Compound C₂, a Product of the Reaction of Oxygen and the Mixed-Valence State of Cytochrome Oxidase

OPTICAL EVIDENCE FOR A TYPE-I COPPER

By B. CHANCE, C. SARONIO* and J. S. LEIGH, JR.

Johnson Research Foundation, University of Pennsylvania, Philadelphia, PA 19104, U.S.A.

(Received 12 July 1978)

Compound C₂ is a product of the reaction of O₂ and the mixed-valence state of cytochrome oxidase. The mixed-valence state of membrane-bound cytochrome oxidase is obtained at –24°C, by using either ferricyanide or yeast peroxidase complex ES as oxidants, and the configurations of oxidized haem *a* and its associated copper ($a^{3+}Cu_{a_3}^{2+}$) and of reduced haem *a*₃ and its associated copper ($a_3^{2+} \cdot CO \cdot Cu_{a_3}^{+}$) are obtained. The mixed-valence-state cytochrome oxidase mixed with O₂ at –24°C and flash-photolysed at –60 to –100°C reacts with O₂ and initially forms an oxy compound (A₂) similar to that formed from the fully reduced state (A₁). Thereafter the course of the reaction differs from that obtained in the fully reduced state, and absorbance increases are observed at 740–750 nm and 609 nm and a decrease at 444 nm, with no increase in absorbance at 655 nm. One possible attribution of the absorbance increases is to charge-transfer interaction between the iron of haem *a*₃ and the copper associated with haem *a*₃, $Cu_{a_3}^{2+}$, having properties of a type-I ‘blue’ copper. A possible attribution of the decrease in absorbance at 444 nm is to liganding of a_3^{2+} . A related explanation is that the 609 nm absorbance involves a charge-transfer interaction of both iron and copper as a mixed-valence binuclear complex, Cu_{a_3} , having properties of a non-blue copper. Intermediates in addition to Compound C₂ are not yet identifiable by chemical or spectroscopic tests. The kinetic and equilibrium properties of Compound C₂ are described.

The intermediates so far identified in the reaction of completely reduced cytochrome oxidase seem logical from the thermodynamic viewpoint. First formed is an oxy compound (A₁), in which no electrons are transferred, and the second is a peroxy compound (B₂), in which two electrons are transferred, one from iron (*a*₃) and one from copper associated with haem *a*₃ (Cu_{a_3}) (Chance *et al.*, 1975a, 1977; Chance & Leigh, 1977). For nomenclature of intermediates see Chance *et al.* (1977). One of the mixed-valence states of cytochrome oxidase that is readily obtained by ferricyanide treatment of the fully reduced CO-liganded oxidase contains $a^{3+}Cu_{a_3}^{2+}$ and $a_3^{2+} \cdot CO \cdot Cu_{a_3}^{+}$. The mixed-valence-state oxidase is allowed to react with O₂ at –110 to –80°C and forms Compounds A₂ and C₂. The rationale of these studies is to determine whether electron transfer from cytochrome to O₂ and the chemical and physical properties of the reaction product depend upon the valence state of *a* and Cu_{a_3} , i.e. whether Compounds A₁ and A₂, and B₂ and C₂ differ. Large spectroscopic differences are found and are the topic of this paper.

* Deceased 1975.

Physical Methods and Sample Preparation

Two optical configurations are used with the split-beam spectrophotometer. In both cases the principle of split-photolysis is used (Chance *et al.*, 1975b). The first optical configuration consists of a fibre optic coupling longitudinally along the e.p.r. tube from the monochromator to the photodetector. The sample size is 3 mm by 40 mm. The e.p.r. tube is 3 mm diameter and the effective path is somewhat less. This configuration is used for example in Figs. 2 and 3, and thus the absorbance change for a given protein concentration is low. The second configuration employs a rectangular cuvette (30 mm × 20 mm) with a 2 mm light-path. Illumination of the 30 mm × 20 mm area is obtained by fibre optics coupling from the monochromator to the photodetector. In this case, the absorbance change per g of protein is about 3 times larger with the circular e.p.r. tubes as for example as obtained in Fig. 1. The cuvette and light-guide configurations used in the dual-wavelength scanning apparatus (Fig. 8) and in the multichannel time-sharing apparatus (Fig. 7) (Chance *et al.*, 1975a) are similar.

The sample preparations together with the kinetic read-out and evaluation closely follow that described elsewhere (Chance *et al.*, 1975a). In these experiments we have studied the membrane-bound oxidase of intact ox or pigeon heart mitochondria. The oxidase of these membranes responds to low concentrations of ferricyanide and $a^{2+}Cu_a^+$ is oxidized with a half-time of about 6s at -24°C . At this temperature neither dissociation of CO nor oxidation of haem $a_3^{2+}\cdot\text{CO}$ occurs to a significant extent within the 6s interval.

The reduced CO-saturated mitochondria are chilled to -22 to -25°C ; 1–10mM-ferricyanide is added with gentle stirring and allowed to react for 20–30s. Then O_2 is added by stirring or by direct addition of an appropriate volume of O_2 -saturated reaction medium containing 30% ethylene glycol. Thereafter freeze-trapping at -78°C occurs.

