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Studies on Partially Reduced Mammalian Cytochrome Oxidase

REACTIONS WITH CARBON MONOXIDE AND OXYGEN

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A number of methods were used to prepare a species of mammalian cytochrome oxidase (EC 1.9.3.1, ferrocytochrome *c*–oxygen oxidoreductase) in which only cytochrome a_3 is reduced and in combination with CO. The kinetics of CO binding by cytochrome a_3^{2+} in this species is significantly different from that exhibited by cytochrome a_3^{2+} in the fully reduced enzyme. The second-order rate constant for combination was $5\times10^4\,M^{-1}\cdot s^{-1}$ and the 'off' constant was $3\times10^{-2}\,s^{-1}$. The kinetic difference spectra cytochrome a_3^{2+}–cytochrome a_3^{2+}–CO reveal further differences between the mixed-valence and the fully reduced enzyme. The reaction between cytochrome a_3^{2+} and oxygen in the mixed-valence species was followed in flow–flash experiments and reveals a fast, oxygen-dependent ($8\times10^7\,M^{-1}\cdot s^{-1}$ at low oxygen) rate followed by a slow process, whose rate is independent of oxygen but whose amplitude is dependent on $[O_2]$. The fast oxygen-dependent reaction yields as the first product the so-called 'oxygenated' enzyme. We conclude from these experiments that the ligand-binding behaviour of cytochrome a_3 depends on the redox state of its partners, a fact which represents clear evidence for site–site interaction in this enzyme. The fact that oxygen reacts rapidly with this enzyme species in which only one component, namely cytochrome a_3, is reduced represents clear and unequivocal evidence that this is indeed the O_2-binding site in cytochrome oxidase and may indicate that reduction of oxygen can proceed via single electron steps.

Site–site interactions play a central role in the function of a number of important biological macromolecules, of which haemoglobin (Wyman, 1948; Perutz, 1970) is perhaps the best characterized example. Cytochrome oxidase is an enzyme whose activity is fundamental to aerobic life and is known to contain four redox centres (Muijsers *et al.*, 1972), two haem *a* groups and two copper atoms. On the basis of their spectral and ligand-binding properties the two haem *a* groups have been identified as belonging to cytochromes *a* and a_3 (Keilin & Hartree, 1939), and the two copper atoms in the molecule are also distinguishable on spectral grounds (Beinert *et al.*, 1962). The reasons underlying these differences between both the haem *a* groups and the copper atoms may reside, not only in local differences in the binding site, but also in possible site–site interaction. The unusual properties (Yong & King, 1972; Beinert *et al.*, 1971) of undetectable copper Cu_u and cytochrome a_3 are thought to arise because of interaction between these two centres. It has also been suggested that the ligand-binding properties of cytochrome *a* is influenced by the state of cytochrome a_3 and vice versa (Wilson *et al.*, 1972).

One method of investigating the existence and nature of site–site interactions in cytochrome oxidase is by comparing the properties of enzyme molecules containing both oxidized and reduced centres with those of the enzyme in its fully oxidized or fully reduced state. Such comparisons may reveal that the properties of a specific site depend on the redox state of its partners. In this context Beinert *et al.* (1971) have suggested that only when the interaction between Cu_u^{2+} and cytochrome a_3^{3+} has been broken, by the reduction of Cu_u^{2+}, does the cytochrome a_3^{3+} then exhibit its expected e.p.r.† signal and become free for reduction. This ensures that only cytochrome oxidase molecules containing four electrons are available for reaction with oxygen in the normally functioning enzyme. This paper reports some properties of a derivative of cytochrome oxidase in which the cytochrome a_3 is reduced and the other components are essentially oxidized, a molecule which is referred to as 'mixed-valence' cytochrome oxidase. In this paper we report data on the reaction of reduced cytochrome a_3 with CO and O_2 by rapid-reaction techniques which have proved very useful in similar studies on the fully reduced enzyme (Gibson & Greenwood, 1963). Our present findings clearly show that site–site interactions influence the behaviour of cytochrome a_3^{2+} in

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† Abbreviation: e.p.r., electron paramagnetic resonance.

its reactions with CO and that cytochrome oxidase with only cytochrome a_3 reduced, i.e. without its full complement of electrons, is capable of a rapid reaction with O_2. In this latter case the product of the reaction appears to resemble the so-called 'oxygenated oxidase' (Tiesjema *et al.*, 1972) rather than oxidized oxidase formed as a result of the reaction between the fully reduced enzyme and O_2 (Greenwood & Gibson, 1967).

Experimental

The cytochrome oxidase (EC 1.9.3.1; ferrocytochrome *c*–oxygen oxidoreductase) used in these experiments was prepared from ox heart Keilin–Hartree particles essentially by the method of Yonetani (1960). To ensure the complete removal of adventitiously bound copper, EDTA (10μM) was included in the buffer solutions used in the final fractionation steps. The final product was dissolved in the required buffer, which contained 1% Tween 80 and was stored in small ampoules at liquid-N_2 temperature. All preparations used had a ratio E_{444} (reduced)/E_{424} (reduced) of 2.3 or better (Gibson *et al.*, 1965).

The mixed-valence derivative of cytochrome oxidase was prepared in a number of different ways, all of which take advantage of the specificity of CO to react only with reduced cytochrome a_3, thereby acting as a trapping agent.

(i) After complete reduction of the cytochrome oxidase by using a slight excess of reduced anthraquinone in a sealed vessel, the solution was equilibrated with 1 atmosphere of CO (101kPa). The CO complex formed was back-titrated with ferricyanide under anaerobic conditions and after the oxidation of excess of reductant had been effected it was observed that approximately three equivalents of ferricyanide were required to complete the titration of the enzyme.

(ii) Addition of one equivalent or less of a reductant to an oxygen-free solution of cytochrome oxidase under CO (101kPa) yielded a compound in which cytochrome a_3 was found to be reduced and combined with CO.

(iii) A derivative having the same spectral properties as observed in (i) and (ii) above was formed on incubation of oxidized cytochrome oxidase under CO at room temperature (21°C) in the absence of O_2. This spectral species was formed over the course of several hours, the time depending on the concentration of oxidase, on the degree of oxygen contamination and on the temperature; the mixed-valence enzyme was not formed at low temperatures (4°C). At present we do not know what acts as the reductant under these conditions.

