Low-Temperature Kinetics of the Reaction of Oxygen and Solubilized Cytochrome Oxidase

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The reaction of solubilized cytochrome oxidase in the fully reduced state with O_2 at low temperatures reveals components with characteristics similar to those observed with the membrane-bound oxidase, namely compounds A and B, which are proposed to be 'oxy' and 'peroxy' compounds respectively. Similar species are identified in both solubilized and membrane-bound oxidases; the reaction velocity constant for the reaction with O_2 and the dissociation constant are decreased 2–3-fold in the solubilized preparation as compared with the membrane-bound species, owing to decreased reactivity towards O_2 in the former. The oxidase prepared in the mixed-valence state shows the distinctive absorption band characteristic of compound C, identified in the membrane-bound oxidase. The assignment of the α , β , γ and near-i.r. absorption bands to possible valence states of these compounds is made.

The existence of an O_2 compound of solubilized cytochrome oxidase was postulated by Okunuki *et al.* (1958), Orii & Okunuki (1963), Wainio (1965), Lemberg & Mansley (1966) and Orii & King (1972). Initially, the compound was prepared by oxygenating the dithionite-reduced material, but more rigorous procedures were subsequently developed by Wharton & Gibson (1968). The sluggish kinetics of the compound had raised questions in the early work as to the feasibility of its biological function, and rendered the search for this compound by rapid kinetic methods futile in both the solubilized (Wharton & Gibson, 1968) and membrane-bound (Chance, 1965) systems. Babcock *et al.* (1976) have examined the magnetic circular dichroism of the 'oxygenated compound' and find it to have a 'spectrum identical with that of the resting enzyme, apart from some additional intensity at 452 nm which we attribute to incomplete reoxidation of cytochrome a^{2+} '.

Gibson & Greenwood (1965) evaluated the kinetic constants for the disappearance of the reduced form of cytochromes a_3 and a , and for the appearance of oxidized copper in the reactions of the oxidase with O_2 . The velocity constant for the reaction of the reduced oxidase with O_2 was determined to be between 3×10^7 and $8 \times 10^7 \text{ M}^{-1} \cdot \text{s}^{-1}$, on the basis of the de-

crease in absorbance at 445 nm. Only the ferric and cupric forms were observed; no spectroscopic species characteristic of the intermediate was identified in these studies, and Greenwood & Gibson (1967) concluded that 'it is unnecessary therefore to assign a kinetically significant role to the oxygenated form of the enzyme'. Gibson & Greenwood (1965) did observe a non-linearity in the profile of O_2 concentration versus pseudo-first-order velocity constant at high O_2 concentrations that can now be interpreted in terms of the results presented here as being due to the formation of an O_2 compound in a small and spectroscopically undetectable amount. They noted that the heterogeneity of the oxidase preparation was sufficient to render interpretation of the results difficult (Greenwood & Gibson, 1967). More recently, in rapid-flow studies at -30°C , Erecinska & Chance (1972) found preliminary evidence for a compound similar to that identified in the present paper as 'compound A'.

Greenwood *et al.* (1974) have pursued the study of the O_2 reaction of the soluble oxidase, and found that, after the reaction with O_2 , the ferricyanide-treated CO-inhibited preparation in a mixed-valence state gives an 'oxygenated' product. Their conditions of preparation were similar to those for compound C observed in mitochondria (Chance *et al.* 1975a,b; Chance & Leigh, 1977) and reported here for the solubilized oxidase. However, a lower temperature

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was required for the trapping technique in the study of the O_2 compounds of the membrane-bound oxidase (Chance *et al.* 1974, 1975b) and much better characterized spectra have been obtained (Chance & Leigh, 1977).

Although the use of the soluble oxidase involves assumptions as to the integrity and homogeneity of the preparations (Greenwood & Gibson, 1967) and about the role that the membrane plays in the function of cytochrome oxidase, some advantages over the membrane-bound oxidase include the more favourable optical properties and the possibility of studying the oxidase reaction without endogenous electron donors such as cytochromes *c* and *c*₁ and the iron-sulphur proteins. In addition, as in the mitochondria, the valence state of cytochrome *a* and its associated copper atom may be altered by equilibration with ferricyanide in the presence of CO.

Several studies of the CO compound of solubilized cytochrome oxidase have appeared recently, and titration with reducing equivalents and redox-equilibrium studies show that the CO compound accepts two electrons (Lindsay & Wilson, 1974; Wever *et al.*, 1977; Rosen *et al.*, 1977). However, oxidative titrations show that the solubilized oxidase donates three electrons (Hartzell & Beinert, 1976). Both haem and copper components of cytochrome *a*₃ seem to be active in electron-transport reactions of the mixed-valence-state oxidase (Chance & Leigh, 1977).

