell & Beinert, 1976). Fig. 2(c) shows the e.p.r. spectrum resulting from thawing the sample of Fig. 2(b) and bubbling O_2 through it for some 30 s followed by refreezing. We now observe that the rhombic $g = 6$ signal has disappeared and the signal assigned to Cu_B (Karlsson & Andréasson, 1981) has appeared.

Fig. 2. *Slow-freeze e.p.r. samples of cytochrome oxidase* (a) Control (191 μM -oxidase). (b) 191 μM -oxidase, 200 μM -cytochrome *c* completely reduced with 10 mM-ascorbate under N_2 and then exposed to a brief (10 s) stream of O_2 before freezing. (c) Sample from (b) thawed, O_2 bubbled through for 30–40 s and then re-frozen. Solutions were made up in 50 mM-Hepes buffer, pH 7.4, containing 0.5% Tween 80. The conditions of e.p.r. spectroscopy were: microwave power, 2 mW; microwave frequency, 9.254 GHz; modulation amplitude, 2 mT; temperature, 16 K.

Karlsson & Andréasson (1981) postulate that this signal arises on trapping ferrous cytochrome a_3 as a true oxygen adduct, thus uncoupling it from cupric Cu_B . The e.p.r. parameters of Cu_B have been given by these authors as g_x 2.052, g_y 2.112, g_z 2.278 and line widths 7.5, 5.0 and 3.0 mT. Carithers & Palmer (1981) have also reported the appearance at high pH values (pH 10) of an e.p.r. signal, which they attribute to Cu_B . The parameters under these conditions are somewhat different, however (e.g. g_z 2.295). These differences possibly arise from the conditions employed and are discussed by Carithers & Palmer (1981). Cytochrome *a* and Cu_A appear to remain partially reduced.

Although the $g = 6$ and Cu_B signals can, therefore, both be observed under turnover conditions by slow-freeze-techniques the point we wish to stress here is that these signals were never observed when rapid-freeze methods were used (see below). We thus conclude that the species of oxidase responsible for the spectra in Figs. 2(b) and 2(c) are not present in significant quantities in the catalytic cycle as such, but may be forms lying on side branches off this cycle (see Fig. 7) and that true 'turnover' intermediates are displaced into these cul-de-sacs by perturbation of temperature-dependent equilibria.

Such perturbations are possible using slow freeze, which takes some seconds to achieve liquid nitrogen temperatures, but do not occur when freezing is rapid.

Rapid-freeze experiments

These experiments were performed in two ways. Either (a) mixing a solution of 'resting' oxidized cytochrome oxidase with one containing ferrocycytochrome *c* in the presence of ascorbate and TMPD, or (b) mixing a solution of reduced cytochrome oxidase, ferrocycytochrome *c*, ascorbate and TMPD with aerobic buffer. In the first class of experiments one examines the steady-state species starting from the 'resting' enzyme, whereas in the second class, the starting material is oxidized 'pulsed' oxidase. The kinetic and spectral characteristics of these forms of the enzyme have been described elsewhere (Antonini *et al.*, 1977; Brunori *et al.*, 1979).

In both types of experiment, the optical absorbance was monitored at 550 nm and 605 nm, wavelengths characteristic for cytochrome *c* and cytochrome oxidase respectively. Fig. 3 shows a number of kinetic progress curves obtained. At 550 nm rapid bleaching of the absorbance indicating cytochrome *c* oxidation gave way to a steady-state in which little absorbance change occurred. Similar behaviour was observed at 605 nm. Normalization of the absorbance changes at 550 nm resulting on going from the steady-state to complete reduction to the maximum absorbance change possible (i.e. fully oxidized to fully reduced) gives a direct measure of the reduction levels of cytochrome *c* during steady-state. At 605 nm the situation is complicated by both cytochromes *a* and a_3 contributing to the spectrum, but given a knowledge of the relative sizes of these contributions, a similar normalization procedure is possible. Owing to variations in O_2 concentration in the different experiments shown in Fig. 3, the lengths of the steady-states cannot be compared. However, in independent experiments (not shown) we have determined that the level of the steady state was independent of the initial oxygen concentration over the oxygen concentration range explored.