A typical experiment uses a 3mm-bore e.p.r. tube and the split-beam split-photolysis apparatus (Chance *et al.*, 1975c). Frozen-thawed ox heart mitochondria are suspended in 30% ethylene glycol, saturated with CO, and chilled to -20 to -24°C . $200\mu\text{M}\text{-O}_2$ is added

by stirring 20 times, and additional oxidants may be included: ferricyanide, yeast peroxidase and H_2O_2 . Immediately after the additions have been made and the stirring procedure is completed, the sample is freeze-trapped in solid CO_2 /ethanol at -78°C . Subsequently, the samples are put into the split-beam split-photolysis spectrophotometer, and the bottom portion of the tube is flash-photolysed (see Fig. 1).

The spectra obtained in this way are referred to the spectrum of the CO compound, which exhibits a peak at 590nm and a trough at 608–612nm as seen in Fig. 1. On flash photolysis, the 590nm band disappears and that at 608–612nm appears. When O_2 is present, subsequent reaction with O_2 to form oxy compounds A_1 and A_2 increases the absorbance at 590nm and decreases the absorbance at 608–612nm. Subsequently Compounds A_1 and A_2 are converted into Compounds B_2 or C_2 with decreased absorbance at 590nm and respective decreases or increases in absorbance at 608–612nm. Thus the spectra of Compounds B_2 and C_2 are superimposed upon the difference spectrum of haem $a_3^{2+}\cdot\text{CO}$ minus the reduced state. However, subtraction of successive difference spectra eliminates the difference spectrum of the haem $a_3\cdot\text{CO}$ compound as shown below in Figs. 2 and 3.

Fig. 1. Cytochrome oxidase–CO compound(s)

Comparison of the difference spectra for the CO compound of cytochrome a_3 formed from the fully reduced state (top trace) and the mixed-valence state (bottom trace). The change of gain occurs at approx. 520nm. Conditions are 10mg of ox heart mitochondrial protein suspended in 1ml of 30% ethylene glycol/0.16M-sucrose/0.075M-mannitol/3mM-Tris/ H_2SO_4 , pH7.4, and supplemented with 0.06M-succinate, 1.2mM-CO and 0.7mM- O_2 . The flash photolysis and spectroscopic recording are done near the temperature of liquid N_2 . The absorbance changes are in the upward direction when CO combines with cytochrome a_3 , and the baseline for these spectra is the fully reduced or half-reduced oxidase. Absorbance decreases are in the upward direction.

Results

Effect of ferricyanide upon the CO compound of cytochrome oxidase

The photolysis of cytochrome $a_3\cdot\text{CO}$ was found earlier (Chance, 1953) and serves as a precise method for determining absorbance difference spectra of the fully reduced oxidase and its CO compound. The question of the effect of the valence state of aCu_a on the difference spectrum arises. A number of inconsistencies have appeared in connection with the effect of ferricyanide upon cytochrome oxidase. In mid-potential titrations of membrane-bound electron-transfer components and in evaluations of the 'sidedness' of submitochondrial particles and ox heart mitochondria, ferricyanide has proved to be an effective oxidant and has given results consistent with those obtained by yeast peroxidase and H_2O_2 . However, two problems seem to have arisen. First, Keilin (1961) warned against the photodecomposition of ferricyanide to cyanide, and Lanne *et al.* (1977) have shown that ferricyanide at 4–20mM concentrations binds and alters cytochrome oxidase, distorting the experimental results. These effects, which may be due to a 'shielding effect' of the ferricyanide molecules about the soluble cytochrome oxidase, do not seem to be detectable in membrane-bound species, where identical results can be obtained with peroxidase complex ES (see below). Secondly, Wikström *et al.* (1976) suggest that oxidation of cyto-

chrome *a* by ferricyanide diminishes the contribution of haem *a*₃ to the absorbance change at 605 nm observed in the photolysis of the haem *a*₃·CO compound. This observation is not confirmed here.

The results of experiments carried out with the mixed-valence-state preparation procedure described above for oxidizing *a*²⁺Cu_a⁺ to *a*³⁺Cu_a²⁺ is also illustrated in Fig. 1. The control experiment with the fully reduced state-CO compound shows the absorbance difference spectrum (absorbance decrease upwards) obtained by photolysis at approx. -190°C. The difference spectrum shows clear α -, β - and γ -bands at the appropriate positions for *a*₃²⁺·CO. The γ/α ratio, as judged from the troughs at 444 and 612 nm, is 4:1. This value is less than the value at room temperature, because of the smaller enhancement factor for the Soret band than for the α -band in the frozen turbid suspension of mitochondria.

When the mixed-valence-state cytochrome oxidase-CO compound is photolysed the amplitude is equal to that of the fully reduced state (within 5%) and the corresponding γ/α ratio is 4.2:1. Owing to the absorbance of the added ferricyanide, it was not possible to record accurately the amplitude of the 428 nm band. The positions of the absorption bands in the CO+ferricyanide case are slightly shifted to the blue, as pointed out and observed independently by M. K. F. Wikström (personal communication).

These data show that it is possible to form a cytochrome *a*₃²⁺·CO compound in the presence of ferricyanide that has very nearly the same absorbance difference spectrum as that in the absence of ferricyanide and that the valence state of *a*Cu_a does not significantly affect the absorbance difference spectrum of *a*₃²⁺Cu_a³⁺·CO in this wavelength region, as now confirmed by M. K. F. Wikström (personal communication).

Effect of ferricyanide concentration upon Compound B₂ formation

In the case of Compound B₂ formation in the fully reduced and mixed-valence states, large spectroscopic differences are observed. Fig. 2 illustrates the spectroscopic changes due to the transition from Compound B₂ to Compound C₂, as increasing concentrations of ferricyanide together with a constant concentration of O₂ are added at -20°C to the fully reduced CO-saturated mitochondria.