As detailed in the Results section, the properties of mixed-valence oxidase are independent of the method of preparation, and in view of the convenience of method (iii) above, this method was used as a routine. The spectral and functional properties of the mixed-valence enzyme under CO were unchanged after storage for up to 5 days at 4°C. Oxidase concentrations were measured by using an extinction coefficient of 21 $mM^{-1} \cdot cm^{-1}$ at 605nm for the fully reduced protein (Yonetani, 1961).

Superoxide dismutase (bovine) was the kind gift of Dr. G. Bannister (Department of Biochemistry, Radcliffe Infirmary, Oxford, U.K.). All the gases used in these experiments were obtained from the British Oxygen Company (Deer Park Rd., London S.W.19, U.K.) the O_2 being of a medical grade and used without further treatment. CO and O_2-free N_2 were dispensed from the cylinder and stored in glass vessels over an alkaline solution of dithionite-reduced anthraquinone sulphonate before use. All the chemicals used were of AnalaR grade and were obtained from British Drug Houses Ltd. (Poole, Dorset, U.K.), except for Tween 80 and the sodium salt of dimethylarsinic acid (sodium cacodylate), which were obtained from Sigma (London) Chemical Co. (Kingston upon Thames, Surrey, U.K.). This latter compound was used as a buffer in experiments where the Cr^{2+} ion was used as reductant. Solutions containing Cr^{2+} were prepared by dissolving electrolytically pure chromium metal in deoxygenated HCl in an O_2-free N_2 atmosphere (Dawson *et al.*, 1972). Appropriate dilutions of this stock $CrCl_2$ were prepared with deoxygenated cacodylate buffer.

Static spectrophotometric observations were made by using a Pye–Unicam SP.700A recording spectrophotometer. For anaerobic spectrophotometry, cuvettes equipped with vaccine caps (Subaseal) were used. Stopped-flow measurements were made by using an instrument identical with that described by Gibson & Milnes (1964) equipped with 2cm and 2mm light-path cells having 'dead times' of 3ms and 1ms respectively. Flash-photolytic experiments were conducted by using the instrumentation and methods described by Greenwood & Gibson (1967). A Bausch and Lomb monochromator (500mm grating; 1200 grooves/mm; f = 4.4) was used in conjunction with the flash-photolysis apparatus.

E.p.r. measurements were carried out at liquid-N_2 temperatures with a Varian E.3 instrument. The mixed-valence enzyme in combination with CO was prepared directly in 3mm-internal diameter quartz e.p.r. tubes. The spectrum of the enzyme in these tubes was obtained before freezing by using a split-beam spectrophotometer.

Results

Characterization of mixed-valence oxidase

Fig. 1 shows the spectral characteristics of the mixed-valence oxidase in the presence of CO in the Soret, α and near-i.r. regions, compared with those of

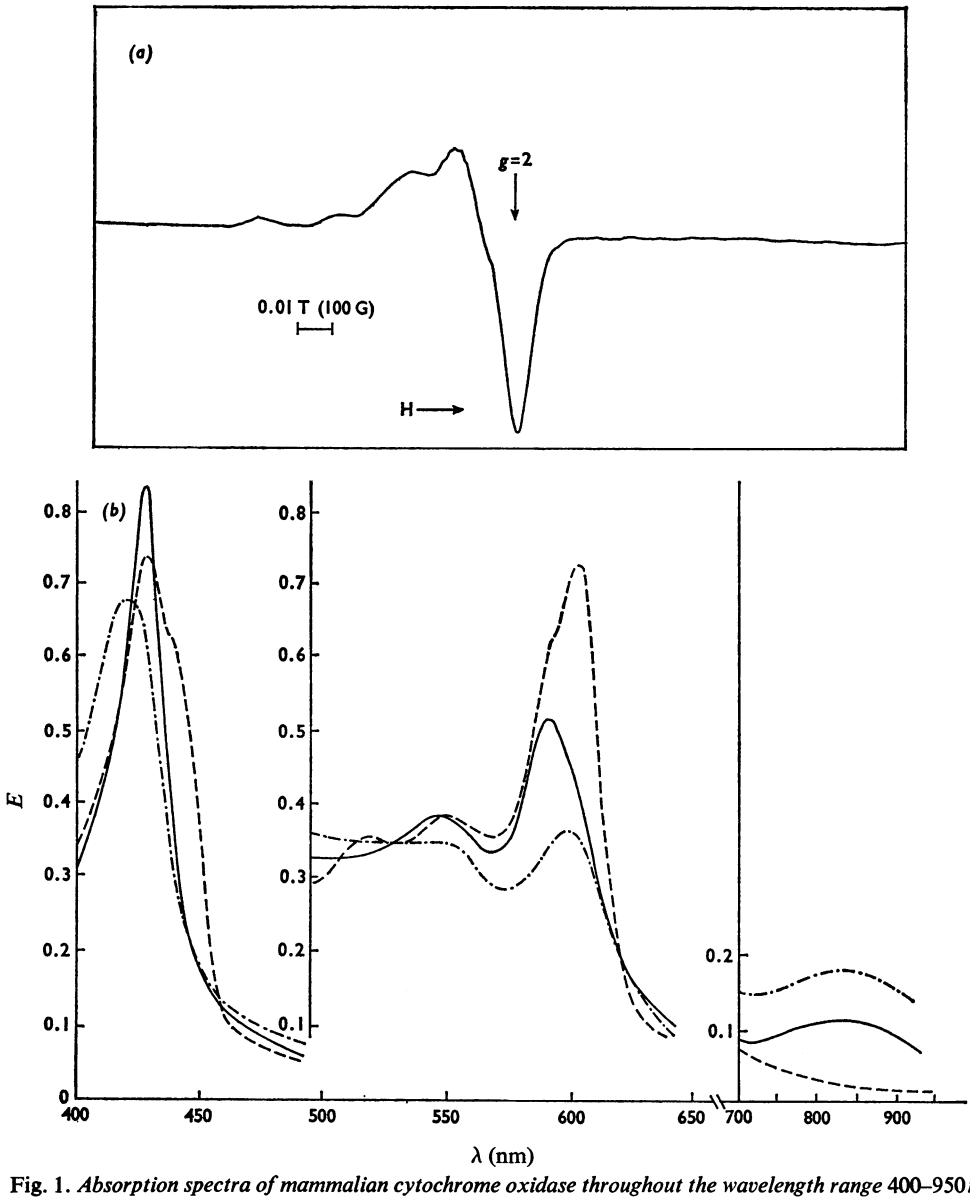


Fig. 1. *Absorption spectra of mammalian cytochrome oxidase throughout the wavelength range* 400–950 *nm*