Fig. 4 reports, through the absorbance changes, the redox levels of the steady-states achieved from either 'resting' or 'pulsed' oxidase as a function of the concentration of TMPD. It is clear that the redox level during steady-state, as expected, moves towards reduction as the TMPD concentration increases. Also, the 'pulsed' enzyme maintains both cytochrome *c* and *a* more oxidized than the resting enzyme. These results are in good agreement with those reported by Brunori *et al.* (1979).

Fig. 3 also shows that, starting from the resting enzyme, the reduction process, monitored at 605 nm, which follows the collapse of the steady state, takes place in two stages. The faster of these two, com-

Fig. 3. Progress curves for the reaction between cytochrome *c* and oxidase monitored at 550 nm and 605 nm

Experiments in the upper panels were performed by mixing aerobic 'resting' cytochrome oxidase with ferrocytochrome *c* in the presence of ascorbate and TMPD. The concentrations after mixing were as follows: cytochrome oxidase, 52 μ M; ferrocytochrome *c*, 40 μ M; ascorbate, 12.5 mM; O_2 , in equilibrium with air. Experiments in the lower panels were performed by mixing a solution containing cytochrome oxidase, ferrocytochrome *c*, ascorbate and TMPD with aerobic buffer. The concentrations after mixing were: cytochrome oxidase, 52 μ M; ferrocytochrome *c*, 46 μ M; ascorbate, 15.5 mM; O_2 , in equilibrium with O_2 gas. The TMPD concentrations are given in parentheses (in mM). The vertical line in each panel corresponds to 0.1 absorbance units and the horizontal line to 2 s. Where shown, the vertical arrows indicate the time of the second 'push', displacing the 'steady-state' reaction mixture into cold isopentane. The reactions were performed in 50 mM-Hepes buffer at pH 7.4, containing 0.5% Tween 80. The temperature was 20°C. Upward deflections at 550 nm and 605 nm correspond to reduction of cytochrome *c* and cytochrome oxidase respectively. The initial downward deflection for each trace is due to previous solution being swept out of the observation cell and to the establishment of a steady state.

prising approx. 80% of the total absorbance change in the absence of TMPD (not shown), decreases at high TMPD concentrations and practically vanishes at the highest TMPD concentrations used (see Fig. 3). This behaviour is consistent with the reduction of cytochrome *a*, which makes the major spectral contribution at the wavelength, and which, being in rapid electron-transfer equilibrium with cytochrome *c*, should follow the steady-state-reduction level of this component.

The amplitude of the slower reduction process observed at 605 nm was TMPD concentration-

Fig. 4. Dependences of Δ absorbance changes (from steady-state to the fully reduced condition) on the TMPD concentration

Conditions were as described in the legend to Fig. 3. (a) $\circ$, Resting enzyme; $\bullet$, pulsed enzyme. The absorbance values, measured at 550 nm, have been normalized to correspond to the same cytochrome *c* concentration in the experiments with resting and pulsed oxidase. (b) $\circ$ shows the total absorbance change at 605 nm observed for the resting enzyme, and $\square$ and $\triangle$ report the absorbance changes taking place in the fast and slow phases, respectively. $\bullet$ refers to the pulsed enzyme.

independent. We conclude that in this slow process, we are monitoring the reduction of the O_2 -binding site, cytochrome a_3 - Cu_B , upon onset of anaerobicity. Thus, even when the electron flux from cytochrome c into the enzyme ensures that in the steady-state cytochrome a is almost fully reduced, the steady-state level of the oxygen-binding site (cytochrome a_3) lies strongly towards the oxidized condition. This conclusion is consistent with the behaviour of the 'pulsed' enzyme, in which no slow phase of reduction is observed at 605 nm (Fig. 3). This is in keeping with the known enhancement of the internal electron transfer rate in this enzymic form (Antonini *et al.*, 1977; Brunori *et al.*, 1979) so that the reduction of cytochrome a_3 by internal electron transfer from cytochrome a is faster. Nevertheless, even at high TMPD concentrations, the steady-state level of the 605 nm absorbance was not held at the fully reduced position, suggesting once more than cytochrome a_3 is not significantly reduced in turnover in the 'pulsed' enzyme. Calculations based on Figs. 4(a) and 4(b) (using for cytochrome c $\Delta\epsilon_{530} = 20\,000\text{ M}^{-1}\cdot\text{cm}^{-1}$ and for cytochrome a $\Delta\epsilon_{605} = 18\,000\text{ M}^{-1}\cdot\text{cm}^{-1}$) also reveals that during steady-state more cytochrome a is reduced than cytochrome c , indicating that under these conditions (and assuming quasi-equilibrium

