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CO BINDING TO MITOCHONDRIAL MIXED VALENCE STATE CYTOCHROME OXIDASE AT LOW TEMPERATURES

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Summary

The kinetics and thermodynamics of the reaction of mixed valence state membrane-bound cytochrome oxidase with CO over the 178—203 K range has been studied by multichannel optical spectroscopy at three wavelength pairs (444—463 nm in the Soret region, and 590—630 and 608—630 nm in the α region) and analysed by non-linear optimization techniques. As in the case of the fully reduced membrane-bound cytochrome oxidase-CO reaction (Clore, G.M. and Chance, E.M. (1978) Biochem. J. 175, 709—725), the normalized progress curves at the three wavelength pairs are significantly different indicating, on the basis of Beer's law, the presence of a minimum of three optically distinct species. The only model that satisfies the triple statistical requirement of a standard deviation within the standard error of the data, a random distribution of residuals and good determination of the optimized parameters, is a two species sequential mechanism: flash photolysis of the mixed valence state cytochrome oxidase-CO complex (species II_{MC}) yields unliganded mixed valence state cytochrome oxidase (species E_M) and free CO which then recom-

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Supplementary data to this article are deposited with, and can obtained from, Elsevier/North-Holland Biomedical Press B.V., BBA Data Deposition, P.O. Box 1345, 1000BH Amsterdam, The Netherlands. Reference should be made to No. BBA/DD/125/47804/590 (1980) 34—49. The supplementary information includes: Proof that the analysis and interpretation of transient kinetic data acquired using the dual wavelength technique in terms of the number of intermediates, models and rate constants is independent of the choice of monitoring and reference wavelengths (providing these are different) and does not require the latter to be an isosbestic point for any of the species; correlation matrix relating the optimized rate constants at the reference temperature T_D (188 K) and zero-point activation energies for the mixed valence state membrane bound cytochrome oxidase-CO reaction.

Abbreviations: MCD, magnetic circular dichroism; $S.D._{ln}$, standard deviation of the natural logarithm of the optimized parameter.

bine to form species I_{MC}; species I_{MC} is then converted into species II_{MC}. All the thermodynamic parameters describing the model are calculated and compared to those obtained for the fully reduced membrane-bound cytochrome oxidase-CO reaction (Clore and Chance (1978) Biochem. J. 175, 709—725). Although there are some qualitative similarities in the kinetics and thermodynamics of the reactions of mixed valence state ($a_3^{2+}Cu_B^+ \cdot a^{3+}Cu_A^{2+}$) and fully reduced ($a_3^{2+}Cu_B^+ \cdot a^{2+}Cu_A^+$) cytochrome oxidase with CO, there are large and significant quantitative differences in zero-point activation energies and frequency factors; over the temperature range studied, the mixed valence state cytochrome oxidase-CO reaction is found to proceed at a significantly slower rate than the fully reduced cytochrome oxidase-CO reaction. These differences indicate that changing the valence states of cytochrome *a* and Cu_A has a significant effect on the CO binding properties of cytochrome a_3 and possibly Cu_B.

Introduction

The minimum functioning unit of mammalian cytochrome oxidase (EC 1.9.3.1) is thought to consist of two A-type haems differing only in the nature of their axial ligands, cytochromes *a* and a_3, and two copper atoms, Cu_A and Cu_B [1]. There has been considerable interest in the way in which changes in the properties of one or more of the metal centres affect the properties of the others. These interactions have been studied by redox titrations [2], EPR [3,4], MCD [5,6] and ligand binding reactions [7]. However, these experiments were essentially of a qualitative nature and their interpretation has been the object of considerable dispute (for recent reviews see Refs. 1, 8 and 9).

In a previous paper [10] we examined the mechanism of CO binding to fully reduced membrane-bound cytochrome oxidase over the 178—203 K range. The normalized progress curves obtained simultaneously at 444—463, 590—630 and 608—630 nm were found to be significantly different indicating, on the basis of Beer's law, the presence of a minimum of three optically distinct species. By analysing the data at the three wavelength pairs simultaneously at six temperatures, we found that the only model that statisfied the triple statistical requirement of a standard deviation within the standard error of the data, a random distribution of residuals and good determination of the optimized parameters [10—13], was a two species sequential mechanism stated as:

$$\underbrace{E + CO_p \underset{k_{-1}}{\overset{k_{+1}}{\rightleftharpoons}} I_C \underset{k_{-2}}{\overset{k_{+2}}{\rightleftharpoons}} II_C}_{h\nu \text{ (flash photolysis)}} \qquad (1)$$

where species E (fully reduced unliganded cytochrome oxidase) and CO are the species populated by photolysis of species II_C (the final fully reduced cytochrome oxidase-CO complex). (The subscript p is used to label the free CO molecules present in the 'pocket' of cytochrome oxidase containing the CO binding site.) In earlier work on fully reduced soluble cytochrome oxidase, Sharrock and Yonetani [14] also found that the recombination of CO involved an intermediate species. However, because they only monitored a single wavelength pair, assumed that species E and I_C were optically indistinguishable

and analysed their data at each temperature independently, they were not able to determine whether the species produced on photolysis of species II_C was E or I_C.

Greenwood and coworkers [7,15] compared the rates of CO binding at room temperature to fully reduced and mixed valence state cytochrome oxidase, and found that the apparent second-order rate constant was approx. 20% smaller in the latter ($6 \cdot 10^4$ $M^{-1} \cdot s^{-1}$ for the former and $5 \cdot 10^4$ $M^{-1} \cdot s^{-1}$ for the latter). At room temperature only a single exponential is seen under pseudo-first-order conditions so that for the model given by Eqn. 1 $k_{+2} >> k_{-1} + k_{+1} \cdot [CO]$ and k_{-2} is small. As a result, the maximal concentration of species I_C achieved during the reaction would be very small (less than 1% of the total oxidase concentration). Given that the apparent second order rate constants for CO recombination are large, it would then not be possible to distinguish the two steps given in Eqn. 1. It is therefore of considerable interest to compare the kinetics and thermodynamics of the recombination of CO with fully reduced and mixed valence state cytochrome oxidase in a temperature range over which the two steps can be easily separated.