(*a*) E.p.r. spectrum for the mixed-valence complex (525 μM) in combination with CO recorded at 100 kHz modulation frequency, at 20 mW microwave power and at a temperature of −140°C. The signal amplitude (ordinate) is plotted as a function of the magnetic field H (abscissa), the direction of increase of which is indicated by the arrow. In (*b*) 8.5 μM-oxidase was used in the Soret region, whereas in the α-band region this was raised to 36.5 μM and was 80 μM in the near i.r. The enzyme was, in all cases, dissolved in 0.1 M-potassium phosphate buffer, pH 7.4, containing 1% Tween 80, and unless otherwise reported, was at a temperature of 21°C and the light-path was 1 cm.

fully oxidized enzyme and the fully reduced CO complex. Fig. 1(*a*) shows the e.p.r. spectrum of the mixed-valence compound around $g = 2$ which is consistent (Fig. 1*b*) with fully oxidized copper in the enzyme. It is clear that the spectrum (Fig. 1*b*) of the mixed-valence enzyme is quite distinct from that of either the oxidized or the fully reduced enzyme in the presence of CO. In the Soret and α-band region the spectrum is consistent with that of the enzyme in which cytochrome *a* is oxidized. Additionally, the presence of the

absorption band at 830nm and the e.p.r. signal shows that the visible copper, Cu_d (Beinert *et al.*, 1971), is also oxidized.

Although the 830nm band is observed in the mixed-valence species we have consistently found that the background absorbance in this region is lowered, possibly suggestive of a haem contribution in this region.

The spectral features in Fig. 1, coupled with the observed photosensitivity of the mixed-valence CO–enzyme (see below) lead us to conclude that the cytochrome a_3 is in the fully reduced state in combination with CO, and in the presence of oxidized cytochrome *a* and visible copper. Addition of reductants, e.g. Cr^{2+}, to the mixed-valence CO compound clearly shows progressive reduction of cytochrome *a* and visible copper, resulting in the spectrum of fully reduced CO–enzyme (see Fig. 1). In experiments in which oxidized oxidase was incubated in the presence of N_2 for 2 days, a different form of the enzyme resulted (see Fig. 2, curve *a*). The extinction in the α region is consistent with the presence of largely reduced cytochrome *a* ($\approx 90\%$), whereas the peak at 430nm and the very prominent shoulder at 440nm, seen in the Soret region, suggest that cytochrome a_3 is oxidized. On addition of CO (≈ 1 mM) to this species a slow spectral change occurs leading to the formation of a species *d* (Fig. 2), having the spectral characteristics of the mixed-valence CO complex. The rate of the transition $a \rightarrow d$ observed in Fig. 2 ($k \approx 2 \times 10^{-3} s^{-1}$) is very much slower than the rate of combination of CO with reduced cytochrome a_3 (see below). This process must therefore reflect the slow electron transfer from cytochrome a^{2+} to cytochrome a_3^{3+} under these conditions, and the rate is in close agreement with that observed for the reduction of cytochrome a_3^{3+} when oxidized oxidase is treated with dithionite (Lemberg *et al.*, 1968). We are unable to monitor the fourth redox site, Cu_u, but in the mixed-valence species prepared by method (i) above three equivalents of oxidizing agent are consumed. All this evidence leads us to the conclusion that mixed-valence oxidase in the presence of CO may be formally described as $[(a^{3+}Cu_d^{2+}Cu_u^{2+})(a_3^{2+}CO)]$.

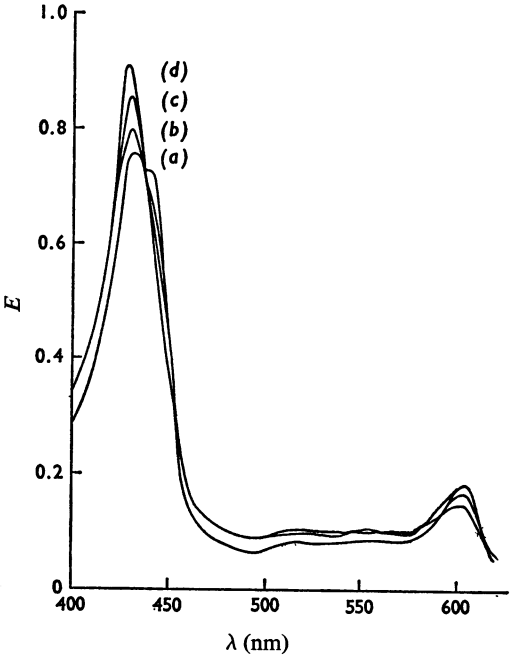


Fig. 2. *Effect of storage of cytochrome oxidase under N_2 followed by the addition of CO*

Curve (*a*) shows the absorption spectrum of mammalian cytochrome oxidase (9.5μM-total haem) after storage under N_2 for several days at 21°C. Curves (*b*), (*c*) and (*d*) show the effect of replacing the N_2 atmosphere with CO (101kPa): curve (*b*), 1min after addition of CO, curve (*c*), after 10min, and curve (*d*), after 40min. The temperature at which the spectra were recorded was 21°C and the light-path was 1cm; 0.1M-potassium phosphate buffer, pH7.4, containing 1% Tween 80 was used.

Kinetics of CO binding to mixed-valence oxidase

As with the fully reduced enzyme–CO complex the mixed-valence oxidase–CO material was found to be photosensitive. After a brief photolytic flash the recombination of CO with the reduced cytochrome a_3 corresponded at all wavelengths, CO concentrations (0.1–1mM) and all oxidase concentrations (2–50μM) to a simple process, i.e. a single exponential relaxation.