The main effect of increasing ferricyanide concentrations from 10 to 500 μ M upon the difference spectra is the progressive replacement of the trough at 603–608 nm due to Compound B₂ by the large peak at about 610 nm due to Compound C₂. The trough at 590 nm that remains in this transition is initially due to the photolysis of the cytochrome *a*₃²⁺·CO compound and the ligand replacement with O₂ to form Compound A₁, causing a re-establishment of ab-

Fig. 2. Formation of Compound C₂

Titration of the formation of Compound C₂ with ferricyanide is recorded with the split-beam split-photolysis apparatus using a 3 mm-diameter e.p.r. tube. The absorbance changes are less than those of Fig. 1 by a factor of approx. 3. The baseline for these spectra is the mixed-valence CO compound. The ferricyanide concentrations are added together with the 200 μ M-O₂ and are listed in the Figure. Temperature of the spectroscopic recording and flash photolysis is -88°C. The recordings are made 100 s after flash photolysis. The suspension contained 13.3 mg of ox heart mitochondrial protein/ml of 40% ethylene glycol/0.085 M-mannitol/0.16 M-sucrose/0.01 M-succinate/0.01 M-glutamate/1.2 mM-CO. Absorbance increases are in the upward direction.

sorbance at 590, and followed by the disappearance of Compound A₁ or A₂, the precursors of Compounds B₂ and C₂ respectively. The traces of Fig. 2 suggest that redox-potential variation is the key to the transition from Compound B₂ to Compound C₂. W. J. Ingledew (unpublished observations) and Chance & Leigh (1977) obtained values of approx. 250 mV for this transition.

In order to detect the extent of Compound C₂ formation in the presence of Compound B₂ in these successive experiments we have obtained difference spectra (for a different experiment) corresponding to 25 μ M- (trace B) and zero (trace A) ferricyanide (Fig. 3a), and 500 μ M- (trace B) and zero (trace A) ferricyanide (Fig. 3b). In both cases the difference spectra are identical to within the experimental error. This might be expected from a system in which each respiratory chain acts independently; those that have reacted with ferricyanide to form the mixed-valence-state compound will produce Compound C₂; other redox combinations do not.

Quantification of results

It is not currently possible to form large amounts of Compound C₂ at room temperature nor is it possible to measure mid-potentials in the frozen

Fig. 3. Compound C_2 formation

Formation of Compound C_2 with low and high ferricyanide concentrations. (a) Trace A, no ferricyanide; trace B, $25\mu\text{M}$ -ferricyanide. (b) Trace A, no ferricyanide; trace B, $500\mu\text{M}$ -ferricyanide. Traces A and B represent the experimental data. Traces B-A represent calculated differences of these two traces in both cases. Absorbance increases are in the upward direction. Experimental conditions were identical with those of Fig. 2.

solid at -88°C . However, the best that can be done is to compare the response of cytochrome *a* to ferricyanide (and O_2) at -28°C , as measured in the liquid state at 603–630 nm, with the subsequent formation of Compound C_2 at -88°C , as obtained by flash photolysis (see Fig. 4). The magnitudes of the absorbance differences are plotted one against the other for various concentrations of ferricyanide. Although initially there is no formation of Compound C_2 , there is a region of linear increase of Compound C_2 formation with the degree of oxidation of cytochrome *a* up to about 30% of the maximum. However, as the reaction with cytochrome *a* nears completion, the yield of Compound C_2 increases further with increasing ferricyanide concentration up to $500\mu\text{M}$, at which the maximal amount seems to be formed. A sufficiently positive potential to oxidize cytochrome *a* completely must be reached before Compound C_2 achieves maximal formation.

Fig. 4. Correlation of Compound C_2 formation and cytochrome *a* oxidation

Comparison of the -28°C oxidation of cytochrome *a* (603–630 nm), as obtained by ferricyanide addition to the CO-blocked mitochondria (abscissa), with the yield of Compound C_2 , as measured by flash-photolysis activation at -88°C (ordinate). Experimental conditions were similar to those of Figs. 2 and 3.

Kinetics of the reaction with ferricyanide

The kinetics of the reaction at low and high ferricyanide concentrations, corresponding approximately to the values of Fig. 3, are illustrated by Fig. 5. In this case, time-sharing multi-wavelength spectrophotometry is used (Chance *et al.*, 1975b). The table of wavelength pairs appears on the right-hand side of the Figure. The experiments are carried out at -24°C so that the reaction of ferricyanide with cytochrome c^{2+} and $a^{2+}\text{Cu}_a^{+}$ can be observed before the flash photolysis and the formation of Compound C_2 . The addition of $40\mu\text{M}$ -ferricyanide and $200\mu\text{M}$ - O_2 to the CO-inhibited reduced components of the mitochondria (left-hand set of traces) shows in trace (d) a deflection corresponding to partial oxidation of cytochromes *a* and *c*. This reaction exhibits a half-time of about 6 s, probably limited by the mixing time (see serrations of the traces due to mixing). Similar times are observed for the other components (traces *a*, *b*, *c* and *e*). Thereafter the traces are stable, indicating equilibrium of the ferricyanide reaction and blockade of the O_2 reaction by the previously added CO (Lindsay & Wilson, 1974). This is verified to be effective by the high ratio of absorbance changes in traces (b) and (c) to that in trace (a). Trace (e) shows a significant response corresponding to a partial oxidation of Cu_a by ferricyanide, as is expected from

Fig. 5. Kinetics of Compound C₂ formation

The kinetics of the ferricyanide reaction together with the subsequent response to flash photolysis at -24°C at low and high ferricyanide concentrations are illustrated. The experiments were carried out in a 2-mm-path rectangular cuvette with light-guide coupling to a time-sharing multi-wavelength spectrophotometer. The wavelengths are indicated on the right side of the Figure together with appropriate absorbance calibrations. The experimental conditions were identical with those of Fig. 2, except that 20 mg of ox heart mitochondrial protein/ml was used. The direction of the absorbance decrease for each trace (a-e) is indicated at the right-hand side.

its apparent mid-potential of 245 mV (Lindsay & Wilson, 1974).