Materials and Methods

Preparations

Phospholipid-sufficient cytochrome oxidase had an activity as described by Kuboyama *et al.* (1972) and Yu *et al.* (1975). The preparation was stored at 20°C. For use, samples of concentrated enzyme are diluted in a medium containing 75% of 0.01M-phosphate buffer, pH 7.5, 25% ethylene glycol, 50mM-ascorbate and 2μM-cytochrome *c*, and, if desired, 5μM-tetramethyl-*p*-phenylenediamine. The reaction medium is saturated with CO before addition of the oxidase. The enzyme (0.5ml of solution) is then transferred to an e.p.r. tube (3.5mm inside diameter): 30min at room temperature is allowed for complete reduction of the enzyme, as checked by optical spectra taken at room temperature, and the tubes are then stored at 0°C. The ethylene glycol concentrations used do not have a significant effect on the respiration as measured by the platinum micro-electrode (B. Chance & C. Saronio, unpublished work).

Protein is determined by the method of Lowry *et al.* (1951), with bovine serum albumin as standard.

Oxygenation of the samples is carried out just above the freezing point of the mixture (−25°C), either by vigorous stirring or by generation of O_2 from the decomposition of H_2O_2 (Chance *et al.*, 1975d). A small amount of anti-foam (Dow Corning Silicone

Antifoam A, Midland, MI, U.S.A.) is added to the stirring rod to prevent bubble formation during the stirring procedure. The effusion of CO from the detergent-solubilized preparation is very rapid, and anti-foam must be used during the stirring procedure to avoid significant loss of CO and consequent oxidation of the reduced cytochrome oxidase at −25°C. This problem is avoided when a small volume of CO-saturated oxidase is oxygenated by dilution with a large volume of O_2 -saturated ethylene glycol (Chance & Leigh, 1977). The oxygenated samples are generally stored at −80°C in the dark until the experiment is to be done.

Considerable variations in the oxidase concentration seemed to have little effect on the results, since the O_2 reactions are pseudo-first-order with respect to O_2 . The absorbance changes did not increase linearly with the oxidase concentrations at the higher values (Chance *et al.*, 1972).

In the studies of compounds B and C, prepared at different redox potentials, the preparation (4μM-oxidase, 30% ethylene glycol, 0.2M-mannitol, 5mM-phosphate buffer, pH 7.2) is gassed with argon/CO (1:1) at 23°C; 30μM-tetramethyl-*p*-phenylenediamine is added and reduced by small additions of $Na_2S_2O_4$, giving the dye intermediate, Wurster's Blue, at a potential sufficiently negative to reduce the oxidase and cause it to combine with CO. The potential is then progressively increased by addition of ferricyanide. The samples are then rapidly transferred to the e.p.r. tube by a 1mm catheter and cooled to −20°C, oxygenated as above, and chilled and stored at −80°C.

In a somewhat improved technique (cf. the charts of Fig. 7), the sample of cytochrome oxidase is equilibrated at −20°C as above in a CO-saturated atmosphere. The effluent gas is allowed to exit through the sample tube and into the optical cuvette (see below) or the e.p.r. tube mentioned above. The sample is then transferred under pressure of CO via a 0.5m length of stainless-steel tubing (0.3ml volume) coiled in a −23°C cooling bath and extending into the chilled (−23°C) cuvette. The chilled sample now occupies only one-third the volume of the 1mm cuvette (2mm optical path), and injection of 0.7ml of O_2 -saturated (2mm at −23°C) chilled ethylene glycol (30%) now completes the filling of the cuvette. Oxygenation is thus effected without the need for vigorous stirring, except to mix the contents of the cuvette, which requires about 5s at −23°C. Mixing is continued after the transfer of the sample to a −78°C ethanol/solid CO_2 mixture and until freezing takes place. The sample is then transferred to an appropriate spectrophotometer for spectroscopic (wavelength-scanning) or kinetic (dual-wavelength) studies.

In Fig. 6(a) below, the kinetics were studied with 10μM-cytochrome oxidase reduced with 5mM-ascorbate and 5μM-tetramethylphenylenediamine.

The oxidase solution (80 μM) is saturated with CO and then diluted at -20°C with 7 vol. of aqueous solution containing 30% ethylene glycol that had previously been air-saturated at 23°C . The oxidase preparation and the air-saturated medium are mixed for 10 s and then transferred to a freezing bath at -78°C . The sample is contained in a 2 mm-optical-path cuvette with an aperture approx. 2 cm^2 , which is then transferred to the kinetic spectrophotometer, and the darkened sample equilibrated to -97°C with a thermoregulator. When thermal equilibrium is obtained, a 200 J flash lamp activates the reaction, leading to the kinetics observed at 591–630 nm, 444–463 nm and 830–940 nm.