To compare the reaction between reduced cytochrome a_3 and CO in the mixed-valence oxidase and in fully reduced oxidase we prepared the mixed-valence oxidase–CO complex (4ml total volume) in a sealed Thunberg cuvette, which contained a few mg of $Na_2S_2O_4$ in the side arm. On completion of the photochemical experiments on the mixed-valence complex the cuvette was tipped and an identical series of experiments performed on the now fully reduced material.

The second-order rate constant for the recombination of CO with mixed-valence oxidase was $5 \times 10^4 M^{-1} \cdot s^{-1}$, i.e. approx. 20% lower than that of the fully reduced enzyme (see also Gibson & Greenwood, 1963). This difference, although small, is reproducible.

Comparison of the kinetic difference spectra, cytochrome a_3^{2+}–CO–cytochrome a_3^{2+} for the mixed-valence compound and fully reduced enzyme showed that these were broadly similar and in agreement with the static difference spectrum reported by Yonetani (1960). Close examination of the two sets of kinetic

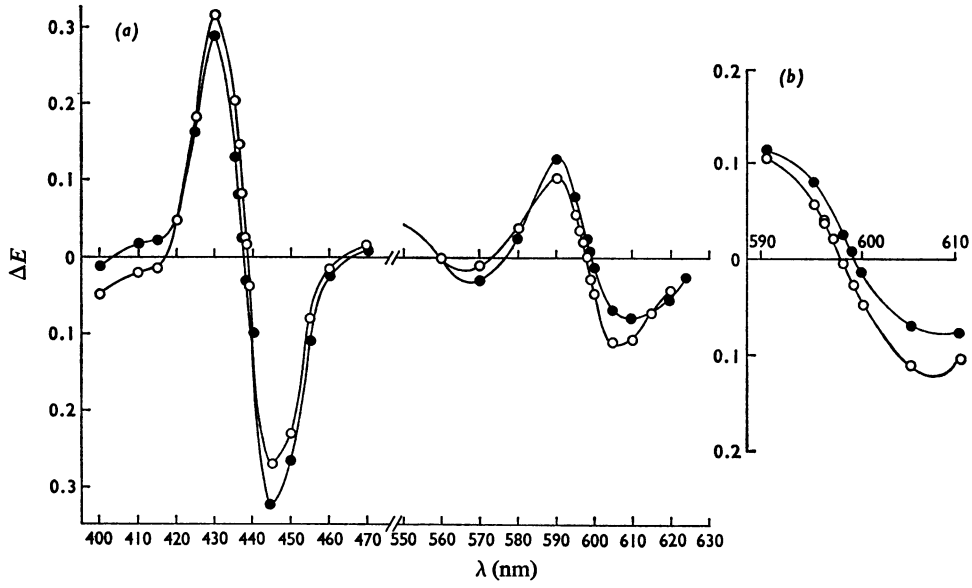


Fig. 3. *Kinetic difference spectra cytochrome a_3^{2+}–CO–cytochrome a_3^{2+} obtained in flash-photolytic experiments over the wavelength region 400–630 nm*

○ corresponds to the difference spectrum observed with the mixed-valence complex with CO, and ● shows the difference spectrum obtained after complete reduction, with $Na_2S_2O_4$, of the mixed-valence complex. For convenience the positions of the isosbestic points in the α-band region for the two conditions are shown on an expanded abscissa (590–610nm) (*b*). The concentration of cytochrome oxidase used in the Soret region was 10 μM-total haem and for the α-band region 37.6 μM. The experiments were carried out in glass Thunberg cells (1 cm light-path) equilibrated with CO to give a concentration of 1.03 mM. The nominal band-width of the monitoring light used was 1.7 nm and the temperature was 22°C. All experiments were performed in 0.1 M-potassium phosphate buffer, pH 7.4, containing 1% Tween 80.

difference spectra, however, reveals small but distinct differences (see Fig. 3). These differences are especially obvious in the position of the isosbestic points, which were found to be independent of the band-width of the observing light (0.8–3.4 nm). However, the position of the isosbestic point for the mixed-valence enzyme was influenced by the buffer system used (see Table 1). Both the rate of combination and the position of the isosbestic point for the mixed-valence oxidase were unchanged by the addition of up to five equivalents of ferricyanide. Under these conditions we would expect that all redox centres other than cytochrome a_3 were

Table 1. *Dependence of the position of the α-region isosbestic point in the kinetic difference spectrum cytochrome a_3^{2+}–CO –cytochrome a_3^{2+} on state of reduction, pH and buffer*

For details see the text. The reported isosbestic point in 0.1 M-potassium phosphate buffer, pH 7.4, represents the mean of three different experiments, but the others represent single experiments. All the experiments were performed by using enzyme protein obtained in a single preparation from three hearts. Assuming that the values are normally distributed the mean values for the isosbestic points in the two columns are significantly different at the 1% confidence limit.

	Isosbestic point (nm)	
Conditions	Mixed-valence oxidase	Fully reduced oxidase ($+Na_2S_2O_4$)
Potassium phosphate, pH 6.5, 0.1 M	598.0	599.4
Potassium phosphate, pH 7.4, 0.1 M	598.0	599.4
Sodium cacodylate, pH 6.5, 0.1 M	597.5	599.4
Sodium cacodylate, pH 7.0, 0.1 M	597.2	599.1
Sodium cacodylate, pH 7.0, 0.1 M	597.5*	599.4†

* Less than 1 equivalent of Cr^{2+} added to oxidized oxidase under CO.
† Fully reduced with Cr^{2+}.

oxidized. The fact that the behaviour of the mixed-valence complex is unmodified by ferricyanide also implies that the redox state of cytochrome a_3 is unchanged either in combination with CO or during the time that it is unliganded after the flash.

Photolysis experiments were conducted during the course of a reductive titration of oxidized oxidase under CO with Cr^{2+} as reductant. The results obtained in such an experiment were complicated by the length of time required to reach equilibrium after each addition of Cr^{2+}. Immediately after each addition to the oxidase the absorption spectrum showed a preferential reduction of cytochrome *a*. At equilibrium, where less than 1 equiv. of Cr^{2+} had been added, the cytochrome *a* was largely oxidized and the electron was resident on cytochrome a_3, which was in combination with CO. During the attainment of this equilibrium, the isosbestic point in the α region had moved from that characteristic of the fully reduced material to one typical of the mixed-valence species.