Flash-photolysis activation can cause a ligand exchange and the formation of Compound B₂ in the respiratory chains unaltered by ferricyanide (about 60%) and the formation of Compound C₂ in the respiratory chains oxidized by ferricyanide (about 40%) (Hill & Chance, 1978). Thus the preponderating effect is Compound B₂ formation at low ferricyanide concentrations. The absorbance therefore decreases in traces (a), (b), (c) and (d), and increases in trace (e) corresponding to the oxidation of cytochromes *a* and *a*₃ (traces *a*, *b* and *c*), of cytochrome *c* (trace *d*) and of Cu (trace *e*). However, only partial oxidation occurs under these conditions because Compound B₂ can accept electrons from the reduced components of the respiratory chain, and the added O₂ is expended in the next 2 min, causing Compound B₂ to disappear and the cytochrome *a*₃²⁺·CO compound to re-form. Ferricyanide is, however, less rapidly reduced, and conditions appropriate to the formation of small amounts of Compound C₂ obtain. Thus trace (b), for example, exhibits an increased absorbance at the end of about 200 s due to this reaction.

On the right-hand side of Fig. 5 the reaction is repeated with sufficient ferricyanide (800 μM) to oxidize completely and rapidly ($t_{\frac{1}{2}} = 3$ s at -24°C) the cytochrome components *c* and *a* and Cu_a (trace *d*, traces *a*, *b* and *c*, and trace *e*, respectively, and as determined by independent controls). The traces are stable after the addition of 800 μM-ferricyanide and 80 μM-O₂. Flash photolysis now causes no response of cytochrome *c* (trace *d*) (it is completely oxidized) and causes the formation of Compound C₂, as indicated by the increased absorbance in traces (b) and (c). A small net absorbance change occurs at 591 nm (trace *a*), but the actual change is large and

Fig. 6. Compound C₂ formation with two different oxidants. The effect of 800 μM-ferricyanide (trace A) is compared with that of 0.16 μM-yeast peroxidase and 40 μM-H₂O₂ (trace B), and with 200 μM-O₂ only (trace C). The characteristic peak near 610 nm identifies Compound C₂ formation to an equal extent with both oxidants. Other conditions were as in Fig. 2. Absorbance increases are in an upward direction.

involves the sequential photolysis of *a*₃²⁺·CO (absorbance decrease) followed by the combination of *a*₃²⁺ with O₂ to form Compound A₂ (absorbance increase), and then the decomposition of Compound A₂ to form Compound C₂ (absorbance decrease). In trace (e) at 830 nm, only a small increase in absorbance is observed due to oxidation of Cu_a³⁺. This is because the peak of the absorption spectrum for Cu_a³⁺ under these conditions is at 740 nm, not 830 nm [see Fig. 3 of Chance & Leigh (1977)]. Also, the absorbance change due to the formation of Compound C is relatively smaller at -24°C than at lower temperatures (B. Chance, unpublished observation).

Fig. 7. Compound B₂ and C₂ formation

Kinetics of formation of Compound B₂ (top four traces, no ferricyanide; -87°C) are compared with those of the formation of Compound C₂ (bottom four traces, $400\mu\text{M}$ -ferricyanide, -91°C) for the α - and γ -bands of the haem components, together with the i.r. absorption attributable to copper. The conditions are similar to those of Figs. 2 and 3, except for 1.0mM-O_2 , with 10mg of ox heart mitochondrial protein/ml. A 2mm -path rectangular cuvette was used. Absorbance decreases are indicated by the directions of the arrows on the right-hand side.

Use of yeast peroxidase complex ES to form Compound C₂

In our previous studies of the accessibility of cytochrome *c* to exogenous oxidants, it was found that the intact outer membrane of pigeon heart mitochondria, but not of ox heart mitochondria, protected the cytochromes from oxidation by added Complex ES (yeast peroxidase and H_2O_2) (Yonetani, 1965). Thus Fig. 6 shows that Complex ES formed from $0.16\mu\text{M}$ -yeast peroxidase and $40\mu\text{M-H}_2\text{O}_2$ (trace B) is nearly as effective as $800\mu\text{M}$ -ferricyanide in causing formation of Compound C₂, as contrasted with the control trace (C), where in the absence of these oxidants Compound B₂ is formed.

Therefore the possible dangers of alteration of the properties of cytochrome oxidase by ferricyanide (Keilin, 1961; Lanne *et al.*, 1977) are obviated and a verification of the pathway of electron transfer of eqns. (1)–(3) below is obtained.