The change in pH with temperature has been shown by Williams-Smith *et al.* (1977) to be largely abolished by the presence of large concentrations of serum albumin (approx. 40 mg/ml). Similar effects are observed with mitochondria where the system equilibrated with Phenol Red at 0°C shows no large shift of indicator on cooling to -30°C even with only 3.6 mg of mitochondrial protein/ml. Presumably, the higher protein concentrations (5–25 mg of protein/ml) used in these studies avoid significant pH shifts with temperature (Chance, 1975).

Physical methods

The usual dual-wavelength technique and a split-beam wavelength-scanning spectrophotometer (Chance *et al.*, 1972) were used to record the data of Figs. 1 and 4. The baseline corrections for the scanning spectrophotometer were stored in a digital memory. The kinetics of absorbance changes (Figs. 6a and 6b) were recorded with a multi-channel dual-wavelength apparatus (Chance *et al.*, 1975e).

Samples poised with redox mediators were also studied in the split-beam split-photolysis apparatus (Chance *et al.*, 1975c; Chance & Leigh, 1977). At -88°C one half of the sample tube is flash-photolysed and the absorbance recorded with respect to the unflashed portion. The sample is then chilled to -196°C and the optical spectra at the top and bottom of the tubes are recorded (Figs. 8a and 8b).

Results

Compound A

The kinetic and spectral properties of the compound formed by flash photolysis of the CO compound of the reduced solubilized oxidase are shown in Fig. 1. Fig. 1(b) illustrates the kinetics of the reaction.* The 1 mM- O_2 concentration used here is the maximum that can be obtained with the stirring technique described above, and the half-time is 1.0 s at -86°C .

* The reference number for each experiment is included in the Figure legends, so that further information may be obtained on request from the authors (B. C.).

This may be compared with the reaction of the membrane-bound oxidase, which has a half-time of 1.0 s at -100°C (Chance *et al.* 1974, 1975a). Fig. 1(b) indicates that a compound of approximately the same absorbance is formed by flash photolysis of the CO compound in the presence as in the absence of O_2 (note that the levels a and c are nearly equal). The resulting compound cannot be dissociated by light-intensities adequate to photolyse the CO compound completely (approx. 10 mJ at 591 nm; Stark & Chance, 1969).

Low-temperature trapping of the reaction product of Fig. 1(b) is also possible if, for example, the material at point c of Fig. 1(b) is chilled rapidly to the temperature of liquid N_2 (chilled isopentane may be used to accelerate the chilling); at -196°C , the reaction proceeds no further. In this case, the baseline stored in the memory of the spectrophotometer corresponds to the absorbance of a reduced sample at -196°C . In the absence of O_2 , point b after photolysis represents the spectrum of reduced cytochrome a_3^{2+} . The spectrum of the reaction product at point c is subtracted from that at point b to give the difference spectrum of what we have termed 'compound A' (Chance *et al.*, 1974, 1975a,b) with a peak at 591 nm and a trough at 611 nm. The difference spectrum corresponding to point b minus point a is recorded separately and its amplitude is 25% less than that displayed here for compound A. The difference may be greater than that shown here, since maximal occupancy of compound A with O_2 is scarcely reached at the highest O_2 partial pressures obtained by using the stirring technique.

By adding various amounts of O_2 with the stirring technique, it is possible to obtain and analyse the results as the equilibrium and kinetics of compound A, as shown in Fig. 2. In these calculations, the O_2 concentration is taken to be that at the moment of freeze-trapping. The equilibrium is plotted according to the logarithmic form of the mass-law equation, where the slope corresponds to the stoichiometric coefficient, and the value at equal concentrations of the free and bound forms corresponds to the dissociation constant. A line corresponding to a 1:1 stoichiometry of O_2 to iron and a dissociation constant, K_d , of $340\text{ }\mu\text{M-O}_2$ at -86°C has been drawn on Fig. 2 and fits the data within the accuracy of the experiment; it may be compared with the somewhat smaller values for the membrane-bound oxidase. The reversible equilibrium for the combination of cytochrome oxidase (a_3) and O_2 at low temperatures can be written as:

Fig. 1. Difference spectrum (a) and kinetics of formation (b) of compound A in solubilized cytochrome oxidase. The baseline for spectrum (a) corresponds to point b in the kinetic diagram, and was obtained in a separate experiment from which O_2 was absent. The species represented in the difference spectrum corresponds to the difference between point c and point b, as indicated in the kinetic trace (temperature for O_2 addition, $-25^\circ C$; for kinetic recording, $-86^\circ C$; for spectral recording, $-196^\circ C$). Other conditions were: $40 \mu M$ -solubilized cytochrome oxidase, $5 \mu M$ -cytochrome c, $5 mM$ -ascorbate, $5 \mu M$ -tetramethylphenylenediamine, 20% ethylene glycol, $10 mM$ -phosphate buffer, pH 7.4, $1 mM$ -CO (saturated at room temperature), $1 mM$ - O_2 added at $-25^\circ C$. LDL, Liquid dye laser. Further information may be obtained by use of reference number 1508-1^v (see footnote to p. 789).