A combination of optical spectroscopy in the visible and near-infrared regions, EPR, X-ray absorption edge spectroscopy and potentiometric studies [4,16—22] have shown that the addition of ferricyanide to the mitochondrial or soluble fully reduced cytochrome oxidase-CO complex results in the formation of a mixed valence state cytochrome oxidase-CO complex in which cytochrome a_3 and Cu_B are in the ferrous and cuprous states, respectively, and cytochrome a and Cu_A in the ferric and cupric states, respectively. The use of the mixed valence state species permits one to obtain further insight into the reaction of cytochrome oxidase with CO and the interactions of its four metal centres.

In the present paper, we have therefore investigated the kinetics and thermodynamics of CO recombination following photolysis of the mixed valence state membrane-bound cytochrome oxidase-CO complex ($Cu_A^{2+}a^{3+} \cdot Cu_B^{+}a_3^{2+} \cdot CO$) in intact mitochondria over the 178—203 K range. The values of the thermodynamic parameters governing the four transitions which describe the recombination of CO with fully reduced and mixed valence state cytochrome oxidase are compared, and the effect of altering the valence states of cytochrome a and Cu_A on the CO binding properties of cytochrome a_3 and possible Cu_B evaluated quantitatively.

Materials and Methods

The preparation of the mixed valence state membrane-bound cytochrome oxidase-CO complex is identical to that for the fully reduced membrane-bound cytochrome oxidase-CO complex described by Clore and Chance [10] with the exception that potassium ferricyanide, at a final concentration of 1 mM, is added under anaerobic conditions at 253 K to the mitochondrial suspension containing the fully reduced membrane-bound cytochrome oxidase-CO complex 30 s prior to rapid freezing in an ethanol/solid CO_2 bath at 195 K [12,20].

All spectroscopic measurements were made using a Johnson Foundation dual wavelength multichannel spectrophotometer [23] as described previously

[10]. The wavelengths monitored simultaneously were the same as those used by Clore and Chance [10] so that a direct comparison of the data could be made. These were: 444—463, 590—630 and 608—630 nm. It should be noted that the analysis and interpretation of the kinetics (in terms of the number of intermediates, models and rate constants) are not dependent on the choice of monitoring and reference wavelengths and do not require the latter to be an isosbestic point for any of the species*.

The reaction was activated by a 200 J xenon flash with a pulse width of 1 ms which was approx. 99% saturating. The measuring beam was provided by a tungsten iodide lamp; the intensity of the measuring beam was not sufficient to perturb the measured kinetics, measuring beam photolysis proceeding with a rate constant of less than 10^{-5} s^{-1}.

Each sample was used for measurements at all temperatures and three recordings were made at each temperature. Between each recording the sample was warmed to 223 K, a temperature at which all CO molecules rebind so that a uniform initial state is established [10]. A total of three different samples was used, and sample to sample variation was less than 2%. The temperature used were the same as those used by Clore and Chance [10], namely 178, 183, 188, 193, 198 and 203 K.

The data were digitized by the method of Clore and Chance [11] and normalization was carried out with respect to the states before and immediately after photolysis. The overall standard error of the data, given by the weighted mean of the standard errors of the individual progress curves, was 2.0 ± 0.36%. The numerical techniques and the method of data evaluation were the same as those described previously [10—13,25].

Results and Discussion

In Fig. 1 typical progress curves at 444—463, 590—630 and 608—630 nm, recorded on a slow time scale, comparing the kinetics of the mixed valence state and fully reduced membrane-bound cytochrome oxidase-CO reactions are shown at 188 K. It is easily seen that at 188 K the mixed valence state cytochrome oxidase-CO reaction is significantly slower than the fully reduced cytochrome oxidase-CO reaction.

In Fig. 2 the normalized absorbance changes (normalized with respect to the total absorbance change produced on flash photolysis) versus log t at 444—463, 590—630 and 608—630 nm are shown over the 178—203 K range for the mixed valence state membrane-bound cytochrome oxidase-CO reaction. As in the case of the fully reduced cytochrome oxidase-CO reaction [10], the normalized curves at the three wavelength pairs are significantly different. It therefore follows immediately from Beer's law that a minimum of three optically distinct species must be present.

The observation that the kinetics of the reactions of cytochrome oxidase with both O_2 [11,12,20] and CO [14,26] in the frozen state are pseudo-first order, together with the demonstration of gas exchanges in the active site at liquid helium temperatures [27], suggests that a 'pocket' [10,28] for a popula-

* The proof is available from the BBA Data Bank.