between these components) the redox potential of cytochrome a is more positive than that of cytochrome c by approx. 30–50 mV. This result agrees with previous reports (Andréasson, 1975; Brunori *et al.*, 1979).

Double-push experiments, in which a sample of the steady-state mixture was first monitored optically and then dispelled into cold isopentane, allowed us to monitor the e.p.r. spectra of the enzyme poised at known steady-state levels. Fig. 5 shows an illustrative set of spectra obtained for the 'resting' and 'pulsed' enzyme and in the absence of, and at high concentrations of, TMPD. It is clear that both the low-spin ferric signal at $g = 3$ and the copper signal around $g = 2$ decrease with increasing TMPD concentration, indicating that cytochrome a and copper Cu_A are held at increasingly high reduction levels in steady-state as the electron flux into cytochrome c is increased. Fig. 6(a) shows the relative size of the $g = 3$ e.p.r. signal as a function of TMPD concentration. The low-amplitude signals exhibited by oxidase in its various oxidized states also tend to disappear once the enzyme enters its catalytic cycle. Thus the $g = 1.69$, 1.78 and 5 signal, characteristic of the enzyme, which has undergone a cycle of reduction and oxidation by molecular oxygen, is

Fig. 5. *E.p.r. spectra of freeze-trapped oxidase under turnover*

(a) and (b) refer to 'resting' and (c) and (d) to 'pulsed' oxidase. The concentrations of components were as follows: (a) 52 μM -oxidase, 40 μM -cytochrome c , 12.5 mM-sodium ascorbate; (b) as (a) with 0.27 mM-TMPD; (c) 53 μM -oxidase, 49 μM -cytochrome c , 15.5 mM-sodium ascorbate; (d) as (c) with 0.72 mM-TMPD. These concentrations are correct for the solutions in steady-state before freezing. The actual concentrations in the e.p.r. tubes will be less, owing to the presence of frozen isopentane. The buffer was 50 mM-Hepes, pH 7.4, containing 0.5% Tween 80. The insets to (c) and (d) show the $g = 1.69$, 1.78 signal and the radical signal respectively. The g -value of the radical was 2.0048 with a linewidth of 0.8 mT. Microwave power was 2 mW, microwave frequency was 9.251 GHz, modulation amplitude 2 mT and temperature 16 K. For the inset to (d), however, the corresponding values were 0.2 mW, 9.251 GHz, 0.5 mT and 77 K respectively.

clearly developed in the 'pulsed' low-turnover system (Fig. 5c). However, the signal was found to decrease in size on increasing the turnover rate of the enzyme by addition of TMPD and we were unable to detect its presence under high-turnover conditions. Similarly the $g = 12$ signal (not shown) also appeared to become smaller at higher TMPD concentrations. The species giving rise to these signals do not, therefore, seem to be significantly populated under conditions when the catalytic cycle is operating at maximal rates.

The high-spin ferric $g = 6$ signal and the new copper signal around $g = 2$, observed in partially reduced species (see also Fig. 2), were never observed in the steady-state enzyme. This means either that the cytochrome a_3 and Cu_B sites are reduced, a conclusion unlikely in the light of the optical data discussed above, or that these sites remain coupled in turnover or alternatively that any species in which uncoupling occurs must be present in low concentrations.