The kinetic difference spectra cytochrome a_3^{2+}–CO–cytochrome a_3^{2+} and the observed rate of recombination of cytochrome a_3^{2+} with CO, clearly show that the behaviour of reduced cytochrome a_3 is influenced by the redox state of other sites in the oxidase molecule.

O_2, cf. myoglobin and haemoglobin (Ainsworth & Gibson, 1957). At 'zero light' the CO 'off' rate was $0.03\,s^{-1}$ at 20°C and pH 7.4, and this value taken in connexion with the 'on' rate yields an apparent affinity constant of $1.7\times10^6\,M^{-1}$ compared with $3\times10^6\,M^{-1}$ for the fully reduced enzyme (Gibson & Greenwood, 1963).

Exposure of the mixed-valence CO complex to air yields spectral changes which result from the formation of the so-called 'oxygenated complex' (see Fig. 4). The same spectral species has been observed to form in stopped-flow experiments where the mixed-valence CO–enzyme is rapidly mixed with air-equilibrated buffer. As shown in Fig. 5, the displacement is a simple exponential process which is complete in about 1 min. The kinetic difference spectrum associated with this process indicates that the product of the replacement reaction is the 'oxygenated' oxidase (see also Fig. 4). As shown in Fig. 5 at 610nm the replacement is followed by a much slower reaction which occurs over a much extended time range, varying from 30min to 2h depending on the temperature. The species formed in this second slow phase corresponds to one characterized by the typical absorption spectrum attributed to oxidized cytochrome oxidase. The following sequence of reactions:

Oxidized oxidase → mixed-valence enzyme–CO complex → 'oxygenated' → oxidized oxidase

Reaction of mixed-valence CO complex with O_2

As with the fully reduced enzyme (Gibson & Greenwood, 1964), CO may be displaced from the mixed-valence enzyme by mixing this with a large excess of O_2. This displacement reaction is most conveniently followed by using the stopped-flow apparatus and corresponded to a first-order reaction, the rate constant of which was found to be oxygen-independent (see Table 2). The rate of displacement was found to depend on the incident light intensity, which implies that the quantum yield for CO is greater than that for

Table 2. *Oxygen dependence of the rate of dissociation of CO from the CO derivative of mixed-valence oxidase*

The concentration of cytochrome oxidase was 3.5 μM dissolved in 0.1 M-sodium phosphate buffer, pH 7.4, containing 1% Tween 80. The enzyme was equilibrated with 1 atmosphere of CO (101 kPa) and the displacement reaction was followed at 430nm using a very low intensity of incident light.

$[O_2]$ (μM)	$10^2\times k$ (s^{-1})
5.5	2.75
27	3
135	3
680	3.2

has been repeated on the same sample many times with identical results, indicating the lack of cumulative effects. It is perhaps worth noting at this point that addition of superoxide dismutase had no effect on the decay from 'oxygenated' to oxidized oxidase.

Reaction of mixed-valence oxidase with O_2

By rapidly mixing the CO complex of the mixed-valence enzyme with O_2-containing buffer and exposing the mixture to a very brief but very intense photolytic flash, it is possible to follow the reaction between reduced cytochrome a_3, now unliganded, and O_2. Experiments of this type are made technically possible because, in the absence of the photolytic flash, the reaction between cytochrome a_3^{2+}–CO and O_2 is slow, and rate limited by the CO 'off' constant (see above).

The results of such a flow–flash experiment at 430nm are seen in Fig. 6. Two events, one in the millisecond and one in the second time-range, were observed. The fast process was O_2-concentration-dependent and CO-concentration-independent. Fig. 7 shows the dependence of the rate of the fast reaction as a function of O_2 concentration and is in close agreement with the values obtained by Greenwood & Gibson (1967) for the fully reduced enzyme in identical experiments. At low oxygen concentrations the

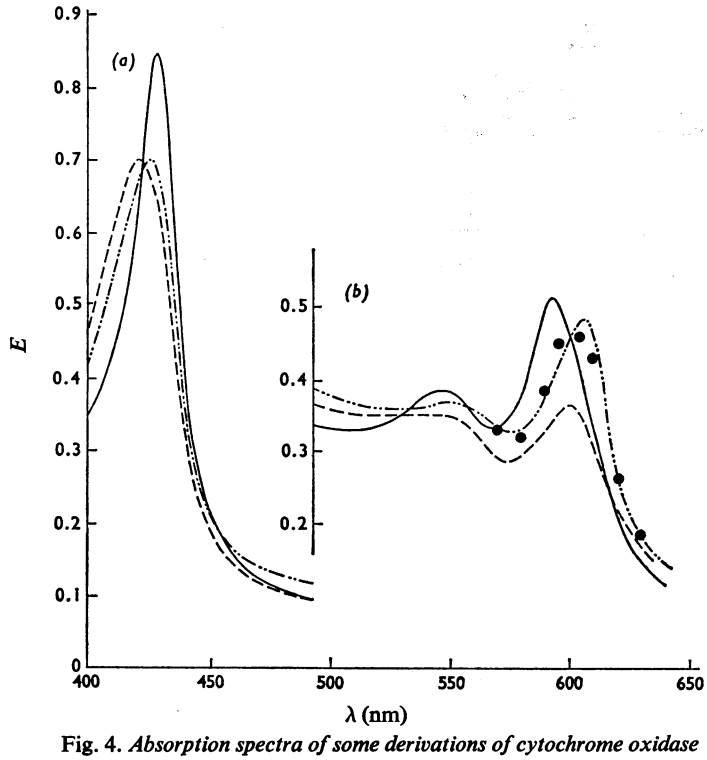


Fig. 4. *Absorption spectra of some derivations of cytochrome oxidase*

Absorption spectra of oxidized (---), mixed-valence enzyme–CO (——), and the so-called 'oxygenated' forms (-··-··) of mammalian cytochrome oxidase. The concentration of enzyme used was 8.9 μM in the Soret region and 36 μM in the α-band region, and in each case the path length was 1 cm. The filled circles were obtained in stopped-flow experiments where the mixed-valence material was mixed with O_2-containing buffer. They thus represent the intermediate formed at the end of the CO-displacement phase. All experiments were carried out in 0.1M-potassium phosphate buffer, pH 7.4, containing 1% Tween 80.

