Studies on Partially Reduced Mammalian Cytochrome Oxidase Reactions with Ferrocycytochrome *c*

By COLIN GREENWOOD and THOMAS BRITTAIN

School of Biological Sciences, University of East Anglia, Norwich NR4 7TJ, U.K.,

and MICHAEL WILSON

Department of Chemistry, University of Essex, Colchester CO4 3SQ, U.K.,

and MAURIZIO BRUNORI

Istituto di Chimica, Facoltà di Medicina e Chirurgia, Università di Roma, Roma, Italy

(Received 22 March 1976)

The kinetics of the electron-transfer process which occurs between ferrocycytochrome *c* and partially reduced mammalian cytochrome oxidase were studied by the rapid spectrophotometric techniques of stopped flow and temperature jump. Stopped-flow experiments showed initial very fast extinction changes at 605 nm and at 563 nm, indicating the simultaneous reduction of cytochrome *a* and oxidation of ferrocycytochrome *c*. During this 'burst' phase, say the first 50 ms after mixing, it was invariably found that more cytochrome *c* had been oxidized than cytochrome *a* had been reduced. This discrepancy in electron equivalents may be accounted for by the rapid reduction of another redox site in the enzyme, possibly that associated with the extinction changes observed at 830 nm. During the incubation period in which the partially reduced oxidase was prepared, the rate of reduction of cytochrome *a* by ferrocycytochrome *c*, at constant reactant concentrations, decreased with time. Temperature-jump experiments showed the presence of two relaxation processes. The faster of the two phases was assigned to the electron-transfer reaction between cytochrome *c* and cytochrome *a*. A study of the concentration-dependence of the reciprocal relaxation time for this phase yielded a rate constant of $9 \times 10^6 \text{ M}^{-1} \cdot \text{s}^{-1}$ for the electron transfer from cytochrome *c* to cytochrome *a*, and a value of $8.5 \times 10^6 \text{ M}^{-1} \cdot \text{s}^{-1}$ for the reverse reaction. The equilibrium constant for the electron-transfer reaction is therefore close to unity. The slower phase has been interpreted as signalling the transfer of electrons between cytochrome *a* and another redox site within the oxidase molecule.

Cytochrome oxidase is an enzyme containing 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 by spectroscopy (Beinert *et al.*, 1962). Although there is now a fairly general consensus about the relative contributions of cytochromes *a* and *a*₃ at the characteristic wavelengths (Yonetani, 1960; Nicholls & Peterson, 1974), the problem of assigning a particular kinetic event to a specific chromophore is very complex. One method of minimizing this type of complication is to prepare an enzyme species in which one or more of the contributing chromophores has been fixed in a particular redox state and therefore can probably be neglected. Following this idea, derivatives of cytochrome oxidase partially reduced and in combination with CO have been prepared and char-

acterized in their ligand-binding and redox properties (Greenwood *et al.*, 1974; Lindsay *et al.*, 1975; Chance *et al.*, 1975).

Studies on the reduction of fully oxidized oxidase with ferrocycytochrome *c* under anaerobic conditions have led to somewhat controversial results. Gibson *et al.* (1965) found evidence that the reduction of cytochrome *a* was associated with the reduction of a second site which, on the basis of previous investigations (Beinert & Palmer, 1964), they suggested might be e.p.r.*-detectable copper. The work of Wilson *et al.* (1975) confirmed the earlier findings of Gibson *et al.* (1965), and on the basis of kinetic observations at 830 nm (a wavelength at which 'detectable' copper makes a contribution to the absorption spectrum of the enzyme) suggested that cytochrome *a* and visible copper are both involved in the early stages of the reduction of fully oxidized

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

oxidase. Andrsson *et al.* (1972) claim to have evidence that, under strictly anaerobic conditions, only cytochrome *a* is reduced in the initial phase and that in some way the presence of oxygen facilitates the reduction of the second site. In this respect the use of the mixed-valence (or partially reduced) species, which can be prepared in a number of ways (Greenwood *et al.*, 1974), is very relevant. The method of preparation chosen in these studies (autoreduction in the presence of CO) requires that the enzyme be strictly anaerobic, and recent potentiometric work by Lindsay *et al.* (1975) has indicated that in this species both cytochrome *a*₃ and the invisible copper atom are reduced.