Kinetics of absorbance changes

The multi-channel time-sharing spectrophotometer can be operated at sufficiently low temperatures (-87 and -91°C for the two parts of Fig. 7) that the kinetics of formation of Compounds B₂ and C₂ are readily recorded as in Fig. 7. Of considerable interest is the correlation of Compound C₂ as measured at 608 – 630nm (trace *c*) with the kinetics of absorbance changes due to the copper component(s) at 830 – 940nm (trace *d*) and the kinetics of cytochrome a_3 ,

as recorded at 444 – 463nm (trace *f*). The greatest contrast in the kinetics of formation of Compound B₂ (no ferricyanide) and Compound C₂ ($400\mu\text{M}$ -ferricyanide) is the change in sign of trace (*c*) at 608 – 630nm , where a slight decrease in absorbance occurs on formation of Compound B₂ and a large increase occurs on formation of Compound C₂. The second notable effect is in the response of Cu^{2+} at 830 – 940nm (trace *d*). Pretreatment with sufficient ferricyanide to oxidize Cu_a completely ($400\mu\text{M}$ affords a ferri-/ferro-cyanide ratio of over $10:1$ and a potential of nearly 500mV) leaves $\text{Cu}_{a_3}^{+}$, which gives a significant optical response at 830nm attributable to the formation of $\text{Cu}_{a_3}^{+}$. The spectral and kinetic data (Chance & Leigh, 1977) indicate that the ratio of absorbance change at 740nm to that at 830nm is 3 in Compound C₂ formation. The kinetics at 830nm and in Fig. 3 of Chance & Leigh (1977) appear to be indistinguishable from those at 609nm , identifying the two absorption bands as either belonging to the same component or to two different components with kinetics that are identical to within the experimental error. In the kinetics of oxidation of copper in the formation of Compound B₂ there is some biphasicity, as noted previously (Chance *et al.*, 1975a, 1977; Chance & Leigh, 1977). The kinetics at 444 – 463nm are of the same sign in Compound B₂ and C₂ formation, as distinct from trace (*c*) at 608 – 630nm , and show a relatively more rapid initial phase in Compound B₂ formation. Also, the amplitude at

444 nm is smaller in Compound C₂ formation than in Compound B₂ formation; the nature of these differences is clarified by Fig. 8.

The absorbance changes at 596–630 nm (trace *a* of Fig. 7) are less well resolved on the time scale presented here and are complicated by the photolysis of cytochrome $a_3^{2+} \cdot \text{CO}$. There is a decrease in absorbance due to the formation of Compound A₂, and an increase in absorbance due to the formation of Compound B₂ or C₂. Their formation causes respectively large and small decreases in absorbance due to the different natures of their spectra.

Time-resolved spectroscopic study of the kinetics of formation of Compound C₂

Fig. 8 affords a wavelength scan of the 500–650 nm region and clarifies the kinetic study of Fig. 7; a dual-wavelength scanner is used as in Fig. 1. The reference wavelength is 630 nm. The conditions are similar, except that the temperature is -104°C in order to

Fig. 8. Kinetics of Compound C₂ formation

Use of the dual-wavelength scanning spectrophotometer for recording the kinetics of formation of Compound C₂ in wavelength region 500–650 nm is shown. The optical path length is 2 mm with light-guide coupling to a rectangular cuvette. The baseline corresponds to the mixed-valence-state CO compound. The reference wavelength is 570 nm. Trace 1 corresponds to the flash photolysis of CO from cytochrome a_3 ; the remaining traces indicate the formation of Compound C₂. Conditions are 13 mg of ox heart mitochondrial protein/ml of 0.2 M-mannitol/5 mM-Tris/H₂SO₄ buffer (pH 7.1)/0.22 mM-glutamate/0.22 M-malate/20 mM-phosphate (pH 7.1)/0.33 mM-ferricyanide/160 μM -CO/1.5 mM-O₂/30% ethylene glycol, at a temperature of -104°C . Absorbance increases are in the upward direction. The trace labelled 5 is the last of the series of 5 of increasing absorbance (top traces). The corresponding trace 5 at 588 nm is that of the least absorbance; 37 s intervenes between traces 1 and 5, and 20 s intervenes between traces 6 and 12.

afford a slower sequence of reactions appropriate to the 37 s scan period. At scan 1 Compound A₂ is already present, the reaction having progressed through the absorbance decrease at 588 nm due to flash photolysis and the absorbance increase due to Compound A₂ formation. Thus the absorbance decrease in traces 1–5 at 580 nm is due to the decomposition of Compound A₂. At 608 nm traces 1–5 show a progression of absorbance increases due to the formation of Compound C₂; there is an isosbestic point at 600 nm, suggesting that only two species are identifiable spectroscopically, Compounds A₂ and C₂ (Denis, 1977).

Traces 6–12 are initiated after an interval of approx. 2 min in which the traces are displaced as illustrated, and a stable end point of reaction is obtained.

From Fig. 2 we can assign trace 1 of Fig. 8 to the above-mentioned sequence of reactions leading to the formation of compound A₂. As the reaction proceeds (trace 12), the 608 nm-absorbing peak is 3-fold in excess of the intensity of that in trace 1 at 588 nm and much larger than those reported by Nichols (1978). The traces show no increase in absorbance at 650 nm attributable to cytochrome a_3^{3+} (Denis, 1977).

A correlation with the lower traces (*a*) and (*c*) of Fig. 7 is apparent when it is recognized that the measured wavelength of trace (*a*) of Fig. 7 is at 596 nm, i.e. on the long-wave side of the 594 nm isosbestic point, and thus the net absorbance change after Compound C₂ formation shown in Figs. 7 and 8 is an increase. In trace (*c*) of Fig. 7, there is a distinctive initial jump at 608 nm due to the photolysis of the CO compound. However, the trace does not return towards the initial baseline as in trace (*a*), but continues in the direction of increasing absorption to form a 608 nm absorption band.