Fig. 2. Equilibrium and kinetic properties of the formation of compound A of solubilized cytochrome oxidase. (a) Mass-law plot in log-log co-ordinates for determining the stoichiometry and equilibrium constants; (b) kinetic plot for determining k_{+1} , k_{-1} and the equilibrium constant. Conditions were generally as in Fig. 1, with flash activation occurring at $-80^\circ C$. The O_2 concentration was varied over the range indicated by addition of H_2O_2 in the presence of catalase (Chance *et al.*, 1975d). Reference number CS-10.

The second-order constant for the forward reaction and an evaluation of the first-order constant for the reverse reaction are given by the plot of Fig. 2(b), of the pseudo-first-order velocity constant versus the O_2 concentration on a linear scale. k_{+1} corresponds to $1.6 \times 10^3 M^{-1} \cdot s^{-1}$ at $-86^\circ C$; thus k_{-1} corresponds to approx. $0.55 s^{-1}$ at this temperature, and K_4 from the kinetic plot agrees well with that from the equilibrium titration ($340 \mu M$). The inclusion of the second step of eqn. (1) is based on data that follow in the paragraphs below.

A comparison of the value of k_{+1} for the solubilized oxidase prepared from ox heart mitochondria with that for k_{+1} in intact ox heart mitochondria, and corrected to the same temperature, indicates that the solubilized oxidase reacts more slowly than the membrane-bound oxidase of ox heart (and pigeon heart) mitochondria (Chance *et al.*, 1974, 1975a). In summary, compound A formed from the solubilized oxidase is qualitatively similar to but not quantitatively identical with that formed from the membrane-bound oxidase.

Compound B

As the temperature is raised, compound A becomes unstable and is converted into a second form in which electrons may be transferred from cytochrome oxidase to O_2 . Fig. 3 records the kinetics of the conversion at $-105^\circ C$ and $-88^\circ C$ at $400 \mu M$ - O_2 .

The reaction is initiated by flashing the liquid dye laser, and the kinetics at 591 nm follow those of Fig. 1, but are slower, owing to the lower temperature; the amount of compound A is also smaller because of the lower O_2 concentration. The time scale is more compressed in Fig. 3 than in Fig. 1. After the formation of compound A (upward deflection of trace A at $-105^\circ C$) there is a slow downward deflection as the absorption band of compound A slowly disappears; the trace reaches a plateau in about 5 min. We view this transition as the conversion of compound A into compound B (Chance *et al.*, 1974, 1975a).

In trace (C) at 608 nm and $-105^\circ C$, the disappearance of the CO compound after the photolysis flash is not clearly shown; instead, a rapid decrease in absorbance at 608 nm characteristic of the formation of compound A is recorded. A further decrease in absorbance at 608 nm occurs due to the formation of compound B, which has less absorbance at this wavelength than either the reduced species, cytochrome a_3^{2+} , or compound A. Trace (B), at 604 nm, is close to the isosbestic point between the CO compound and compound A, so that it shows absorbance changes similar to those of trace (C) rather than those of trace (A).

Recording in the i.r. region is possible, and trace (G) indicates, by a delayed downward deflection, an increase in absorbance that follows the kinetics of

Fig. 3. Effect of temperature on the formation and disappearance of compound A and the formation of compound B. Two experiments are illustrated, one at $-105^\circ C$ and (a) the other at $-88^\circ C$ (b). Time proceeds from left to right and the wavelengths and the sense of the absorbance changes are indicated on the diagram. Conditions were: $40 \mu M$ -solubilized oxidase, $2 \mu M$ -cytochrome *c*, $10 mM$ -ascorbate, $5 \mu M$ -tetramethylphenylenediamine, 20% ethylene glycol, $10 mM$ -phosphate buffer, pH 7.6, $1.2 mM$ -CO added at $20^\circ C$, $400 \mu M$ - O_2 added at $-25^\circ C$. LDL, Liquid dye laser. Reference number 1575.

formation of compound B. This is interpreted as a partial oxidation of copper in the formation of compound B and affords support for eqn. (1).

When the temperature is raised to -88°C , the transformation of compound A into compound B proceeds so much more rapidly that the formation of compound A is scarcely resolved, and traces (A) and (B) at 592 and 603 nm show only the completion of the formation of compound B. Traces (C) and (G) now show similar kinetics. Trace (B) now proceeds more slowly than traces (C) and (G), possibly owing to the formation of a further unidentified intermediate (see also Fig. 10 of Chance *et al.*, 1975b).