The present paper seeks to investigate further the reaction between cytochrome *c* oxidase and its physiological reductant ferrocytochrome *c*. In particular, it is an attempt (i) to determine the rate constants for electron transfer between cytochrome *a* and cytochrome *c*, by making use of the temperature-jump-relaxation method to resolve the initial stages of the process, which is known to be very fast, and (ii) to determine whether one or more than one electrons enter the partially reduced oxidase molecule in the first rapid phase of its reduction by ferrocytochrome *c* and thus whether the reduction of cytochrome *a* is dynamically linked to another redox site, possibly 'visible' copper present, in the oxidase molecule.

Materials and Methods

Cytochrome oxidase (EC 1.9.3.1; ferrocytochrome *c*-oxygen oxidoreductase) was prepared from ox heart by a modification of the method of Yonetani (1960), in which 10 μ M-EDTA was included as a chelating agent in the last fractionation steps to ensure removal of adventitiously bound copper, which is inactive in electron transfer (Lemberg, 1968). The concentration of cytochrome oxidase samples (in terms of haem *a*) was determined by using $\epsilon = 21000 \text{ M}^{-1} \cdot \text{cm}^{-1}$ at 605 nm for the fully reduced enzyme (Yonetani, 1960). All preparations used had a ratio of ϵ_{445} (reduced)/ ϵ_{424} (reduced) of 2.3 or better (Gibson *et al.*, 1965).

The mixed-valence derivative of cytochrome oxidase was prepared by incubating an O₂-free solution of oxidized cytochrome oxidase under an atmosphere of CO at room temperature (21°C) for approx. 24 h (Greenwood *et al.*, 1974).

Cytochrome *c* [Sigma type III; Sigma (London) Chemical Co., Kingston-upon-Thames, Surrey, U.K.] was used without further purification. The reduced protein was prepared by passing the dithionite-reduced material through a column (20 cm $\times$ 1.5 cm) of Sephadex G-25 to remove excess of dithionite and its oxidation products. The reduced cytochrome *c* prepared by this method contained less than 5% of the oxidized protein on leaving the column, and was

subsequently stored under O₂-free N₂ to prevent autoxidation. The concentration of cytochrome *c* samples was calculated using $\epsilon = 27600 \text{ M}^{-1} \cdot \text{cm}^{-1}$ at 550 nm for the reduced protein (Schejter *et al.*, 1963). The buffer used throughout was 0.1 M-potassium phosphate, pH 7.4 plus 1% Tween 80 (Sigma Chemical Co.).

Stopped-flow experiments were performed by using an apparatus identical with that described by Gibson & Milnes (1964) equipped with either 2 cm- or 2 mm-light-path cells, having dead-times of 3 ms and 1 ms respectively. For some experiments a modified stopped-flow apparatus, built according to the design of Dr. S. Ainsworth (personal communication) and having a dead time of 1 ms for a 2 cm light-path, was used.

Temperature-jump experiments were carried out by using an instrument built by Messanlagen Gesellschaft (Gttingen, Germany). The conventional temperature-jump cell of 7 ml capacity and 1 cm light-path was modified for anaerobic use by providing it with a short connecting tube from the main compartment to the upper lid vent hole. This was fitted with a self-sealing vaccine cap, which permitted the cell to be filled under an argon atmosphere before evacuation of the remaining gas phase and equilibration with 101 kPa (1 atm) of CO.

A discharge of 30 kV, corresponding to a temperature increase of 4.5–5.0°C, was generally used. Spectrophotometry was carried out in either a Cary 118 or a Cary 14 instrument modified to take the temperature-jump cell, thus allowing direct observation of the equilibrium state of the mixture.