Absorbance changes in the region of the Soret band

The predominance of cytochrome *a* in the region of the α -bands makes it essential to record the formation of Compound C₂ in the region of the α - or Soret band to determine whether the absorbance increase near 609 nm is also observed.

Fig. 9 illustrates the progress of absorbance changes that occurs during the formation of Compounds A₂ and C₂ at -110°C . The first scan gives an approximate inverted difference spectrum of the cytochrome $a_3^{2+} \cdot \text{CO}$ compound and thus serves as a calibration of the absorbance changes that follow over the 10–20 min interval. The formation of Compounds A₂ and C₂ causes a progressive decrease in the 444 nm peak with very little net change at 429 nm, in agreement with the α -band, which shows that Compound C₂ has about the same absorbance at 590–630 nm as does cytochrome $a_3^{2+} \cdot \text{CO}$.

Fig. 9. Kinetics of Compound C_2 formation. Kinetics of absorbance changes in the region of the Soret band corresponding to Fig. 8. The reference wavelength is 470 nm. Other conditions were identical with those of Fig. 8, except that the temperature was -110°C . Absorbance increases are in an upward direction; 37 s intervenes between traces.

Fig. 10. Kinetics of Compound B_2 formation. Kinetics of absorbance changes in the region of the Soret band corresponding to the formation of Compound B_2 . Other conditions were identical with those of Fig. 9, except that the ferricyanide was omitted and the temperature was -104°C . Absorbance increases are in an upward direction; 37 s intervenes between traces.

The progress of the reaction is indicated by the partial disappearance of the Soret band of cytochrome a_3 to an extent that is 82% of that caused by liganding with CO. Studies at higher temperatures indicate that this is the maximum extent of disappearance obtainable at 444 nm.

A comparison with Compound B_2 formation is useful at this point, and is afforded by the difference spectra of Fig. 10 (Chance *et al.*, 1975c), in which the reaction is started at approximately the same temperature in a preparation without a supplement of ferricyanide. The initial trace is similar to that of Fig. 9 and shows the cytochrome $a_3^{2+} \cdot \text{CO}$ minus reduced difference spectrum, in inverted form because of the photolysis reaction. Cytochrome a_3^{2+} is

successively converted into Compounds A_1 and B_2 . The extent of disappearance of the 444 nm band in Compound B_2 formation is 146% of the absorbance difference between cytochrome $a_3^{2+} \cdot \text{CO}$ and cytochrome a_3^{2+} as compared with 82% for Compound B . Complete oxidation of cytochromes $a + a_3$ corresponds to about 200%.

One interpretation of the spectra is that oxidation of cytochrome a_3 proposed in the formation of Compound B_2 does not occur in Compound C_2 formation and that the haem a_3 remains in liganded ferrous state. This is supported by these observations of the Soret-band data, which show only an 18% difference between the CO-liganded cytochrome a_3^{2+} and Compound C_2 .

Discussion

Spectroscopic studies of the cytochrome oxidase- O_2 reaction at very low temperatures affords identification of three spectroscopically distinct compounds. Two of them, Compounds A_1 and B_2 , are observed in the 'normal' course of electron transfer in which the reaction is started from the completely reduced state. Compound C_2 differs from the other two in that oxidation of a cytochrome by prior treatment with ferricyanide is required in order to cause formation of Compound C_2 . Furthermore, since ferricyanide treatment oxidizes electron donors to Compound C_2 , it appears to be the most stable of the three compounds and has been observed over the widest range in temperatures, i.e. from -80 to -100°C . The characteristics of the compound are unusual; the absorption bands at 609 and 740 nm of the compound are observed. The band at 609 nm is more intense than that of the fully reduced species. There is also an increase in e.p.r. signal at $g = 2.17$.

The pathway of electron transfer in Compound A_2 depends upon the availability of electron donors. If cytochromes a and c and copper are available as electron donors, the reduction of Compound B_2 according to the cycle of Fig. 1 (Chance *et al.*, 1975c) can occur. If, however, no electron donor is available and cytochrome $a_3^{2+} \cdot \text{CO} \cdot \text{Cu}_{a_3}^+$ contains the only components sufficiently positive to resist oxidation by ferricyanide, then, according to previous discussions, cytochrome $a_3^{2+} \cdot \text{Cu}_{a_3}^+$ itself can be the only available electron donor to O_2 operative in the conversion of Compound A_2 into other intermediates. Possible explanations that have been advanced for this response have been the establishment of a higher valency state of iron in a peroxy-cytochrome oxidase intermediate (Chance *et al.*, 1975a; Chance & Leigh, 1977) or, as described here, a mixed-valence-state binuclear complex with haem a_3 reduced and its associated copper in the oxidized state, both interacting with bound and partially reduced O_2 .

Compound C₂ is the most unusual of the three intermediates observed spectroscopically in low-temperature studies of the reaction of cytochrome oxidase with O₂ in both membrane-bound and solubilized preparations. The incongruity of its properties is the opposite sign of absorbance changes in the regions usually attributed to the α - and γ -bands of haem a₃. This converse change of these two components is sufficiently large that no reasonable assignment of cytochrome *a* in the α -band region and cytochrome a₃ in the γ -band region is capable of resolving the dilemma. Furthermore, an additional difficulty in resolving this dilemma is that Compound C₂ appears to be difficult to form at room temperature. The experiments of Greenwood *et al.* (1974), which involve conditions appropriate to the formation of this compound, give only a fraction of the increase in absorbance that we are able to attribute to it at low temperature (Chance *et al.*, 1975a). Furthermore, a progression of studies of the absorption difference spectrum of the compound in the region towards -8°C again only gives a small fraction of that obtainable at low temperatures (B. Chance, unpublished observation).