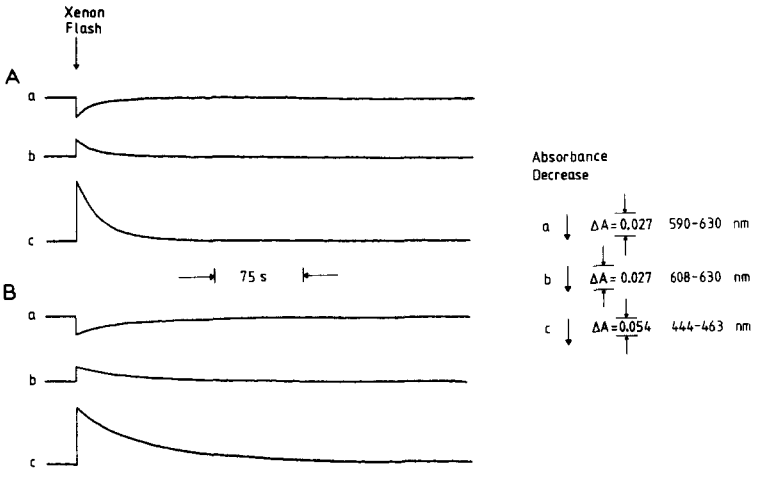

Fig. 1. Comparison of the kinetics of the binding of CO to fully reduced (A) and mixed valence state (B) membrane-bound cytochrome oxidase at 188 K following flash photolysis of the respective CO complexes recorded simultaneously at three wavelength pairs on a slow time scale. The direction of the absorbance changes is indicated by the arrows on the figure as are the magnitude of the absorbance calibrations and the wavelength pairs used in the recording. The time scale is linear and proceeds from left to right at the indicated sensitivity. The experimental conditions are: (A) 21 mg/ml bovine heart mitochondria, containing 7 μM cytochrome oxidase calculated from $\epsilon^{605-630}_{red-ox} = 24.0\ mM^{-1} \cdot cm^{-1}$ [24], 30% (v/v) ethylene glycol, 0.1 M mannitol, 50 mM sodium phosphate buffer, pH 7.2, 5 mM succinate and 1.2 mM CO; (B) as in (A) plus 1 mM potassium ferricyanide.

tion of ligand molecules that is proportional to that of the solvent at the time of freezing, exists near the active site. The linearity of the Arrhenius plots from very low temperatures towards the melting point of the solvent in the reaction with O_2 [20] suggests that the constant of proportionality is 1. We have therefore assumed, on the basis of the above evidence, that the concentration of CO in the 'pocket' of cytochrome oxidase (i.e. the initial CO concentration) is equal to that in the solvent, namely 1.2 mM.

As discussed in the case of the fully reduced cytochrome oxidase-CO reaction [10], two models can be written on the basis of a three species minimum hypothesis:

$$E_M + CO_p \underset{k_{-1}}{\overset{k_{+1}}{\rightleftharpoons}} I_{MC} \underset{k_{-2}}{\overset{k_{+2}}{\rightleftharpoons}} II_{MC} \qquad \text{Model 1} \qquad (2)$$

($h\nu$ (flash photolysis))

$$E_M + CO_p \underset{k_{-1}}{\overset{k_{+1}}{\rightleftharpoons}} I_{MC} \underset{k_{-2}}{\overset{k_{+2}}{\rightleftharpoons}} II_{MC} \qquad \text{Model 2} \qquad (3)$$

($h\nu$ (flash photolysis))

where E_M is unliganded mixed valence state cytochrome oxidase, and species II_{MC} the final mixed valence state cytochrome oxidase-CO complex. Photolysis of species II_{MC} results in photodissociation of the bond between CO and the haem iron. In model 1 (Eqn. 2) photolysis yields species E_M and one molecule of free CO which is indistinguishable from the other CO molecules in the 'pocket' containing the CO binding site (which are termed CO_p). In model 2

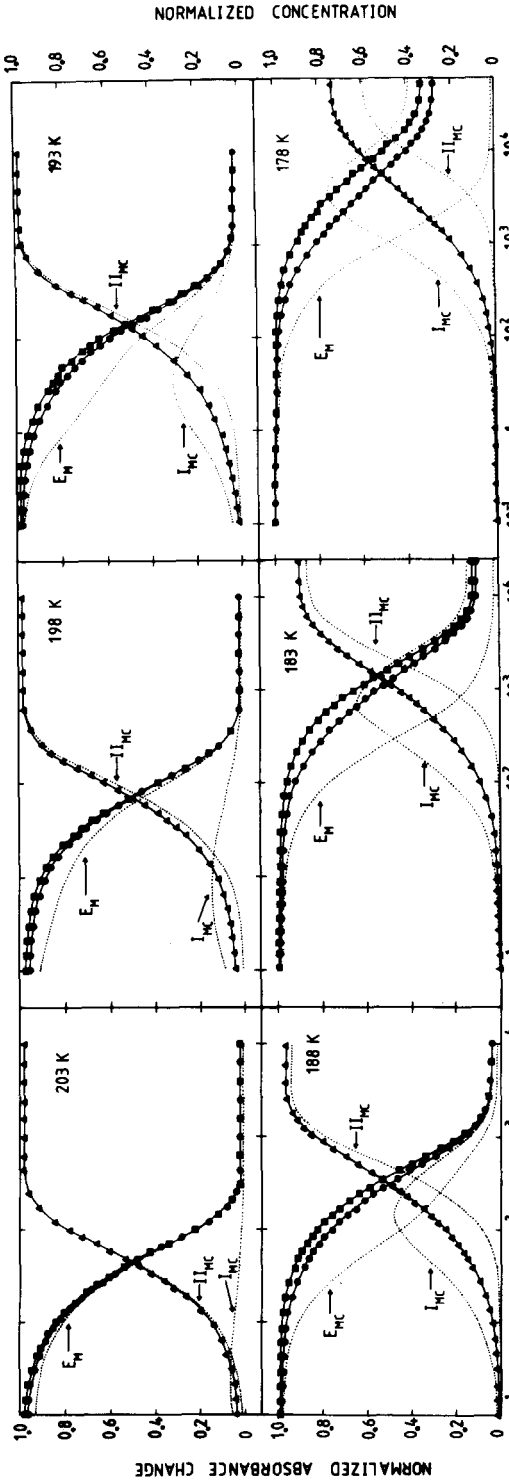

Fig. 2. Comparison of the observed kinetics of the reaction of mixed valence state membrane-bound cytochrome oxidase with CO with computed kinetics for model 1 (Eqn. 6). The experimental points are shown as: ■, 444—463 nm; ▲, 590—630 nm; ●, 608—630 nm. ———, the theoretical normalized absorbance changes, and - - - - - -, the computed time courses of species E_M, I_{MC} and II_{MC}. The overall S.D. of the fit is 1.95% compared to the overall standard error of the data of 2.0 ± 0.36%. The distribution of residuals is random. Initial conditions (i.e. immediately after the xenon flash at t = 0 s) are: 7 μM unliganded mixed valence state cytochrome oxidase (E_M) in the presence of 1.2 mM CO. The experimental conditions are the same as in Fig. 1B.