CO and O₂-free N₂ were obtained from British Oxygen Co. (London S.W.19, U.K.) or from Societ Italiana Ossigeno (Roma, Italy). They were dispensed from the cylinder and stored in glass vessels over an alkaline solution of dithionite-reduced anthraquinonesulphonate before use (Greenwood *et al.*, 1974).

As described in a previous paper (Wilson *et al.*, 1975) the monitoring wavelengths were chosen so as to permit, as near as possible, the separation of the spectral contributions of the various chromophores and thus minimize the need for subsequent correction. In view of the high concentration of ferrocytochrome *c* used in these stopped-flow experiments, the wavelength 563 nm was chosen and a difference in extinction coefficients ($\Delta\epsilon$) of $3600 \text{ M}^{-1} \cdot \text{cm}^{-1}$ obtained from static difference spectroscopy, was used. The wavelength chosen to monitor cytochrome *a* change was 605 nm and a difference in extinction coefficients ($\Delta\epsilon$) of $18000 \text{ M}^{-1} \cdot \text{s}^{-1}$ derived from Fig. 10 of Greenwood *et al.* (1974) was used. The changes at 830 nm were calculated by using the extinction coefficient ($\Delta\epsilon$) of $2200 \text{ M}^{-1} \cdot \text{cm}^{-1}$, after correcting for the contribution of cytochrome *c* at this wavelength. Alternatively the wavelength of

740 nm, which is isosbestic for cytochrome *c*, was selected and a $\Delta\epsilon$ of $1000\text{M}^{-1}\cdot\text{cm}^{-1}$ was used. Both the extinction coefficients in the near-i.r. region have been obtained from spectra published by Greenwood *et al.* (1974).

Results

Temperature-jump studies

Fig. 1 (curve *a*) shows the spectrum of the mixed-valence complex with CO, as prepared in the anaerobic temperature-jump cell, over the range 500–650 nm. The anaerobic addition of ferrocytochrome *c* leads to the equilibrium situation shown by the spectrum *b* (in Fig. 1), which clearly shows that a large amount of reduced cytochrome *c* is present and some cytochrome *a* has been reduced.

When this equilibrium mixture was subjected to rapid temperature perturbation, a fast relaxation process was observed. This had the spectral characteristics of the electron transfer between cytochrome *c* and cytochrome *a*, and could therefore be followed conveniently at 550 and 605 nm. The absorbance changes for a 5°C temperature increase were very small, typically of the order of $0.005 E_{605}$ unit or less, and indicated the oxidation of cytochrome *a* and simultaneous reduction of cytochrome *c*. This suggests that the ΔH° associated with the electron-transfer reaction between cytochromes *c* and *a*, as written in eqn. (1), is small and negative.

Fig. 2(a) is a typical temperature-jump record obtained at 547 nm. The amplitude of the chemical relaxation, measured at a fixed wavelength and for a constant temperature rise, changed with the extent of cytochrome *a* reduction, being smallest at very low or very high extents of reduction.

Logarithmic plots of the temperature-jump data (Fig. 2b) indicated that two processes were present, a faster relaxation with a half-time of the order of 1 ms representing approx. 80% of the total change, and a slower process with a half-time approx. 10 times longer. We suggest that these two relaxations arise from the coupling between the intermolecular electron-transfer reaction, i.e. between cytochromes *c* and *a*, and an intramolecular electron transfer within the oxidase molecule, i.e. between cytochrome *a* and another redox site. This model may be written as follows:

where *a* and *c* represent cytochromes *a* and *c* respectively, the subscripts R and O denote reduced and oxidized components respectively, and *a'*_O represents the oxidase molecule in which cytochrome *a* has

become oxidized by passing an electron on to another redox component.