The method of preparing the sample for wavelength scanning is illustrated in Fig. 4(b), in which the kinetics of formation of compound B measured at 605 nm and at -67°C are shown. These kinetics are similar to those of trace (C) of Fig. 3 at -88°C . Flash photolysis causes the usual kinetics, i.e. splitting the CO compound (point b), followed by the cycle of compound A formation and its conversion into

compound B, with a half-time of approx. 2 s. At point c on the trace, the sample is frozen in liquid N_2 (compound B is stable at these temperatures) and the difference between these spectra and that of the reduced oxidase, obtained as described above, is displayed in Fig. 4(a). Considerable absorbance has been lost at 608 nm. There is a shoulder in the region from 580 to 600 nm, which is more pronounced than in the difference spectrum of compound B of the membrane-bound oxidase (Chance *et al.*, 1974, 1975a).

Fig. 5 illustrates the nature of the consecutive reactions of compound A and compound B (see eqn. 1), showing an O_2 -dependence of the rate of formation of compound B; the pseudo-first-order velocity constant for the formation of compound B is smaller than that of its precursor, compound A. Thus a linear plot of the initial slope of the reaction kinetics versus the O_2 concentration gives an initial linear portion and then a plateau with a saturation value of 1.7 s at -65°C (Fig. 5a). A double-reciprocal

Fig. 4. (a) Difference spectrum corresponding to compound B (the reference spectrum is the reduced oxidase) and (b) kinetics of formation of compound B

The experimental conditions for determining the difference spectrum are given in Fig. 3; the baseline corresponds to point b as determined in a separate experiment in which photolysis in the absence of O_2 occurred. The material used for recording the spectrum of compound B was trapped at -196°C at point c at the end of the trace. The kinetics for formation of compound B (Fig. 4b) were recorded at -67°C . Conditions were: 40 μM -solubilized cytochrome oxidase, 2 μM -cytochrome c, 10 mM-ascorbate, 5 μM -tetramethylphenylenediamine, 20% ethylene glycol, 5 mM-phosphate buffer, pH 7.4; 1 mM-CO added at 20°C , 200 μM - O_2 added at -25°C . LDL, Liquid dye laser. Reference number 1508-3^v.

Fig. 5. Kinetic and equilibrium properties of compound B, measured at -65°C by using $20 \mu\text{M}$ -solubilized cytochrome oxidase (a) Velocity constant for the initial phase of the formation of compound B plotted versus the O_2 concentration. (b) Double-reciprocal plot of the pseudo-first-order velocity constant (k) versus the O_2 concentration. Reference number 1508-4^v.

plot of this initial portion gives an apparent dissociation constant of $295 \mu\text{M}$ (Fig. 5b).

Comparison of the reactivity of the solubilized and membrane-bound oxidases towards O_2

Fig. 6 shows recordings of the reactivity towards O_2 of the two preparations of cytochrome oxidase under identical conditions ($210 \mu\text{M}$ - O_2 at -97°C) with identical time and amplitude scales on the recorders. Nearly identical concentrations of membrane-bound and solubilized oxidase are used; slight differences in the conditions for reducing the oxidase have been described in the Materials and Methods section. The kinetics of the reactions of haem a_3 iron are recorded in this chart at two wavelength pairs: trace (A), cytochrome $a_3^{2+} \cdot O_2$ at 591–630 nm, and trace (D), cytochrome a_3^{2+} at 444–463 nm, corresponding respectively to the decreased absorbance caused by the decomposition of the cytochrome $a_3 \cdot CO$ at 591 nm and to the increased absorbance due to the recombination of cytochrome a_3^{2+} and O_2 at 444 nm. The kinetics of the copper component are recorded at 830–940 nm in trace (F).

With the solubilized oxidase (Fig. 6a), the formation of oxycytochrome oxidase is clearly resolved at 591 nm and reaches a plateau as the disappearance of

Fig. 6. Comparison of the kinetics of the reaction with O_2 of solubilized (a) and membrane-bound (b) oxidase

(a) $10 \mu\text{M}$ -Solubilized oxidase, 5 mM-ascorbate and 5 μM -tetramethylphenylenediamine; (b) ox heart mitochondria (5 mg of protein/ml), 5 mM-succinate. In both (a) and (b) conditions included: 200 mM-mannitol, 5 mM-Tris/sulphate, pH 7.4, 1.2 mM- CO , 1.76 mM- O_2 ; temperature, -97°C ; optical path, 2 mm. Reference number 2011-5,6^v.

the reduced oxidase at 444 nm proceeds exponentially to a steady state. The 830–940 nm trace shows no discontinuity on photolysis, and only a very slow progress, which continues as the chart comes to an end after approx. 10 min.