(Eqn. 3) photolysis of species II_{MC} yields species I_{MC} in which the photodissociated CO molecule occupies a region that is distinct from the 'pocket' and has a maximum occupancy of one CO molecule. As in the case of the fully reduced cytochrome oxidase-CO reaction, only model 1 (Eqn. 2) satisfies the triple statistical requirement [11] of a S.D. within the standard error of the data (i.e. less than 2%), a random distribution of residuals and good determination of the optimized parameters. Model 2 (Eqn. 3) fails to satisfy two out of the three criteria: the S.D. of the fit (3.5%) is greater than the standard error of the data (2.0 ± 0.36%) and there are systematic errors in the distribution of residuals (for details of the method of analysis of model 2 see Ref. 10 where the same model was applied to the fully reduced cytochrome oxidase-CO reaction and similarly rejected). (It should be noted that de Fonseka and Chance [29] have recently examined the kinetics of the fully reduced and mixed valence state membrane-bound cytochrome oxidase-CO reactions at two temperatures (177 and 189 K for the former reaction, 177 and 184 K for the latter reaction) under conditions where photolysis by the xenon flash was incomplete and where measuring light beam caused significant photolysis during the course of the reaction. The data at each temperature were only analysed at a single wavelength pair. These authors rejected model 1 for both reactions on the basis of poor determination of all the optimized parameters, and suggested that both reactions proceeded by a single reversible step mechanism. They noted, however, that there were systematic errors in the distribution of residuals when the data were fitted to a single-step mechanism. Further, no experimental details were given, no experimental data and no computed curves were shown. Their failure to determine all the parameters for model 1 is due to the fact that the information content of a single progress curve in this system is insufficient to determine six unknown parameters: two forward and two backward rate constants, the relative absorption coefficient of species I_{MC} or species I_C at the monitored wavelenght pair, and an additional rate constant to account for photolysis by the measuring light beam during the course of the reaction. A single-step reversible step mechanism, of course, fails to account for the observation that the normalized progress curves at three wavelength pairs are significantly different (see Fig. 2 of this paper and Fig 1 of Ref. 10). This is true whether the measuring light beam does or does not cause photolysis. When a single step reversible mechanism is fitted to all our data simultaneously, the S.D. of the fit is greater than 10% and there are large systematic errors in the distribution of residuals.)

All the data at the three wavelength pairs and six temperatures (178, 183, 188, 193, 198 and 203 K) were fitted simultaneously by optimizing the rate constants $k_{+1}(T_D)$, $k_{-1}(T_D)$, $k_{+2}(T_D)$ and $k_{-2}(T_D)$ at a reference temperature T_D (chosen at 188 K), the corresponding zero-point activation energies $E^0_{A_1}$, $E^0_{A_{-1}}$, $E^0_{A_2}$ and $E^0_{A_{-2}}$ (i.e. the difference in energy between the zero-point levels of the initial and transition states at absolute zero), and the relative absorption coefficients of species I_{MC} at the three wavelength pairs. The temperature dependence of the rate constants was assumed to be described by classical activated complex theory with temperature independence of the pre-exponential component. The justification for this approach over the 140—240 K range for proteins has been discussed in detail in Ref. 10. The normalized

absorbance change, $N_i(t)$, at the ith wavelength pair, is given by:

$$N_i(t) = \sum_l \alpha_i(l)\, F_l(t)/B \tag{4}$$

where B is the total concentration of oxidase and $F_l(t)$ the concentration of the lth species at time t obtained by numerical integration of the coupled simultaneous differential equations derived from Eqn. 2 (model 1) with the initial conditions:

$$[E_M] = 7\mu M \qquad [CO_p] = 1.2\ mM \qquad [I_{MC}] = [II_{MC}] = 0 \tag{5}$$

The relative absorption coefficient, $\alpha_i(l)$, of species l at the ith wavelength pair is defined as

$$\alpha_i(l) = \Delta\epsilon_i^T(l - z)/\Delta\epsilon_i^T(x - z) \tag{6}$$

where $\Delta\epsilon_i^T(l - z)$ and $\Delta\epsilon_i^T(x - z)$ are the molar difference absorption extinction coefficients at a temperature T between species l and x, and species x and z, respectively, at the ith wavelength pair. Species x and z are reference species and from Eqn. 6 they have relative absorption coefficients of 1.0 and 0, respectively. The data are normalized with respect to the difference in absorbance between the states before and immediately after (i.e. at $t = 0$ s) photolysis so that the reference species are E_M and II_{MC} (for further details of the data analysis see Ref. 10).

The comparison of the experimental and computed curves for model 1 are shown in Fig. 2. The overall S.D. of the fit is 1.95% (compared to a standard error of the data of 2.0 ± 0.36%) and the distribution of residuals is random. The values of the optimized rate constants at the reference temperature T_D (188 K) together with their $S.D._{ln}$ and 5—95% confidence limits are given in Table I.

Looking at Fig. 2 we see that as the temperature is decreased so the maximal and equilibrium concentrations of species I_{MC} rise and the difference between the normalized absorbance changes at the three wavelength pairs increases. As the temperature is increased the reverse occurs so that at 203 K, the amount of species I_{MC} formed during the reaction is so small that the 444—463 and 608—630-nm curves are seen to be superimposable from 1 s onwards. As a consequence, at temperatures above 203 K the system behaves as a single exponential process.