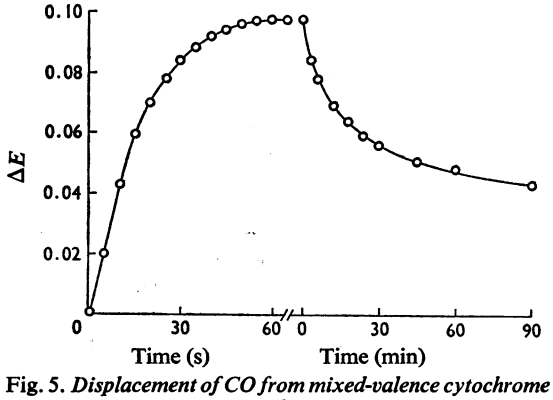
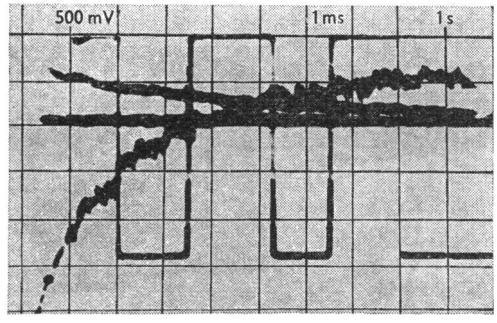


Fig. 5. *Displacement of CO from mixed-valence cytochrome oxidase*

The reaction was observed at 610nm on mixing the mixed-valence enzyme–CO complex (42 μM before mixing) with air-equilibrated 0.1 M-potassium phosphate buffer, pH 7.4, containing 1% Tween 80. The reaction was carried out in the stopped-flow apparatus fitted with a 2 cm light-path cell and by using light of nominal band-width 1.8 nm. The temperature in this experiment was 20°C.

second-order rate constant is 8.5×10^7 M$^{-1}\cdot$s^{-1}, but this value is not maintained, and falls to 3×10^7 M$^{-1}\cdot$s^{-1} at the highest oxygen concentration used. In contrast the rate of the slow phase was independent of O_2 concentration; however, its amplitude decreased with increasing O_2 concentration (see Fig. 8). This slow process could not be attributed to the replacement of CO by O_2, as it occurred under conditions of full photodissociation where no mixed-valence compound would exist, and in contrast with the replacement reaction was found to be independent of incident-light intensity. The slow phase was observed, albeit at somewhat decreased amplitude, in the presence of an excess of potassium ferricyanide, and thus it seems unlikely that it represents the oxidation of some additional partially reduced site, other than cytochrome a_3. This conclusion is also supported by the observation that the amplitude of the slow phase was oxygen dependent.

The wavelength dependence of the two phases is presented in Fig. 9 in the Soret region, with only the initial phase in the α-band region. The fast phase of



Time 1ms/cm, ascending trace; 1s/cm, descending trace

Fig. 6. *Flow–flash kinetic record of mixed-valence enzyme reacting with O_2*

Oscilloscope trace of the reaction between mixed-valence oxidase (2 μM-haem *a*) and O_2 (6.5 μM), after the displacement of CO from the enzyme by a photolytic flash (350J, 200 μs duration). Reaction was carried out in the presence of 25 μM-potassium ferricyanide in 0.1 M-potassium phosphate buffer, pH7.4, containing 1% Tween 80. The path length was 5cm and the wavelength of observation 430nm with a band width of 3.4nm. Corning filters 7.59 and 4.97 were used on the observation beam.

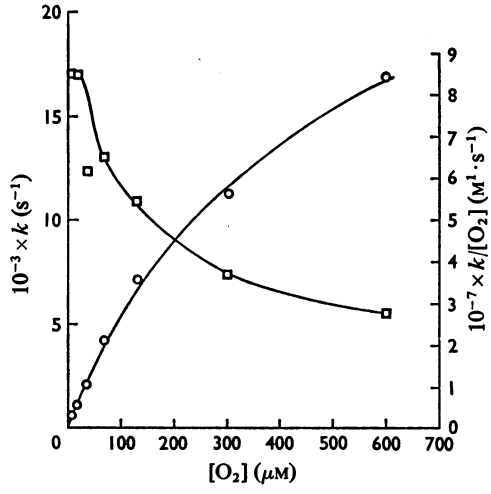


Fig. 7. *Dependence of the rate of the initial reaction as a function of oxygen concentration when the CO complex of the mixed-valence enzyme was photolysed in the presence of O_2*

The left-hand ordinate shows the pseudo-first-order rate (○). The right-hand ordinate represents the second-order rate constant (□). The oxidase concentration used in this experiment was 1.9 μM after mixing and the CO was 0.5mM after mixing. The light-path of the cell used in this experiment was 6cm, the wavelength and the nominal band-width of the light were 445nm and 3.4nm respectively. A photolytic flash of 425J was used to achieve over 90% breakdown of the CO complex of reduced cytochrome a_3. The temperature was 22°C.

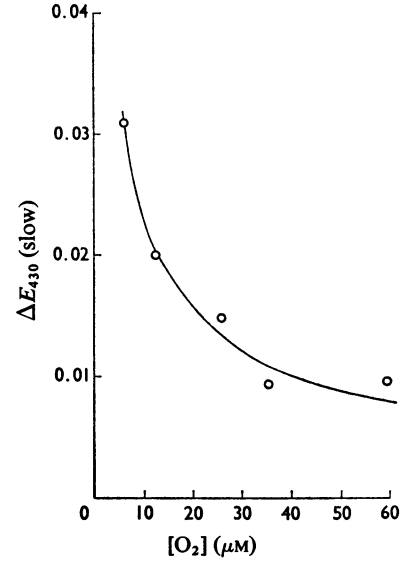


Fig. 8. *Oxygen-concentration dependence of the amplitude of the slow process after photolytic displacement of CO from the mixed-valence enzyme*

Cytochrome oxidase (8 μM-haem *a*) dissolved in 0.1 M-potassium phosphate buffer, pH7.4, containing 0.5% Tween 80 was used in the presence of 25 μM-potassium ferricyanide. The path length was 1 cm and the monitoring wavelength and nominal band-width 430 and 1.7nm respectively. Corning filters 7.59 and 4.97 were used on the observation beam and the 350J flash used gave 100% breakdown of the mixed-valence enzyme–CO complex.