The overall conclusion, therefore, is that, in terms

Fig. 6. E.p.r. signal amplitudes as a function of TMPD concentration

Signal amplitude is expressed as a percentage of the maximum value observed. Open symbols refer to 'resting' and filled symbols to 'pulsed' enzyme. Concentrations and e.p.r. conditions are as in Fig. 5. (a) Low-spin ferric cytochrome a $g=3$ signal (corrected for cytochrome c contribution); (b) radical signal at $g=2$.

of previously described e.p.r. signals, cytochrome oxidase during turnover possesses an almost featureless spectrum. There is one signal, however, which is observed during turnover, which can amount to as much as 5% of the oxidase concentration and which becomes more pronounced under high-turnover conditions. In some samples this value could be increased to at least 10% by warming the sample to 200 K. This signal, at $g = 2$, clearly stems from free-radical species. Its form is complex and appears to be a superimposition of two radical signals. However, storage of the rapid-freeze samples even at liquid- N_2 temperatures led to a form in which only a single radical species was detectable. Such radical signals have been noted previously (Beinert, 1974) and have been discussed briefly in terms of intermediates of the 'turnover' enzyme by Jensen *et al.* (1981). The origin of this signal is not known. The most obvious candidate is a radical formed from the reductant used, i.e. ascorbate (Hartzell & Beinert, 1976) or TMPD. However, Jensen *et al.* (1981) report an identical $g = 2$ signal with that reported here, resulting when the enzyme is reduced without ascorbate (say by NADH and phenazine methosulphate). Thus in contrast, in a recent paper (Carithers & Palmer, 1981) mediators seem to be excluded as the source of the radical. Although TMPD may be excluded as the source of the radical, as it is produced even in the absence of this mediator, the signal amplitude increases with TMPD concentration, i.e. with increasing electron flux into the enzyme (Fig. 6b). One intriguing possibility is that we are observing a protein radical, the behaviour of which is consistent with it being a feature of an intermediate in the catalytic cycle. To investigate this possibility further, we performed some experiments in which ferrocyanochrome c (1 mM), freed of excess ascorbate by passage down a Sephadex G-25 column, equilibrated with oxygenated buffer, was oxidized directly in an e.p.r. tube by addition of $25 \mu\text{M}$ -cytochrome oxidase. At known times throughout the course of this oxidation, monitored optically at 695 nm, the assay mixtures were frozen by immersing the tubes in isopentane cooled by liquid N_2 . The e.p.r. spectra revealed that ferrocyanochrome c in the oxygenated buffer and a sample that had been completely oxidized by the addition of oxidase and allowed to stand for 20 min before freezing possessed no radicals. However, samples frozen during the enzyme turnover clearly showed a radical signal, which amounted to approx. 1% of the enzyme concentration. This signal may be the broad component of the signal described above and in the inset to Fig. 5(d). Although as yet we cannot absolutely exclude the possibility that the observed radical is derived from the reductant tightly bound to cytochrome c the available evidence is also consistent with the radical

being a feature of a species of the enzyme under turnover.

A catalytic cycle for cytochrome oxidase

The reaction between cytochrome *c* oxidase and molecular oxygen has been investigated at room temperature by Greenwood & Gibson (1967) starting from the fully reduced enzyme and from the 'mixed-valence' enzyme by Greenwood *et al.* (1974). At such temperatures, intermediates in the reactions are so short-lived that they are undetectable. To trap intermediates, low-temperature flash-photolytic methods were developed by Chance *et al.* (1975), who were able to identify a number of transient species on spectral grounds. By using a similar approach allied to e.p.r. methods to probe the nature of the O₂-binding site during the reaction, Clore *et al.* (1980*a,b*) were able to monitor electron transfer from reduced cytochrome *a* and Cu_A sites to the O₂-binding site and were thus able to assign the formal valence state to this unit, although they were unable to determine the electron distribution within it.

These authors have proposed a catalytic cycle for the enzyme. It is difficult, however, to be sure that species identified at low temperatures are on the catalytic pathway as it operates at higher temperatures.