TABLE I

OPTIMIZED VALUES OF THE RATE CONSTANTS AT THE REFERENCE TEMPERATURE T_D (188 K) TOGETHER WITH THEIR $S.D._{ln}$ AND CONFIDENCE LIMITS FOR THE MIXED VALENCE STATE CYTOCHROME OXIDASE-CO REACTION (MODEL 1)

Parameter	Dimensions	Optimized value	$S.D._{ln}$	Confidence limits	
				5%	95%
k_{+1} (T_D)	$M^{-1} \cdot s^{-1}$	85.4	0.118	70.4	104
k_{-1} (T_D)	s^{-1}	0.0519	0.152	0.0404	0.0666
k_{+2} (T_D)	s^{-1}	0.0269	0.0521	0.0247	0.0293
k_{-2} (T_D)	s^{-1}	0.00103	0.116	0.000851	0.00124

TABLE II

COMPARISON OF THE RELATIVE ABSORPTION COEFFICIENTS OF SPECIES I_{MC} AND I_C

The S.D. are shown in parentheses. The relative absorption coefficients of species I_C are taken from Ref. 10. The reference species for species I_{MC} are the unliganded mixed valence state cytochrome oxidase (E_M) and the final mixed valence state cytochrome oxidase-CO complex (II_{MC}). The reference species for species I_C are the unliganded fully reduced cytochrome oxidase (E) and the final fully reduced cytochrome oxidase-CO complex (II_C). At 444—463 and 608—630 nm $\alpha_i(E_M) = \alpha_i(E) = 1.0$ and $\alpha_i(II_{MC}) = \alpha_i(II_C) = 0$; at 590—630 nm $\alpha_i(II_{MC}) = \alpha_i(II_C) = 1.0$ and $\alpha_i(E_M) = \alpha_i(E) = 0$.

Relative absorption coefficient	I_{MC}	I_C
$\alpha_{444-463}(l)$	0.830 (± 0.025)	0.780 (± 0.028)
$\alpha_{608-630}(l)$	0.649 (± 0.025)	0.639 (± 0.030)
$\alpha_{590-630}(l)$	0.370 (± 0.025)	0.483 (± 0.032)

Comparison of the contributions of species I_{MC} and I_C at the three wavelength pairs

In Table II the optimized values of the relative absorption coefficients of species I_{MC} are compared to those of species I_C, the corresponding species in the fully reduced cytochrome oxidase-CO reaction [10]. From Table II, we see that

$$\alpha_{444-463}(I_{MC}) > \alpha_{608-630}(I_{MC}) \approx 1 - \alpha_{590-630}(I_{MC})$$
$$\alpha_{444-463}(I_C) > \alpha_{608-630}(I_C) > 1 - \alpha_{590-630}(I_C)$$
$$\alpha_{444-463}(I_{MC}) = \alpha_{444-463}(I_C) \qquad \alpha_{608-630}(I_{MC}) = \alpha_{608-630}(I_C) \tag{7}$$
$$\alpha_{590-630}(I_{MC}) < \alpha_{590-630}(I_C)$$

From the definition of $\alpha_i(l)$ given in Eqn. 6, it follows that the absorbance changes at 444—463 nm for the E_M-to-I_{MC} and I_{MC}-to-II_{MC} transitions, relative to the absorbance change for the E_M-to-II_{MC} transition, are approximately the same as those for the E-to-I_C and I_C-to-II_C transitions, respectively, relative to the absorbance change for the E-to-II_C transition. At 590—630 nm, however, the absorbance change for the E_M-to-I_{MC} transition, relative to the absorbance change for the E_M-to-II_{MC} transition, is significantly smaller than that for the E-to-I_C transition, relative to the absorbance change for the E-to-II_C transition.

Comparison of the temperature dependence of the rate constants and free energies of activation for the mixed valence state and fully reduced membrane-bound cytochrome oxidase-CO reactions over the 140—240 K range

The temperature dependence of the rate constants and observed rate constants are shown in Fig. 3, and the temperature dependence of the free energies of activation, $\Delta G_i^{\ddagger}$, in Fig. 4 for both the mixed valence state and fully reduced membrane-bound cytochrome oxidase-CO reactions (the values for the latter reaction are taken from Ref. 10). (Note that the values from 140 to 178 K and 203 to 240 K are extrapolated from the values obtained by optimized over the experimental range, 178—203 K inclusive; the justification for such an approach has been discussed in detail by Clore and Chance [10].)