The companion recording (Fig. 6b) is obtained with ox heart mitochondria (approx. 5 mg of protein/ml), in this case reduced with 5 mM-succinate, saturated with CO, cooled to -20°C , and mixed with air-saturated reaction medium, as in the experiment of Fig. 6(a). The rate of formation of compound A at 591 nm is about threefold faster than the reaction at 444 nm, as judged from the initial slope and half-time. A second distinctive feature is that compound A does not attain its maximum concentration, but rather reaches a steady state and then decays, owing to a more rapid conversion into compound B. The absorbance change at 830–940 nm is, however, similar; there is no fast reaction on photolysis, and the trace continues to progress to completion as the 10 min of the recording ends.

In summary, the principal differences between the solubilized and the membrane-bound oxidases are a threefold more rapid reaction with O_2 , and a more

rapid electron donation to reduce O_2 , in the membrane-bound oxidase.

Polyphasic-reaction kinetics

If the temperature is raised high enough for the conversion of compound A into compound B to approach completion, multiple phases of the reaction are recorded, as shown in Fig. 7 at -30°C . As above, the initial fast portion contains components assignable to compounds A (200 ms/division) and B (1 s/division). A slow phase of the kinetics at 605 nm follows, which is at present assigned to cytochrome *a* oxidation. However, the log plot (Fig. 7c) indicates two readily resolvable reactions, one with a rate constant of 2.5 s^{-1} with an amplitude of 20–25% of the total, and the other with a rate constant of 0.2 s^{-1} with an amplitude of 35–40% of the total. In a separate Arrhenius plot (not shown) the temperature-dependence of these two phases gives an energy of activation of 88 kJ/mol in the range from -50°C to -20°C for the faster phase, and 142 kJ/mol in the range from -40°C to -20°C for the slower phase. The slower phase may be due to heterogeneity in the solubilized oxidase, since the effects are not observed in the membrane-bound preparation.

Fig. 7. Illustration of biphasicity in the kinetics of formation of compound B recorded on two time scales (a and b) and analysed in a semi-logarithmic plot (c)

Conditions included: $40\text{ }\mu\text{M}$ -solubilized cytochrome oxidase, $2\text{ }\mu\text{M}$ -cytochrome *c*, 10 mM-ascorbate, 20% ethylene glycol, 10 mM-phosphate buffer, pH 7.4, 1.2 mM-CO added at 20°C , $200\text{ }\mu\text{M}$ - O_2 added at -25°C . Temperature was -30°C . LDL, Liquid dye laser. Wavelengths are 605–630 nm. Reference number 1505.

Fig. 8. Absorption spectra of the formation of compound B at low mid-potentials (+93 mV in spectrum A, +190 mV in spectrum B) and of compound C at +325 mV in spectrum C, all at -88°C

Traces (A) – (B) and (A) – (C) are also provided. Absorbance decreases are in an upward direction for spectra (A), (B) and (C), whereas the convention used in subtraction gives an absorbance increase as an upward deflection in the spectra (A) – (B) and (A) – (C). The potentials are referred to the calomel electrode and are indicated in the diagram. Conditions included: $40\text{ }\mu\text{M}$ -solubilized cytochrome oxidase, $30\text{ }\mu\text{M}$ -tetramethylphenylenediamine. Dithionite and ferricyanide were added as necessary to give the measured potentials. Reaction mixtures also included 0.2 M -mannitol, 30 mM -phosphate buffer, pH 7.0, and 30% ethylene glycol. Reference number 1833–1, 2, 4^v.

Compound C

In studies of the membrane-bound oxidase, treatment of the components of the respiratory chain with ferricyanide resulted in the formation of a new species of intermediate compound that is preceded by compound A (A_2 of Chance *et al.*, 1975a; Chance & Leigh, 1977). The same reaction has been followed in the solubilized oxidase, by using both poisoning in an electrometric titration and ferricyanide alone (see the

Materials and Methods section). Samples are transferred from the CO-saturated electrometric titrator, oxygenated at -20°C , and stored at -28°C . They are flash-photolysed in the slit-beam split-photolysis spectrophotometer at -88°C so that the spectra of Fig. 8 represent the difference between the flash-photolysed portion of the sample tube and that containing the unaltered CO compound. The absorbance decrease due to photolysis at approx. 587 nm is indicated as an upward deflection, and the absorbance increase at 608 nm due to the formation of compound C is indicated as a downward deflection in the upper set of traces.