The observations we report on the spectral and e.p.r. properties of the enzyme trapped by rapidly freezing the enzyme during turnover at room temperature we hope allow us to resolve this problem. Our results, although not permitting us to exclude species absolutely from the catalytic cycle, allow us to put some upper limits on their steady-state concentration, which, say, for the compound displaying the Cu_B signal (Reinhammar *et al.*, 1980), must be no more than a few percent of the enzyme as under no circumstances is this species clearly observed. The scheme shown has a number of general features, which should be appreciated before a detailed examination is given. It is now generally agreed that cytochrome oxidase exists in two conformations with different enzyme activity, 'resting' and 'pulsed' (Antonini *et al.*, 1977). Unfortunately, these terms are also used to designate particular species of these conformations with specific valence states and spectroscopic properties. To avoid the resulting confusion it has been suggested by Wilson *et al.* (1981) that the two conformations are named R ('resting') and P ('pulsed') respectively. Thus, the resting enzyme is a particular form of the R conformation, and the pulsed enzyme has P conformation. The important distinction between the R and P conformations appears to be the rate of intramolecular electron transfer to the O₂-binding site (Brunori *et al.*, 1979; Petersen & Cox, 1980) leading to a higher activity for the P conformation. This difference is clearly demonstrated in the progress curves

given in Fig. 3. For the oxidized protein, the stable conformation is R, whereas the fully reduced enzyme is converted into the P conformation.

The catalytic cycle presented here refers to the P conformation, but a complete scheme would include a second catalytic cycle for the R conformation, of which only one species is depicted in Fig. 7. The two cycles may be similar, but the individual species would have other spectroscopic properties (for examples, see below). A 'two-state' model has recently been described by Wilson *et al.* (1981) in which the R and P cycles are linked through both the fully oxidized and reduced forms, but interconversions might conceivably occur at all oxidation levels. However, the relative rates of such conversions are such that under turnover, the P conformation eventually dominates.

In our scheme, certain species are shown as being outside the P cycle. They may not enter the R cycle either, and their conformation is uncertain. All structures refer only to the O₂-binding site. The electrons entering this site come from cytochrome *a* and Cu_A sites, and, for simplicity, these components are not included. Similarly, in the following discussion, all properties described, such as paramagnetism and e.p.r. response, refer to the O₂-binding site only. The nomenclature of the intermediates is that of Clore *et al.* (1980*b*).

Dioxygen reacts with the fully reduced Cu_B¹⁺-cytochrome *a*₃²⁺ site (possibly with Cu_A and cytochrome *a* oxidized; see below) leading to intermediate I_M in which peroxide is bound between the metals, probably forming a non-paramagnetic ground state. This reaction is extremely fast at 20°C. Greenwood & Gibson (1967) have reported a value of 10⁸ M⁻¹·s⁻¹ for the second-order rate constant of the reaction, and an identical value was reported for the reaction of 'mixed-valence' oxidase (Greenwood *et al.*, 1974). Electron transfer from cytochrome *a*₃ and Cu_B sites follows very quickly after O₂ binding at this temperature, and thus we have not included a true O₂ adduct on the pathway, as its concentration must be vanishingly small. Furthermore, the rapidity of the O₂ reaction ensures that the concentration of the reduced site must be extremely small, thus accounting for our optical data. The one-electron reduction of intermediate I leads to a system having an odd number of unpaired electrons, which should give an e.p.r. signal. Indeed, a Cu²⁺ signal with unusual saturation properties has now been seen by Karlsson *et al.* (1981) under somewhat different conditions to those explored here. It seems very unlikely that the reducing equivalents could be retained in the oxidase with the O—O bond intact, and it is suggested that one water molecule leaves the enzyme. Thus the conversion of intermediate I involves breaking of the O—O bond, a mechanistically difficult step. We may expect this

Fig. 7. Proposed catalytic cycle for cytochrome c oxidase

The 'mixed-valence' species is so termed to indicate that while the O_2 -binding site is fully reduced, Cu_A and cytochrome a sites are oxidized. See the text for details. Cytochrome a_3 is abbreviated to a_3 for convenience.

to be slow, certainly slower than the production of intermediate I from the preceding intermediate.

In our rapid-freeze samples we were unable to detect the signal from intermediate II. This may have been due to the relatively low concentration of the enzyme we had to use (approx. $30 \mu\text{M}$ after dilution with the isopentane). It is also likely that intermediate II is present in turnover in low concentration as it is formed by a relatively slow step involving bond breaking and reacts further in a process that may be expected to be faster.