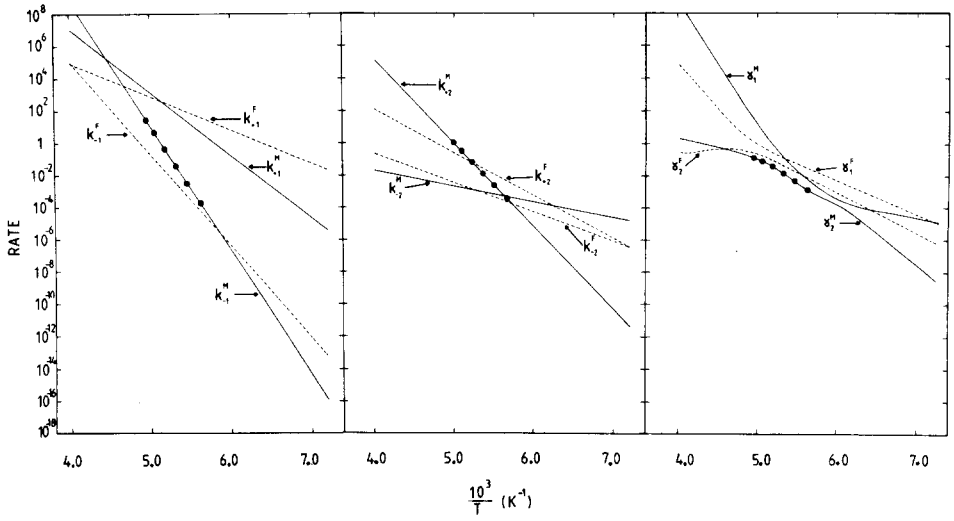

Fig. 3. Comparison of the temperature dependence of the rate constants and observed rate constants for the mixed valence state (———) and fully reduced (- - - - - -) membrane-bound cytochrome oxidase-CO reactions over the 140—240 K range. The superscripts M and F denote the rate constants for the mixed valence state and fully reduced cytochrome oxidase-CO reactions, respectively. γ_1 and γ_2 are the computed 'observed' rate constants for the fast and slow phases, respectively, given by the roots of the quadratic equation

$$\gamma^2 + \gamma(k_{+1}[CO_p] + k_{+2} + k_{-1} + k_{-2}) + k_{+1}[CO_p](k_{+2} + k_{-2}) + k_{-1}k_{-2} = 0$$

taken with reverse signs [10]. The units of k_{+1}^M and k_{+1}^F are $M^{-1} \cdot s^{-1}$; the units of all other rate constants are s^{-1}. •, temperatures at which the experiments were done.

A number of interesting differences exist between the two reactions. Four temperature zones can be distinguished for both reactions but their temperature ranges differ considerably. The temperature ranges and kinetic characteristics of each temperature zone are given in Table III. Zone 1 starts at a

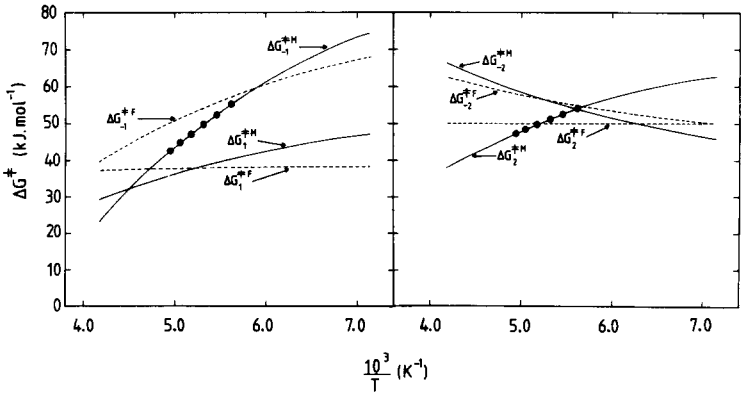

Fig. 4. Comparison of the temperature dependence of the free energies of activation for the mixed valence state (———) and fully reduced (- - - - - -) membrane-bound cytochrome oxidase-CO reactions over the 140—240 K range. The superscripts M and F denote the free energies of activation of the mixed valence state and fully reduced cytochrome oxidase-CO reactions, respectively. •, temperatures at which the experiments were done.

TABLE III

TEMPERATURE ZONES DESCRIBING THE KINETIC BEHAVIOUR OF THE MIXED VALENCE STATE AND FULLY REDUCED CYTOCHROME OXIDASE-CO REACTIONS

The data for the fully reduced cytochrome oxidase-CO reaction are taken from Ref. 10.

	Zone 1	Zone 2	Zone 3	Zone 4
Temperature range (K) for the:				
Mixed valence state cytochrome oxidase-CO reaction	<177	177—185	185—223	>223
Fully reduced cytochrome oxidase-CO reaction	<140	140—202	202—240	>240
Kinetic characteristics	$k_{-2} > k_{+2}$	$k_{+1}[CO_p] > k_{-1}$ $k_{+2} > k_{-2}$ $k_{+2} > k_{-1}$	$k_{-1} > k_{+2}$	$k_{-1} > k_{+1}[CO_p]$ $k_{-2} > k_{+2}$
Relationships of the free energies of activation ($\Delta G_i^{\ddagger}$)	$\Delta G_2^{\ddagger} > \Delta G_{-2}^{\ddagger}$	$\Delta G_{-1}^{\ddagger} > \Delta G_1^{\ddagger}$ $\Delta G_{-2}^{\ddagger} > \Delta G_2^{\ddagger}$ $\Delta G_{-1}^{\ddagger} > \Delta G_2^{\ddagger}$	$\Delta G_2^{\ddagger} > \Delta G_{-1}^{\ddagger}$	$\Delta G_1^{\ddagger} > \Delta G_{-1}^{\ddagger}$ $\Delta G_2^{\ddagger} > \Delta G_{-1}^{\ddagger}$
Comments	At equilibrium: $[I_{MC}] > [II_{MC}]$ $[I_C] > [II_C]$	The reaction proceeds down a free energy gradient towards species II_{MC} and II_C in the mixed valence state and fully reduced cytochrome oxidase-CO reactions, respectively.	CO recombination proceeds by a random walk process: after the formation of species I_{MC} or I_C, the reaction has a larger probability of returning to species E_M or E, respectively, than of proceeding to species II_{MC} or II_C, respectively.	

higher temperature, the temperature range of zone 2 is smaller, and zones 3 and 4 start at lower temperatures in the mixed valence state cytochrome oxidase-CO reaction than in the fully reduced cytochrome oxidase-CO reaction.