In trace (A) of Fig. 8, the potential is +93 mV and the electron carriers of the oxidase preparation are reduced. The formation of compound B is recorded and its difference spectrum is plotted with reference to the CO compound. The most striking features of the difference spectrum of trace (A) are the decrease in absorbance at 585 nm and the increase in the absorbance around 620 nm due to the photolysis of the CO compound. The absorbance at 612 nm appears to be due to the formation of compound B.

In trace (B) the indicated potential has been increased to +190 mV and a significant oxidation of haem *a* and its associated copper component has occurred. The difference spectrum for the flash-photolysis product measured with respect to the CO compound (trace B) shows increased absorbance at $600\text{--}620\text{ nm}$, as also indicated by the difference spectrum for the two states (trace B–A), where increased absorbance is upwards.

In trace (C), the indicated potential is +325 mV and haem *a* and its associated copper are oxidized, whereas haem a_3 and its associated ('invisible') copper remain reduced (Lindsay & Wilson, 1974; Chance & Leigh, 1977). The difference spectrum of trace (A) – (C) clearly shows the 609 nm peak characteristic of compound C. The apparent mid-potential for the conversion of compound B into compound C is approx. 220 mV .

Near-i.r. difference spectra of compounds A, B and C

The absorption-difference spectra of compounds B and C with respect to the CO compound of cytochrome a_3 are shown in Fig. 9. The biochemical preparations are similar to those of previous experiments, with 5 mM -ascorbate and $5\text{ }\mu\text{M}$ -tetramethylphenylenediamine used as reductant. The procedure for binding with CO, cooling to -20°C , oxygenating, further cooling, and freeze-trapping with equilibration at -80°C is also similar to that described above. The poise in Fig. 9(a) is about 150 mV , and over 260 mV in Fig. 9(b).

The i.r.-absorption difference spectra show increased absorbance for both compound B (Fig. 9a) and compound C (Fig. 9b). These spectra differ significantly from those observed by Griffiths & Wharton

Fig. 9. Absorption difference spectra of compounds B (a) and C (b) in the near-i.r. region

In both spectra, conditions included $4\mu\text{M}$ -cytochrome oxidase, 30% ethylene glycol, 0.12M-mannitol, 3 mM-Tris/sulphate, 20mM-Mops (4-morpholinepropanesulphonic acid) buffer, pH 7.0, 1.2mM-CO and 2mM- O_2 . In (a) the system was poised at a low potential ($\sim 150\text{ mV}$), and in (b) at a high potential ($\geq 260\text{ mV}$). Reference number 1898^v.

(1961). The fully oxidized species gives an absorption difference spectrum at 830nm, and the blue shift is approx. 80nm for compound C and approx. 30nm for compound B. If the temperature is raised, however, the absorption difference spectrum of compound B shifts from 790nm to near 830nm for the fully oxidized state. However, there is no evidence that the difference spectrum of compound C shifts to 830nm with increased temperature or that it is a component of the 830nm band. Also the 830nm band is present after ferricyanide treatment, and the results here show no disappearance of absorbance at 830nm in the formation of compound C (Chance & Leigh, 1977).

Discussion

Although the solubilized oxidase has the disadvantages of heterogeneity and unknown electron donors, and lacks both energy coupling and physiological controls on electron transport, it can be used at higher concentrations and with less difficulty caused by spectroscopic interference from either fixed or variable-time components than can the membrane-bound oxidase. Although these latter difficulties are largely avoided at low temperatures, where only a few components of the oxidase are capable of rapid reaction, it is important to determine whether the various types of O_2 compounds identified in the membrane-bound system exist in the solubilized preparation, and whether the kinetics and equilibria of their reactions are altered by the solubilization procedure.

Comparison of the membrane-bound and solubilized oxidases

The data of the present paper afford a more incisive comparison of the activity of the solubilized and membrane-bound oxidases than has been possible in previous work. Here the activities of the two preparations have been tested under identical experimental conditions with identical read-outs of the reaction kinetics. Two distinctive differences are observed, namely a threefold more rapid reaction of the membrane-bound oxidase with O_2 and a greater tendency to form compound B. This may be compared with the earlier data of Chance (1965) and Chance *et al.* (1964), where the second-order velocity constant for the disappearance of the reduced cytochrome a_3 in the membrane-bound oxidase was fourfold smaller than the corresponding value for the solubilized oxidase as measured by Gibson & Greenwood (1965).

It is apparent that the rate-limiting steps may differ vastly. In our experiments at -90°C , the formation of compound A is involved, as is its transformation into compound B. But at higher temperatures the accessibility of O_2 to the active site might be one of the rate-limiting factors, although the reaction velocity constant is not diffusion-limited in a formal sense. Although the discrepancy may be due to experimental error in the above-mentioned works, particularly the inability to compare the two systems under identical conditions as has been possible in the present paper, there nevertheless remains the possibility that the

active site is more 'open' in the solubilized preparation than in the membrane-bound oxidase.