Intermediate III, from which no e.p.r. signal has been found, may, together with intermediate I, comprise the majority of the enzyme during turnover. It decays further to the pulsed form (Shaw *et al.*, 1979). This is at the same oxidation level, but shows the so called $g = 5$ signal and has a 655 nm band. The difference between intermediate III and the pulsed form may lie in the degree of protonation as shown. The concentration of the pulsed form may be lower at high turnover, where the availability of electrons facilitates its conversion into the next intermediate (Fig. 7). This is consistent with the decrease in the $g = 5$ signal observed at high TMPD concentrations.

The reduction of the pulsed form is shown as two successive one-electron steps. Again, the first of these should give an e.p.r.-detectable species. Under turnover, no such signal is observed, which suggests that the transfer rate of the second electron is higher than that of the first. Of the many possible one-electron reduced forms, we have included the one giving a rhombic $g = 6$ signal in the catalytic

cycle. This is based mainly on the observation that this signal is rapidly formed on reacting the fully reduced enzyme, relaxed into the P conformation, with cytochrome c^{3+} (Petersen & Andréasson, 1976) or with O_2 and reducing substrate (Rosén *et al.*, 1977). The axial $g = 6$ signal, on the other hand, forms rapidly on reduction of the resting forms (Rosén *et al.*, 1977) and might therefore be involved in the R cycle. The species giving an e.p.r. signal at $g = 2.6$, observed at higher pH, is in our scheme outside the catalytic cycle. It cannot be excluded, however, that it represents a form in the R cycle at high pH, being produced in a one-electron-reduction of intermediate III.

In a previous report from this laboratory (Karlsson & Andréasson, 1981), the half-reduced form of the O_2 -binding site having cytochrome a_3 reduced was thought to react directly with O_2 . This led to a species, in our scheme labelled 'Cu_B e.p.r. signal', that was believed to take part in the catalytic cycle. However, since we prefer a half-reduced species with cytochrome a_3 oxidized, it seems more likely that the cytochrome a_3 -Cu_B unit must be fully reduced before O_2 reacts. This has the further advantage that O_2 can directly form a bridge between the metal centres, as suggested in the structure of intermediate I. The Cu_B e.p.r. signal, therefore, in our scheme is on a side track, reached in excess of O_2 when the reducing equivalents are exhausted. The intervening species, $\text{Cu}_B^{2+} \text{OH}^-$ -cytochrome a_3^{2+} , is assumed to be present in very low concentration under all conditions, and is not observed in e.p.r.

Although not in the main theme of the present discussion, a few comments on the valence states of cytochrome *a* and Cu_A sites are appropriate. With the rates suggested above, the reduction of the O₂-binding site occurs essentially in two two-electron steps. This implies that when a molecule appears with both cytochrome *a*₃ and Cu_B sites reduced, the other metal centres (or at least one of them) are oxidized. The subsequent reaction with O₂ is faster than the reduction of cytochrome *a* and Cu_A sites. Thus the species reacting with O₂ may be a form of the mixed-valence oxidase, forming intermediate I_M, which then may be reduced to intermediate I. For the species having Cu_B and cytochrome *a*₃ oxidized (at the right hand side of the scheme) it depends on the pH and the turnover rate whether cytochrome *a* and Cu_A sites get reduced in intermediate III or in the pulsed form.

The 'new' radical signal, which may be intrinsic to the 'turnover' enzyme, is not a consequence of the structures written in the scheme. If it is correct that the signal arises from a protein radical in the oxidase, one may speculate that this lies on the electron transfer pathways from the cytochrome *a*-Cu_A side of the complex to the O₂-binding site. Radicals have been implicated in such electron-transfer pathways elsewhere, e.g. cytochrome *c*/cytochrome *c* peroxidase (Poulos & Kraut, 1980). This hypothesis may account for the enhanced amplitude of the radical signal under rapid-turnover conditions, where the electron flux through the enzyme would be greatest and it is noteworthy that the radical is most pronounced in the 'pulsed' enzyme (Fig. 6*b*) in which it has been postulated that such through transfer is more efficient than in the resting state.

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