This model, which is the same as that proposed by Wilson *et al.* (1975) to explain the initial electron-transfer reaction between cytochrome *c* and its oxidase, leads to the following expression for the faster reciprocal relaxation time:

$$\tau_f = k_{+1}([\bar{a}_O] + [\bar{c}_R]) + k_{-1}([\bar{a}_R] + [\bar{c}_O]) + k_{+2} + k_{-2} \quad (3)$$

where the barred quantities denote equilibrium concentrations. Eqn. (3) may be transformed:

$$\frac{1}{\tau_f} \left(\frac{[\bar{a}_O] + [\bar{c}_R]}{[\bar{a}_O] + [\bar{c}_R]} \right) = k_{+1} + k_{-1} \left(\frac{[\bar{a}_R] + [\bar{c}_O]}{[\bar{a}_O] + [\bar{c}_R]} \right) + \left(\frac{k_{+2} + k_{-2}}{[\bar{a}_O] + [\bar{c}_R]} \right) \quad (4)$$

If we assume that $(k_{+2} + k_{-2})/([\bar{a}_O] + [\bar{c}_R])$ is small compared with the other terms in eqn. (4), then a plot of $(1/\tau_f)/([\bar{a}_O] + [\bar{c}_R])$ versus $([\bar{a}_R] + [\bar{c}_O])/([\bar{a}_O] + [\bar{c}_R])$ should yield a straight line with intercept k_{+1} and slope k_{-1} .

Fig. 3 shows such a plot for the faster relaxation process, and there is some doubt as to whether or not

Fig. 1. Spectrum of the mixed-valence CO complex of cytochrome oxidase in the absence and presence of added ferrocytochrome *c*

The solid line (*a*) shows the spectrum of the CO complex of mixed-valence cytochrome oxidase ($40\text{ }\mu\text{M}$ -total haem, i.e. $20\text{ }\mu\text{M}$ -cytochrome *a*). The broken line (*b*) shows the spectrum after the addition of ferrocytochrome *c* (total cytochrome *c* concentration $26\text{ }\mu\text{M}$). From this spectrum the concentrations of each of the components were estimated to be: $[\bar{a}_O] = 15\text{ }\mu\text{M}$; $[\bar{a}_R] = 5\text{ }\mu\text{M}$; $[\bar{c}_R] = 8.5\text{ }\mu\text{M}$; $[\bar{c}_O] = 17.5\text{ }\mu\text{M}$. These spectra were read in the temperature-jump cell (1 cm path-length), before temperature perturbation. Other conditions; 0.1 M -potassium phosphate buffer, pH 7.4, containing 1% Tween 80; temperature 24°C .

Fig. 2. Typical progress curves observed in temperature-jump experiments

(a) Relaxation processes after a 5°C temperature jump. The lower time-scale refers to trace (i) and the upper scale to trace (ii). The reaction mixture contained $41\ \mu\text{M}$ -oxidase (total haem) as the CO complex of the mixed-valence species and $37.5\ \mu\text{M}$ -cytochrome *c* in $0.1\ \text{M}$ -potassium phosphate buffer, pH 7.4, containing 1% Tween 80. The monitoring wavelength was $547\ \text{nm}$ and the initial temperature was 20°C . (b) A logarithmic plot of the data presented in (a); $\circ$ and Δ , data from traces (i) and (ii) respectively.

Fig. 3. Plot of the temperature-jump data for the faster phase seen in temperature-jump studies according to eqn. (4) of the text.

The open and filled symbols refer to different experiments done under the same conditions.

the experimental points are scattered about a straight line. Such uncertainty is to be expected bearing in mind the technical difficulty of the experiments and the errors involved in measuring the equilibrium