This discrepancy of the absorbance changes in the α - and γ -band regions together with an absorption spectrum of the copper component at an atypical wavelength (740nm) and the appearance of a $g = 2.17$ signal (W. J. Ingledew, unpublished observations; Chance & Leigh, 1977) all point to a remarkable change in the properties of cytochrome oxidase when the reactions are initiated from the mixed-valence state rather than from the fully reduced state. This possibility has been presaged by the work of Leigh *et al.* (1974), in which the converse phenomenon occurred, i.e. an effect upon the spin state of haem *a* caused by the change of ligand binding to haem a₃ at temperatures as low as 5 K. We would point to the possibility of reciprocity in the control of the properties of haem a₃Cu_{a3} by the redox state of haem aCu_a.

In identifying which properties of cytochrome a₃Cu_{a3} might be subject to change, the postulation of a model seems appropriate. Emerging evidence favours a cytochrome a₃Cu_{a3} model in which the iron and copper components form a binuclear complex of unusual redox and optical absorption properties. This model seems appropriate to other studies as well, e.g. to the reaction of mercaptans with tyrosinase (Denium *et al.*, 1976), in which the preponderating $g = 2.17$ signal as observed in Compound C₂ is appropriately modelled, and to a binuclear complex of iron and copper linked by an imidazolate ion as suggested by Palmer *et al.* (1976). The ideas proposed in these models plus the experimental observation that the K-edge absorption spectrum of Cu_{a3} closely resembles that of a model compound, stellacyanin, which contains a type-I blue ionic copper (Powers *et*

al., 1979a), seem appropriate to a configuration of iron and copper in Compound C₂ in which the properties of its copper atom are unmasked by a shift of their geometry leading to decreased interaction (Powers *et al.*, 1979b).

On these premises, it is proposed that the anomalous redox state of aCu_a in the mixed-valence state 'uncouples' the interaction between a₃ and Cu_{a3} in the binuclear configuration of Compound C₂, so that: (a) the absorption band of Cu_{a3}²⁺ shifts to 740nm from 790nm as observed in Compound B₂; (b) the 'blue'-type copper absorption band at 609nm appears; (c) the e.p.r. signal of $g = 2.17$ appears; (d) copper becomes a better electron donor to O₂ than iron (in contradistinction to the equality of the donors in the configuration of Compound B₂).

A number of experiments are required to consolidate this viewpoint as anything more than a working hypothesis. Obviously, the important ones are the measurement of the iron-copper distance in the resting states, in the mixed-valence states and in the Compound C₂ configuration, together with a determination of whether ligands other than O₂ will form similar and presumably more stable intermediate compounds.

Reaction with ferricyanide

The reaction of ferricyanide with cytochrome oxidase occurs effectively if cytochrome *c* is available to mediate between the oxidase and the redox couple. Alternatively, mediators such as diaminodurene etc. can be used. However, the reaction observed follows, at least in principle, the reaction scheme of eqns. (1)–(3) below:

This reaction cycle shows that equilibrium is reached, which occurs under usual conditions, with cytochrome aCu_a in the fully oxidized state and cytochrome a₃Cu_{a3} in the reduced state and liganded to CO. The procedure that we had used avoids the competition between the CO reactions and the redox reactions, as must be the case in the titration experiment of Lindsay & Wilson (1974). In that case, the spontaneous dissociation of CO from the oxidase would periodically liberate a₃²⁺, which, in turn, could react directly with the poised couple, be oxidized, and subsequently be reduced. Again CO would compete with the oxidant for the reduced form. In these experiments, the reaction is non-competitive, or more properly uncompetitive, since the half-time for the dissociation from the CO to the oxidase is longer than the time for addition of ferricyanide and O₂ to the chilled suspension of mitochondria. Thus the condi-

tions differ sharply from those of the Lindsay & Wilson (1974) experiments, and a closer approximation to a true mixed-valence state can be obtained.

The non-equilibrium titration of the CO-inhibited oxidase with ferricyanide also differs from the approach of Wever *et al.* (1977), where the reducing equivalents are added to the oxidized oxidase in the presence of phenazine methosulphate as a mediator in the absence of CO. In this case, reducing equivalents can be trapped in a variety of forms of the oxidase, which are best indicated by the configurational notation below and may well include mixtures of fully reduced and fully oxidized with some component of mixed-valence state, i.e. half-reduced and half-oxidized. We prefer the notation of the latter state as a statistical mixed-valence state, the true molecular mixed-valence state being obtained by the titration of the CO compound of ferricyanide at low temperatures.

Configurational representation of reaction

The configurational notation (Hill & Chance, 1978) for these redox states is included in Table 1. It is useful to include six components of the respiratory chain; the oxidized forms are designated by 'O' and the reduced form by 'R'. The sequence runs from Rieske's iron-sulphur protein through cytochromes c_1 and c to cytochrome oxidase to O_2 . List (1) of Table 1 identifies the configuration obtained just after photolysis and identified as R, i.e. fully reduced. Compounds A_1 and A_2 also have fully reduced configurations and O_2 is combined with cytochrome a_3 . List (3) represents the configuration appropriate to Compound B_2 , in which the partial reduction of O_2 is simply designated by R. List (4) indicates the mixed-valence configuration before photolysis. This configuration differs from the above-mentioned statistical mixed-valence state, which would be the arithmetic average of 50% of state R (list 1) and the fully oxidized list (6). Compound C_2 may have the configuration of list (5); the only component in this

configuration that differs from that of the fully oxidized configuration would be cytochrome a_3 . If cytochrome a_3 had undergone a one-electron oxidation-reduction reaction, the configuration of Compound C_2 would be identical with that of the fully oxidized or resting enzyme (list 6). Thus the state of cytochrome a_3 in Compound C_2 must be a very special one that does not permit the formation of the fully oxidized state in spite of the presence of oxidants on both 'sides' of the oxidase itself.