Relation between room- and low-temperature results

The consistent result of kinetic studies with both the membrane-bound and the solubilized oxidase is the complete absence of spectroscopic evidence for a significant concentration of functional intermediate compounds (Gibson *et al.*, 1965) (see the introduction). At -28°C , absorbance changes near 430 nm recorded at 2–5 ms suggest an intermediate compound at low concentration (Erecinska & Chance, 1972), and these and other data suggest near-maximal concentration at lower temperatures (Chance *et al.*, 1975*a,b*). The apparent energies of activation are larger for compound B formation than for compound A formation and thus compound A is less likely to be found at higher temperatures. More recent data show that electron donation from cytochrome *a* becomes activated above -40°C (Chance *et al.*, 1977), and thus the steady-state concentration of compound B may be greatly decreased above -30°C and especially at room temperatures.

Since different electron donors appear to become functional as the temperature is increased, it is not possible to predict precisely which ones, if any, exist at significant concentrations at room temperature. Obviously, the possibility that such intermediates would not exist at room temperature at significant concentrations would testify to the 'success' of cytochrome oxidase as a very efficient 'oxygen reductase' of very high O_2 affinity (Chance, 1965) and of very great rapidity of function.

Compound A

Compound A seems to have qualitatively similar and quantitatively different properties in the two preparations. In common with the two preparations are the general characteristics of a highly dissociated light-insensitive product formed by the photolysis of the CO compound in the presence of various O_2 concentrations. The molecular absorption coefficient of compound A is similar to that of the CO compound, but is difficult to determine exactly, owing to the possibility that incomplete occupancy has been obtained in the formation of compound A. Assuming that saturation has been obtained, the molecular absorption coefficient at the α -band for compound A exceeds that for the CO compound by as much as 25%. In the region of the Soret band, the situation may be reversed. But at present, the data are sufficiently imprecise (on account of light-scattering correction) to give the exact values of the two molecular absorption coefficients.

Compound B

Compound B appears to have characteristics

similar to those of the corresponding compound in the membrane-bound system. There are significant differences in the transformation from compound A into B, this being less rapid in the solubilized than in the membrane-bound preparation. An important feature of the reaction involving the formation of compound B in the solubilized oxidase is the stoichiometric inferiority, or even the complete absence, of cytochrome *c*. A further complication in the solubilized oxidase is afforded by the slow reactions, which appear to be due to components of either haem *a* or haem a_3 that are oxidized much more slowly than are other portions of the oxidase. Two slow phases are observed, one roughly tenfold slower than the other. Since the slower phase is not observed in the reaction of the cytochrome *c*-supplemented membrane-bound oxidase with O_2 , it is considered to be due to some heterogeneity of this preparation. Alternatively, the slow phases may be due to the sluggish reaction of cytochrome *a* observed at low concentrations of cytochrome *c* (Chance *et al.*, 1977).

Compound C

Compound C forms distinctively in the solubilized oxidase as well as in the membrane-bound oxidase and requires a potential for half-maximal transformation from compound B into C of approximately that of cytochrome *a*, suggesting that the fundamental change involved in the formation of compound C is the oxidation of haem *a* and its associated copper, haem a_3 and its associated copper remaining reduced under such conditions owing to the liganding of haem a_3 to CO (Lindsay & Wilson, 1974; Chance & Leigh, 1977).

Characteristics of the i.r. absorption

The attribution of the near-i.r.-absorption bands of cytochrome oxidase to copper is now supported by a number of authors (Griffiths & Wharton, 1961; Chance, 1966; Aasa *et al.*, 1976; Hartzell & Beinert, 1976; Wever *et al.*, 1977). Of greatest interest in the formation of compounds B and C are the anomalous positions of their i.r.-absorption bands, 782 nm and 744 nm respectively, for the particular conditions of Fig. 9. The characteristics of these absorption difference spectra are such that it is unlikely that the shift to 782 nm from the usual 830 nm position of the copper band of cytochrome oxidase is due simply to a combination of varying amounts of two species absorbing at 744 nm and 830 nm. Instead, the data suggest that these are two different maxima for hitherto 'invisible' copper, representing absorption-difference 'signatures' under the two conditions in which haem *a* and its associated copper are reduced (compound B) or oxidized (compound C). Only in the fully oxidized oxidase is the absorption-difference

peak of copper at 830nm. The anomalous absorption band at 609nm has been attributed either to the 'unmasking' of the copper associated with haem a_3 as a 'blue' copper protein with prominent absorption bands at 609 and 740nm as in the copper protein stellacyanine or to a charge-transfer interaction between ferrous haem and cupric copper (Chance & Leigh, 1977).

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