concentrations of the reactants (see Fig. 1) and the relaxation rates. Nevertheless, a clear trend is discernible and extrapolation of the straight line fitted by the least-squares method back to the ordinate yields a value of k_{+1} of $9 \times 10^6\ \text{M}^{-1}\cdot\text{s}^{-1}$. This value is close to those reported in the literature for the electron-transfer reaction from cytochrome *c* to cytochrome *a* (Gibson *et al.*, 1965; Van Buuren *et al.*, 1974). Assuming the final term of eqn. (4) to be negligible, we obtain from the slope of the line in Fig. 3 a value close to $8.5 \times 10^6\ \text{M}^{-1}\cdot\text{s}^{-1}$ for k_{-1} , the electron-transfer rate constant from cytochrome *a* to *c*. The equilibrium constant for the reaction in eqn. (1) is therefore close to unity. Some sets of experiments, however, gave lower values for the rate constants, e.g. k_{+1} approx. $2 \times 10^6\ \text{M}^{-1}\cdot\text{s}^{-1}$, and this may be attributed to slight differences in the salt content of the cytochrome oxidase preparations, as it is known that the electron-transfer rates are very sensitive to ionic strength (Van Buuren *et al.*, 1974). The value of the equilibrium constant was found to lie, for all sets of values, between 0.5 and 1.

The concentration-dependence of the reciprocal relaxation time, $1/\tau_s$, from the slower process could not be analysed in the same detail owing to the small amplitude of the slow process and also to the complexity of the expression for $1/\tau_s$ given by the model in eqn. (1) and (2). However, it was found that $1/\tau_s$ increased with total reactant concentration, rising from approx. $40\ \text{s}^{-1}$ to approx. $100\ \text{s}^{-1}$ on increasing

the cytochrome *c* concentration from 37 to 140 μM (while maintaining the oxidase concentration at 40 μM). Eqns. (1) and (2) predict that the slower relaxation time should exhibit a dependence on reactant concentration, and one would expect $1/\tau_s$ to approach the rate limit ($k_{+2} + k_{-2}$) at high reactant concentrations.

Stopped-flow studies

Fig. 4 shows a set of stopped-flow records at three characteristic wavelengths obtained on mixing ferrocyanochrome *c* with the mixed-valence CO complex of cytochrome oxidase under strictly anaerobic conditions. The rapid oxidation of ferrocyanochrome *c* in a 'burst phase' is accompanied by the formation of the 605 nm band and by the bleaching of the 830 nm band. This behaviour is similar to that observed in the ferrocyanochrome *c* reduction of the fully oxidized oxidase (Wilson *et al.*, 1975).

A set of data obtained on mixing the mixed-valence CO complex with three concentrations of ferrocyanochrome *c* at 20°C is presented in Table 1(a), and some data gathered at 10°C are given in Table 1(b). As previously reported (e.g. Wilson *et al.*, 1975), a great deal of the expected absorbance change is lost in the dead-time of the apparatus. One important point, which is immediately apparent from these data, is that we always see more ferrocyanochrome *c* oxidized than cytochrome *a* reduced (as determined from the changes at 563 nm and 605 nm), the difference being approximately accounted for by the change observed at either 740 or 830 nm.

Table 2 shows the results of stopped-flow kinetic observations made at a constant concentration of ferrocyanochrome *c* during the time of formation of the mixed-valence CO complex. The most striking point to emerge from these results is that the apparent rate of electron transfer from cytochrome *c* to cytochrome *a* falls by a factor of over two during formation of the mixed-valence species. In view of the close similarities between the mixed-valence CO complex and fully oxidized oxidase in their reactions with ferrocyanochrome *c*, we are reasonably confident that cytochrome *a* is the initial electron acceptor in this case. The curve in Fig. 5, which represents the kinetic difference spectrum produced on mixing the mixed-valence CO complex with Cr^{2+} ions, is clearly that of cytochrome *a*, confirming the presence of oxidized cytochrome *a* in the mixed-valence species.

Fig. 4. Progress curves obtained in stopped-flow experiments

The progress curves of the reactions observed in stopped-flow experiments are shown at 830 nm (a), 563 nm (b) and 605 nm (c). The reaction shown in (a) was between 37 μM -enzyme and 75 μM -ferrocyanochrome *c*. (b) and (c) show the reaction between 3.7 μM -enzyme and 25 μM -ferrocyanochrome *c*. The vertical scale represents a change in extinction of 0.005 (a and b) and 0.01 (c). The horizontal scale represents 10 ms/cm (a), 20 ms/cm (lower trace, b and c); and 500 ms/cm (upper trace, b and c). The reactions were carried out at 10°C in 0.1 M-potassium phosphate buffer, pH 7.4, +1% Tween 80 in a 2 mm path-length cell.