Table 1 further emphasizes that Compound B_2 may have a short lifetime owing to the plethora of electron donors that are not only highly competent kinetically at room temperature, but also in stoichiometric excess. Thus the idea that the oxidized form may have a very short lifetime, especially at low O_2 concentrations, seems to be underlined by Table 1.

The question of the catalytic activity of Compound C_2 is of considerable importance, since electron acceptance by this intermediate directly rather than through cytochrome aCu , might be demonstrable and further support the low-temperature data that suggest that it is an electron acceptor from cytochrome c in the configuration of Compound B_2 . Thus Compound C_2 does not at present qualify as an inactive intermediate such as the enzyme-substrate compound of catalase, Compound II (Chance, 1949), or indeed a peroxidase Compound III (Chance, 1952).

References

- Chance, B. (1949) *J. Biol. Chem.* **179**, 1341-1369
 Chance, B. (1952) *Arch. Biochem. Biophys.* **41**, 404-415
 Chance, B. (1953) *J. Biol. Chem.* **202**, 397-406
 Chance, B. & Leigh, J. S. Jr. (1977) *Proc. Natl. Acad. Sci. U.S.A.* **74**, 4777-4789
 Chance, B., Saronio, C. & Leigh, J. S. Jr. (1975a) *J. Biol. Chem.* **250**, 9226-9237
 Chance, B., Graham, N. & Legallais, V. (1975b) *Anal. Biochem.* **67**, 552-579
 Chance, B., Harmon, J. & Wikström, M. K. F. (1975c) in *Electron Transfer Chains and Oxidative Phosphorylation* (Quagliariello, E., Papa, S., Palmieri, F., Slater, E. C. & Siliprandi, N., eds.), pp. 81-95, North-Holland/American Elsevier, Amsterdam and New York
 Chance, B., Leigh, J. S. Jr. & Waring, A. (1977) in *Structure and Function of Energy-Transducing Membranes* (van Dam, K. & van Gelder, B. F., eds.), pp. 1-10, Elsevier/North-Holland Biomedical Press, Amsterdam
 Denis, M. (1977) *FEBS Lett.* **84**, 296-298
 Denium, J., Lerch, K. & Rheinhammer, B. (1976) *FEBS Lett.* **69**, 161-164
 Greenwood, C., Wilson, M. T. & Brunori, J. (1974) *Biochem. J.* **137**, 205-215
 Hill, T. & Chance, B. (1978) *J. Theor. Biol.* **72**, 17-56
 Keilin, D. (1961) *Nature (London)* **190**, 717-718
 Lanne, B., Malström, B. G. & Vänngård, T. (1977) *FEBS Lett.* **77**, 146-150
 Leigh, J. S. Jr., Wilson, D. F. & Owen, C. (1974) *Arch. Biochem. Biophys.* **160**, 476-486

Table 1. Configurations of electron-transfer components

These lists are incomplete in that they do not indicate liganding states of haem a_3 with unprotonated O_2 reduction products, including peroxide O_2^{2-} (Compound B_2), superoxide O_2^- (Compound C_2) and water O^{2-} (in the oxidized or resting state).

Compound	O_2	a_3	Cu	a	Cu	c	c_1	FeS	
R		R	R	R	R	R	R	R	(1)
A_1	O_2	R	R	R	R	R	R	R	(2)
B_2	O_2^{2-}	O	O	R	R	R	R	R	(3)
Mixed valence state		R	R	O	O	O	O	O	(4)
C_2	O^-	R	O	O	O	O	O	O	(5)
O_2	O^{3-}	O	O	O	O	O	O	O	(6)

- Lindsay, A. & Wilson, D. F. (1974) *FEBS Lett.* **48**, 45–59
- Nichols, P. (1978) in *Biochemical and Clinical Aspects of Oxygen* (Caughey, W. J., ed.), Academic Press, New York, in the press
- Palmer, G., Babcock, G. T. & Vickery, L. E. (1976) *Proc. Natl. Acad. Sci. U.S.A.* **73**, 2206–2210
- Powers, L., Blumberg, W. E., Chance, B., Barlow, C. H., Leigh, J. S., Jr., Smith, J., Yonetani, T., Vik, S. & Peisach, J. (1979a) *Biochim. Biophys. Acta* in the press
- Powers, L., Blumberg, W. E., Chance, B., Barlow, C. H., Leigh, J. S., Jr., Smith, J., Yonetani, T., Vik, S. & Peisach, J. (1979b) *Abstr. Annu. Meet. Biophys. Soc.* **23rd** in the press
- Wever, R., van Drooge, J. H., Muijsers, A. O., Bakker, E. P. & van Gelder, B. F. (1977) *Eur. J. Biochem.* **73**, 149–154
- Wikström, M. K. F., Harmon, H. J., Ingledew, W. J. & Chance, B. (1976) *FEBS Lett.* **65**, 259–277
- Yonetani, T. (1965) *J. Biol. Chem.* **240**, 4509–4514