It is noteworthy that when the value of the overall equilibrium constant for the formation of the final cytochrome oxidase-CO complex is extrapolated to room temperature (293 K), values of 0.17 μM^{-1} and 14 M^{-1} are found for the mixed valence state and fully reduced cytochrome oxidase-CO reactions, respectively, compared to values in the range of 3.3—5 μM^{-1} for both reactions found by Greenwood et al. [7]. It should also be noted that when the value of k_{-1} for the mixed valence state cytochrome oxidase-CO reaction is extrapolated to 293 K a value of $2.8 \cdot 10^{13}$ s^{-1} is found which is approximately 4.5 times greater than the maximum expected value of a first-order rate constant at 293 K (i.e. the value when the free energy of activation is zero which is 6.15 ·

10^{12} at 293 K). These observations imply that in the case of both reactions changes in the thermodynamic parameters, in particular the entropies and energies of activation governing the reactions

$$E_M + CO_p \rightleftharpoons I_{MC}$$

and

$$E + CO_p \rightleftharpoons I_C$$

occur during the solid to liquid state transition [10].

Comparison of the thermodynamics of CO binding to mixed valence state and fully reduced membrane-bound cytochrome oxidase in the solid state

Although there exist certain qualitative similarities in the thermodynamics of CO binding to mixed valence state and fully reduced membrane-bound solid state cytochrome oxidase, there exist some major quantitative differences. The entropies of activation ($\Delta S_i^{\ddagger}$) at 188 K, the zero-point energies of activation ($E^0_{A_i}$) and the temperature-independent frequency factors (λ_i) for both reactions are collected in Table IV. In Fig. 5, the zero-point energies of activation, together with the free energies and entropies of activation at 188 K, are plotted as a function of the reaction coordinate for both reactions. In addition, the ratios of the number of quantum states, W_i'/W_i, between two positions that

TABLE IV

COMPARISON OF THE ZERO-POINT ACTIVATION ENERGIES ($E^{\circ}_{A_i}$), THE ENTROPIES OF ACTIVATION ($\Delta S_i^{\ddagger}(T_D)$) AT THE REFERENCE TEMPERATURE T_D (188 K) AND THE TEMPERATURE-INDEPENDENT FREQUENCY FACTORS (λ_i) FOR THE MIXED VALENCE STATE AND FULLY REDUCED CYTOCHROME OXIDASE-CO REACTIONS

The S.D.$_{ln}$ of $E^{\circ}_{A_i}$ and the S.D. of $\Delta S_i^{\ddagger}(T_D)$ and $\log(\lambda_i)$ are shown in parentheses. The S.D. of $\log(\lambda_i)$ and $\Delta S_i^{\ddagger}(T_D)$ for the mixed valence state cytochrome oxidase-CO reaction are calculated from the S.D.$_{ln}$ of the optimized rate constants and zero-point activation energies (given in Table I and this table, respectively) and the correlation matrix relating the optimized rate constants and zero-point activation energies * using Eqns. 1 and 4 of Appendix II from Ref. 10. The data for the fully reduced cytochrome oxidase-CO reaction are taken from Ref. 10.

Parameter	Dimensions	Mixed valence state cytochrome oxidase-CO reaction	Fully reduced cytochrome oxidase-CO reaction
$E^{\circ}_{A_1}$	kJ · mol^{-1}	74.4 (0.0315)	40.9 (0.0224)
$E^{\circ}_{A_{-1}}$	kJ · mol^{-1}	148 (0.00586)	110 (0.0463)
$E^{\circ}_{A_2}$	kJ · mol^{-1}	98.4 (0.0249)	51.9 (0.0255)
$E^{\circ}_{A_{-2}}$	kJ · mol^{-1}	18.8 (0.424)	34.5 (0.216)
$\Delta S_1^{\ddagger}(T_D)$	J · mol^{-1} · K^{-1}	175 (± 12.7)	0.202 (± 4.67)
$\Delta S_{-1}^{\ddagger}(T_D)$	J · mol^{-1} · K^{-1}	513 (± 24.7)	286 (± 23.1)
$\Delta S_2^{\ddagger}(T_D)$	J · mol^{-1} · K^{-1}	244 (± 13.1)	0.569 (± 7.17)
$\Delta S_{-2}^{\ddagger}(T_D)$	J · mol^{-1} · K^{-1}	−206 (± 42.6)	−124 (± 0.847)
kog(λ_1)	M^{-1} · s^{-1}	22.6 (± 1.53)	13.5 (± 0.565)
log(λ_{-1})	s^{-1}	39.8 (± 0.479)	28.0 (± 2.78)
log(λ_2)	s^{-1}	25.8 (± 1.58)	13.1 (± 0.847)
log(λ_{-2})	s^{-1}	2.25 (± 5.11)	5.56 (± 4.72)

* Available from the BBA Data Bank.