Table 1. *Effects of ferrocytochrome c concentration on the reduction of mixed-valence cytochrome oxidase*

The effect of ferrocytochrome *c* concentration on the reaction at 20°C (a) and 10°C (b) of 75 μM -cytochrome *c* oxidase is shown. The extinction change (ΔE) at various wavelengths is occurring in the burst-phase shown together with the number of $\mu\text{equivalents}$ of electrons exchanged as represented by this change, calculated from the extinction coefficients described in the text. The experiments were performed in 0.1 M-potassium phosphate buffer, pH 7.4, +1% Tween 80 in the 2 cm path-length cell of the stopped flow apparatus.

Concn. of ferrocytochrome <i>c</i> (μM)	Changes at 563 nm		Changes at 605 nm		Changes at 830 nm	
	ΔE	$\mu\text{equivalents}$	ΔE	$\mu\text{equivalents}$	ΔE	$\mu\text{equivalents}$
(a)						
36	0.036	10	0.14	7.75	0.0077	3.5
59	0.05	14	0.155	8.5	0.0082	4.2
118	0.063	17.5	0.175	9.75	0.0077	3.5
(b)						
22	0.035	9.8	0.115	6.4	0.008	3.6
68	0.085	23	0.20	11.1	0.013	6
210	0.090	25	0.20	11.1	0.011	5

Table 2. *Effect of the length of incubation on the reduction of cytochrome oxidase*

The effect is shown of the length of incubation of 50 μM -cytochrome *c* oxidase with CO on the apparent pseudo-first-order rate constants observed at 563 and 605 nm together with the apparent ratio of ferrocytochrome *c* oxidized to cytochrome *a* reduced (c^{II}/a). The cytochrome *c* oxidase was made to react with 300 μM -ferrocytochrome *c* at 10°C in the 2 mm-path-length cell of the stopped-flow apparatus in 0.1 M-potassium phosphate buffer, pH 7.4, +1% Tween 80.

Time (h)	c^{II}/a	$k(\text{s}^{-1})$ 563 nm	$k(\text{s}^{-1})$ 605 nm	% of mixed-valence CO complex formed
0.75	1.9	200	290	43
3.0	1.7	183	190	63
6.0	1.6	164	136	83
26.0	2.0	111	111	100

Discussion

The mixed-valence CO complex of cytochrome *c* oxidase offers not only a simplified system for investigating the complex reactions of this enzyme, but also a means of preparing and handling the protein in the strictly anaerobic conditions which are required for temperature-jump studies. Greenwood *et al.* (1974) argued that the species formed after incubation of cytochrome oxidase with CO contained only one site, namely cytochrome a_3 , in the reduced state: on this basis the mixed-valence species should be able to accept three equivalents of electrons in reductive experiments. Work by Lindsay *et al.* (1975) convincingly suggests that binding of CO to the mixed-valence species only occurs after reduction of both invisible copper and cytochrome a_3 , and therefore only cytochrome *a* and visible copper in fact remain oxidized in mixed-valence oxidase.

Fig. 5. *Kinetic difference spectrum*

The kinetic difference spectrum between the mixed-valence complex and the mixed-valence complex reduced by Cr^{2+} ions is shown; 10 μM -protein was reduced by 6.25 mM- Cr^{2+} ions in the 2 mm-path-length cell (Soret region) and 2 cm-path-length cell (α region) of the stopped-flow apparatus at 20°C. The buffer was 0.1 M-sodium cacodylate, pH 7.4.