Fig. 9 is superficially similar to the Soret kinetic difference spectrum observed by Greenwood & Gibson (1967) (see Fig. 5 of their paper which they attributed to the valence change of cytochrome a_3 between fully oxidized and fully reduced enzyme). However, the α-band region of Fig. 9, when taken in conjunction with the static spectra of a number of enzyme derivatives in this region (Fig. 10), suggest a different interpretation. The spectra seen in Figs. 10(*a*)–10(*d*) inclusive represent the static spectra of fully oxidized, fully reduced, mixed-valence enzyme in combination with CO, and the so-called oxygenated compound respectively. The spectrum in Fig. 10(*e*), however, has been constructed from the spectra of the mixed-valence CO complex (curve *c*) and the kinetic difference spectrum for this region, shown in Fig. 3. Fig. 10(*e*) therefore represents the absorption spectrum of the mixed-valence compound stripped of CO, i.e. the species reacting with O_2 in the flow–flash experiments reported above.

From Fig. 10 it is clear that at 610nm for example, the so-called oxygenated complex has a greater extinction than the mixed-valence enzyme, whereas at

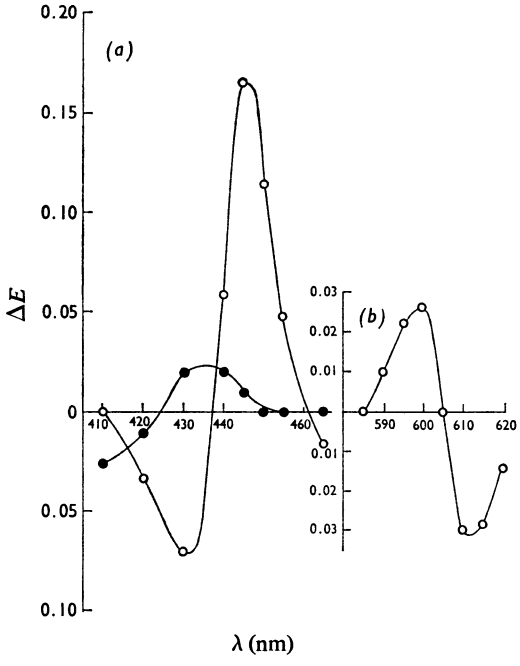


Fig. 9. *Kinetic difference spectra for the reaction of reduced cytochrome a_3 with oxygen*

The kinetic difference spectra (initial minus final absorbances) are shown, (*a*) for the fast phase (○) of the reaction between reduced cytochrome a_3 and O_2 and the slow phase (●) in the Soret region and (*b*) for the initial phase only (○) in the α-band region. The enzyme concentration was 2 μM for the Soret region and 10.5 μM for the α-band region (both concentrations expressed in terms of total haem after mixing). No liquid filter was used in this experiment and the 350J flash was sufficient to produce 100% breakdown of the CO complex. The light-path was 5cm and the nominal band-width used 3.4nm. The experiment was performed in 0.1M-potassium phosphate buffer, pH7.4, containing 1% Tween 80 and was 25 μM in potassium ferricyanide, and the temperature was 22°C. In both regions of the spectrum the enzyme was mixed with buffer containing 13 μM-O_2.

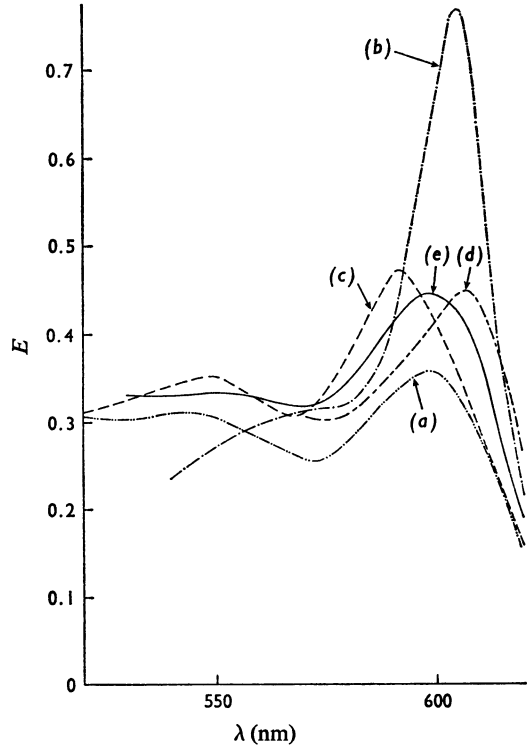


Fig. 10. *Absorption spectra of some cytochrome oxidase derivatives in the α-band region*

(*a*) Fully oxidized oxidase as prepared; (*b*) oxidase fully reduced by addition of $Na_2S_2O_4$; (*c*) mixed-valence CO complex of oxidase prepared by method (iii) of the Experimental section; (*d*) 'oxygenated' oxidase prepared by exposing the mixed-valence CO derivative to air; (*e*) spectrum of mixed-valence oxidase in the absence of CO constructed from spectrum (*c*) and the kinetic difference spectrum cytochrome a_3^{2+}–CO–a_3^{2+} obtained for the mixed-valence enzyme and shown in Fig. 3. The oxidase concentration was 36 μM (haem *a*) and was dissolved in 0.1M-potassium phosphate buffer, pH7.4, containing 1% Tween 80.

the same wavelength the absorption of fully oxidized enzyme is less. Flow–flash experiments at this wavelength clearly show that the rapid reaction leads to a species having a higher extinction than the material immediately after the photolytic flash. As a result of this we therefore conclude that the initial product of reaction with O_2 in this case is not the oxidized species, but rather the so-called oxygenated species, although some minor differences may be present, as for instance that seen at 585nm.