By performing stopped-flow experiments at 563 nm, 605 nm and in the near-i.r. region at 740 or 830 nm [a wavelength assigned, in part at least, to visible copper in the enzyme (Griffiths & Wharton, 1961; Wharton & Tzagoloff, 1964)], it is possible to monitor the redox changes of cytochromes *c* and *a*, as well as visible copper. The reaction records in Fig. 4 clearly show that the rapid changes at these wavelengths bear

a close temporal relationship to one another. The data in Tables 1(a) and 1(b) show (a) that more equivalents of ferrocyanochrome *c* are oxidized than ferricytochrome *a* are reduced, and (b) that the difference in equivalents, particularly at low ferrocyanochrome *c* concentration, may be accounted for by considering the changes at 830 nm. However, this explanation becomes less tenable as the cytochrome *c* concentration increases. These conclusions are consistent with the findings of Gibson *et al.* (1965), and more recent observations of Wilson *et al.* (1975) and Van Buuren *et al.* (1974) on the fully oxidized oxidase. Andréasson *et al.* (1972) and Andréasson (1975), however, reported that under strict anaerobiosis only one equivalent of ferrocyanochrome *c* is oxidized for each cytochrome *a* equivalent reduced starting from the fully oxidized oxidase. They suggest that the presence of oxygen radically alters the kinetics of internal electron transfer within the oxidase molecule and thus induces a change in the stoichiometry of reduction from one equivalent of cytochrome *c* oxidized/equivalent of cytochrome *a* reduced to a value closer to two equivalents of cytochrome *c* oxidized/equivalent of cytochrome *a* reduced. Our results, as presented in Table 1, which indicate that more ferrocyanochrome *c* is oxidized than cytochrome *a* is reduced, suggest that even in the absence of O₂ the mixed-valence CO complex, which only forms after complete enzymic exhaustion of O₂, accepts more than one electron rapidly. It cannot be totally excluded, however, that CO substitutes for O₂ with regard to its postulated function (Andréasson *et al.*, 1972) of influencing the internal electron-transfer rates.

Working with the mixed-valence oxidase the slow processes observed with the fully oxidized oxidase and attributed to the reduction of other redox sites in the enzyme, e.g. cytochrome *a*₃ (Gibson *et al.*, 1965; Wilson *et al.*, 1975), were not observed. There appear to be close similarities between the initial reactions of ferrocyanochrome *c* with both fully oxidized and the mixed-valence CO complex. However, an experiment in which the rate of the electron-transfer reaction between cytochromes *c* and *a* was monitored during the time-course of the formation of the mixed-valence species showed a decrease in this rate. Table 2 shows that as the proportion of mixed-valence CO complex increased from 40% to completion, the rate of electron transfer from ferrocyanochrome *c* to the enzyme fell by a factor of about two. As cytochrome *a* is known to interact with cytochrome *a*₃ (Tiesjema *et al.*, 1973), a tentative explanation of this phenomenon might be that the state of cytochrome *a*₃ is influencing the reactivity of cytochrome *a*, in other words a reciprocal effect to that noted by Greenwood *et al.* (1974), where the redox state of cytochrome *a* modified the reactivity of reduced cytochrome *a*₃ for CO.

In view of the very high second-order rates found for the interaction of ferrocyanochrome *c* with cytochrome oxidase (Gibson *et al.*, 1965; Wilson *et al.*, 1975), the use of flow methods is strictly limited. Van Buuren *et al.* (1974) neatly avoided these constraints by using pulse radiolysis to generate ferrocyanochrome *c* in the presence of cytochrome oxidase and following the subsequent reactions. We have turned to the temperature-jump technique as an additional approach to this problem.

The results of rapidly raising the temperature of an equilibrium mixture of cytochrome *c* and mixed-valence cytochrome oxidase are given in Fig. 2, which shows the presence of two relaxation processes. The initial phase seen in temperature-jump experiments is concentration-dependent, and we identify it with the electron transfer between cytochrome *c* and cytochrome *a*. From Fig. 3, values have been derived for the forward and reverse rate constants, namely $k_{+1} = 9 \times 10^6 \text{ M}^{-1} \cdot \text{s}^{-1}$ and $k_{-1} = 8.5 \times 10^6 \text{ M}^{-1} \cdot \text{s}^{-1}$.

From a number of sets of experiments, the value of the equilibrium constant for the electron-transfer reaction between cytochrome *c* and cytochrome *a* was found to lie in the range 0.5–1.0. These results indicate that the partners differ in redox potential by between 20 and 0 mV (cytochrome *a* being more positive). This estimate is not inconsistent with the values reported for cytochrome *a*, which range from 220 mV (at pH 7.2; Lindsay *et al.*, 1975) to 280 mV (Andréasson, 1975), given the value of 255 mV for cytochrome *c*. The slower relaxation time cannot be unequivocally identified with a specific chemical event. However, it seems very likely that it reflects re-equilibration of the second process (described by eqn. 2), i.e. internal electron shuttle between cytochrome *a* and visible copper. The absolute values of the slower relaxation time (between 40 and 100 s⁻¹), and its concentration-dependence, are qualitatively in agreement with this hypothesis, which, if proved, may provide direct information on this intramolecular reaction step.

C. G. gratefully acknowledges grants from the Royal Society for the purchase of a Cary 118C spectrophotometer and Tektronix oscilloscope type 7514. T. B. thanks the Science Research Council for a Senior Research Associateship. C. G., M. W. and M. B. thank E.M.B.O. and the Royal Society/Academia Nazionale del Lincei for support. We thank Mr. Adrian Thompson for his skilled technical assistance in the preparation of the enzyme used in these experiments. This work was supported by S.R.C. Grant B/RG/8048.9.

References

- Andréasson, L. E. (1975) *Eur. J. Biochem.* **53**, 591–597
- Andréasson, L. E., Malmström, B. G., Strömberg, C. & Vänngård, T. (1972) *FEBS Lett.* **28**, 297–301
- Beinert, H. & Palmer, G. (1964) *J. Biol. Chem.* **239**, 1221–1227

- Beinert, H., Griffiths, D. E., Wharton, D. C. & Sands, R. H. (1962) *J. Biol. Chem.* **237**, 2337–2346
- Chance, B., Saronio, C. & Leigh, J. S. (1975) *J. Biol. Chem.* **250**, 9226–9237
- Gibson, Q. H. & Milnes, L. (1964) *Biochem. J.* **91**, 161–171
- Gibson, Q. H., Palmer, G. & Wharton, D. C. (1965) *J. Biol. Chem.* **240**, 915–920
- Greenwood, C., Wilson, M. T. & Brunori, M. (1974) *Biochem. J.* **137**, 205–215
- Griffiths, D. E. & Wharton, D. C. (1961) *J. Biol. Chem.* **236**, 1857–1862
- Keilin, D. & Hartree, E. F. (1939) *Proc. R. Soc. London Ser. B* **125**, 171–186
- Lemberg, M. R. (1968) *Physiol. Rev.* **49**, 48–121
- Lindsay, J. G., Owen, C. S. & Wilson, D. F. (1975) *Arch. Biochem. Biophys.* **169**, 492–505
- Muijsers, A. O., Tiesjema, R. H., Henderson, R. W. & Van Gelder, B. F. (1972) *Biochim. Biophys. Acta* **267**, 216–221
- Nicholls, P. & Peterson, L. C. (1974) *Biochim. Biophys. Acta* **357**, 462–467
- Schejter, A., George, P., Glauser, S. C. & Margoliash, E. (1963) *Biochim. Biophys. Acta* **73**, 641–643
- Tiesjema, R. H., Muijsers, A. O. & Van Gelder, B. F. (1973) *Biochim. Biophys. Acta* **305**, 19–28
- Van Buuren, K. J. H., Van Gelder, B. F., Wilting, J. & Braams, R. (1974) *Biochim. Biophys. Acta* **333**, 421–429
- Wharton, D. C. & Tzagoloff, A. (1964) *J. Biol. Chem.* **239**, 2036–2041
- Wilson, M. T., Greenwood, C., Brunori, M. & Antonini, E. (1975) *Biochem. J.* **147**, 145–153
- Yonetani, T. (1960) *J. Biol. Chem.* **235**, 845–852