Discussion

The data presented in the Results section on the spectral and photochemical properties of mixed-valence cytochrome oxidase suggest that it is a derivative of the enzyme in which three redox sites are oxidized while the fourth is reduced and in combination with CO. This latter site, being capable of CO binding is, by definition (Keilin & Hartree, 1939), termed cytochrome a_3. The reactions between the mixed-valence species and ligands of ferrous iron may therefore be described formally as follows:

$$[a^{3+}\,Cu_d^{2+}\,Cu_u^{2+}\,a_3^{2+}] + X \rightleftharpoons [a^{3+}\,Cu_d^{2+}\,Cu_u^{2+}\,a_3^{2+}\,X]$$

where X denotes the ligand. Differences in the functional behaviour of any of the sites between the mixed-valence oxidase and fully reduced enzyme must reflect site–site interactions, or perturbations of such interactions involving the redox partners in the enzyme. The reactions between mixed-valence cytochrome oxidase and CO are significantly different from those involving the fully reduced enzyme with this ligand, and we therefore conclude that the properties of the ligand-binding site (reduced cytochrome a_3) depend on the valence state of one or more of the other sites present. The site(s) exerting this influence on the ligand-binding haem *a* group is as yet unidentified, although Cu_u might be considered a likely candidate in view of the work of Beinert *et al.* (1971) quoted in the introduction. The phenomenological conclusion is unequivocal, although the nature of the physical mechanism, whether direct interaction or conformational changes of the type postulated by Tiesjema *et al.* (1973), we do not know at this stage. Some evidence for haem–haem interaction as proposed by Tiesjema *et al.* (1973) is afforded by the photochemical experiments carried out during the course of a reductive titration in the presence of CO, as described in the text, in which the position of the isosbestic point in the kinetic difference spectrum (cf. Fig. 3), tended to reflect the valence state of cytochrome *a*.

In the absence of cytochrome *c*, Tiesjema *et al.* (1973) have found that the midpoint potentials of the two haem groups are identical, and they go on to suggest that, under these conditions, the designation of cytochromes *a* and a_3 is meaningless. Fig. 2 of the present paper would seem to suggest that, under essentially similar conditions, we can produce a species in which only one haem moiety is reduced. This haem does not, however, seem capable of binding CO and this ligand binds only after electron exchange has occurred, presumably from cytochrome *a* to a_3. Our results are therefore better explained in terms of the chemical non-equivalence of cytochromes *a* and a_3, the latter being the binding site for O_2 and CO.

The displacement of CO by O_2 in the mixed-valence complex clearly shows that, in these flow experiments, the so-called oxygenated species is formed when oxidase containing only one reduced site is allowed to react with O_2. Indeed this appears to be a very good way of preparing the oxygenated compound for further study. Our observations on the reaction of mixed-valence oxidase with O_2 in this way are in agreement with similar observations on mixed-valence derivatives prepared by different techniques (Myer, 1972; Tiesjema *et al.*, 1972).

The reaction with oxygen after the photolytic removal of CO is more complex, although the results (see Figs. 9 and 10) allow us to say definitely that the first product observed after the oxygen-dependent fast phase is not oxidized enzyme as prepared and therefore shows, in this respect, a clear distinction from the fully reduced enzyme (Greenwood & Gibson, 1967). However, it is difficult to designate the product of the reaction precisely. Throughout much of the spectrum the species formed at the end of the initial oxygen reaction is similar to the 'oxygenated' form of the enzyme, although some regions of the spectrum may not be entirely consistent with this conclusion. The bulk of the evidence thus points to the fact that, on reaction with O_2 either directly or by CO displacement, the mixed-valence oxidase forms the oxygenated compound.

In stressing the differences between the products formed as a result of the reactions between O_2 and the mixed-valence oxidase or the fully reduced enzyme it is important not to lose sight of the basic similarities. In this context, the absolute rate and oxygen dependence of the initial reaction are very similar in each case and suggest that we are observing a process common to both enzyme derivatives. This common process may well be the initial binding, which Greenwood & Gibson (1967) have proposed is rate-limited by diffusion of O_2 into the oxidase complex.

The interpretation of the slow phase presents difficulties, although it is possible to exclude the explanation that this represents the replacement reaction of undissociated CO complex by O_2 in view of the fact that we were attaining 100% photolysis and that the observed (slow) rate was light-insensitive. In addition it appears highly improbable that the slow phase represents either oxidation of other sites or the decay of the product of the fast reaction to another form of the enzyme. The decrease of amplitude of the slow phase with increasing oxygen concentration seen in Fig. 8 suggests that competing reactions, involving the unliganded mixed-valence enzyme, occur subsequent to photolysis. For example the photoproduct may rapidly decay through a monomolecular process to another species, which is either a conformationally different form or one in which cytochrome a_3^{2+} has been oxidized by other sites while unliganded. Both these explanations of the observed reaction kinetics in the flow-flash experiments would seem to imply that we might expect complex kinetics for the recombination of CO with cytochrome a_3^{2+} after flash photolysis, which was not observed. Indeed the data clearly show that the CO recombination is simple, whereas the O_2 reaction is complex, and thus the slower phase is presumably reflecting events which occur only when oxygen is the ligand of cytochrome a_3^{2+}. Oxygenated oxidase is known to contain four oxidized sites (Tiesjema *et al.*, 1972) and is thought to be a conformational variant of the fully oxidized enzyme (Myer, 1972; Tiesjema *et al.*, 1972). If, as it appears from our experimental findings, the oxygenated species is the product of the O_2 reaction, this implies that we are observing the electron exchange between O_2 and an enzyme species able to donate only one electron. This being so we

might reasonably expect the formation of O_2 radicals, in particular $O_2^{-\cdot}$, which might remain bound to the enzyme and allow us to designate the product of reaction of 'mixed-valence' oxidase with oxygen as $a^{3+}Cu_d^{2+}Cu_u^{2+}a_3^{3+}O_2^{-\cdot}$. There are various ways to look for such radicals; for instance it might be possible to combine flow–flash with rapid-freeze and use the e.p.r. approach developed by Knowles *et al.* (1969). Our experiments using superoxide dismutase, which clearly showed no effect of this enzyme on the decay of the oxygenated to oxidized oxidase nor on the oxygen kinetics, in no way imply that this radical is not produced in the reactions involving mixed-valence oxidase and O_2, but only that the rates are not limited by the decay of this radical in solution.

Whatever the final product of the O_2 reaction of mixed-valence oxidase may be, the data allow us to conclude that reduced cytochrome a_3 is the site which reacts with O_2 and that even when this site is the only one reduced, the reaction with O_2 remains very rapid.

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