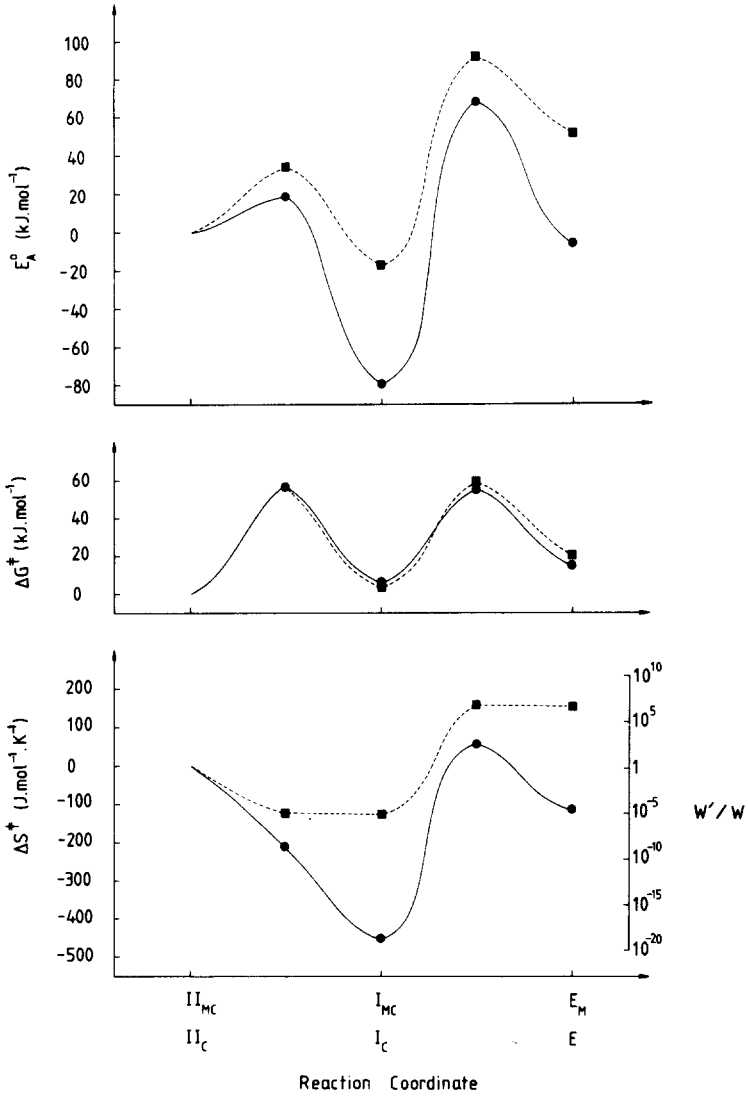

Fig. 5. Comparison of the free energy and entropy of activation at 188 K and the zero-point activation energy as a function of the reaction coordinate for the mixed valence state (●———●) and fully reduced (■- - - - -■) membrane-bound cytochrome oxidase-CO reactions.

differ in entropy by an amount $\Delta S_i^{\ddagger}$, normalized to 1.0 in species II_{MC} and II_C, are also shown in Fig. 5. It should be noted that over the 140—240 K range, the values of $\Delta S_i^{\ddagger}$ remain constant within the errors specified. (The values of the thermodynamic parameters for the fully reduced cytochrome oxidase-CO reaction are taken from Ref. 10.)

The formation of species I_{MC} from species E_M and CO_p is accompanied by a decrease in enthalpy, free energy and entropy. The decrease in entropy (328 J · mol^{-1} · K^{-1} corresponding to a decrease of the order of $1.4 \cdot 10^{17}$ in the number of quantum states) is significantly larger than the decrease in entropy

occurring on the formation of species I_C from species E and CO_p (286 J · mol^{-1} · K^{-1} corresponding to a decrease of the order of $9 \cdot 10^{14}$ in the number of quantum states [10]). The value of $E^0_{A_1}$ is approximately half that of $E^0_{A_{-1}}$ for both reactions so that the activated complex $I^{\ddagger}_{MC}$, like its equivalent in the fully reduced cytochrome oxidase-CO reaction, $I^{\ddagger}_C$, is reached early in the forward reaction process and its electronic structure closely resembles that of the reactants, E_M and CO_p. $\Delta S^{\ddagger}_1$ for the mixed valence state cytochrome oxidase-CO reaction is large (175 J · mol^{-1} · K^{-1}) so that any loss in transitional and rotational freedom of the CO molecule is overcompensated by an increase in translational and rotational freedom in another part of the biomolecule. This is in contrast to the fully reduced cytochrome oxidase-CO reaction where $\Delta S^{\ddagger}_1$ is very small (0.202 J · mol^{-1} · K^{-1}) and the decrease in translational and rotational freedom of the CO molecule is nearly perfectly compensated by a concomitant increase in the translational and rotational freedom in another part of the biomolecule.

The formation of species II_{MC} from species I_{MC} and of species II_C from species I_C is accompanied by an increase in enthalpy and entropy. The formation of species II_{MC} from species I_{MC}, however, is accompanied by a decrease in free energy above 177 K and an increase in free energy below 177 K, in contrast to the formation of species I_C from species II_C where there is a decrease in free energy throughout the 140—240 K range. The increase in entropy on the formation of species II_{MC} from species I_{MC} (450 J · mol^{-1} · K^{-1} corresponding to an increase of the order of $3 \cdot 10^{23}$ in the number of quantum states) is considerably larger than that on the formation of species II_C from species I_C (126 J · mol^{-1} · K^{-1} corresponding to an increase in the number of quantum states of the order of $4 \cdot 10^6$). The formation of the activated complex $II^{\ddagger}_{MC}$ from species I_{MC} is accompanied by a significant increase in entropy ($\Delta S^{\ddagger}_2$ = 244 J · mol^{-1} · K^{-1}), in contrast to the formation of the activated complex $II^{\ddagger}_C$ from species I_C where the increase in entropy is very small ($\Delta S^{\ddagger}_2$ = 0.569 J · mol^{-1} · K^{-1}). Thus the geometric structure of $II^{\ddagger}_{MC}$ is intermediate between those of species I_{MC} and II_{MC}, whereas the geometric structure of $II^{\ddagger}_C$ is very similar to that of species I_C. Further, the difference between $E^0_{A_2}$ and $E^0_{A_{-2}}$ is much larger in the mixed valence state cytochrome oxidase-CO reaction (79.6 kJ · mol^{-1}) than in the fully reduced cytochrome oxidase-CO reaction (17.4 kJ · mol^{-1}). Therefore, $II^{\ddagger}_{MC}$ is reached significantly later in the reaction process than $II^{\ddagger}_C$, and the electronic structure of $II^{\ddagger}_{MC}$ is closer to that of species II_{MC} than that of $II^{\ddagger}_C$ to species II_C.

The chemical nature of species I_{MC} and I_C

The chemical nature of species I_{MC} and I_C is unknown at the present time. In an earlier report [10] we suggested that the n = 2 (i.e. two electron) behaviour of the cytochrome oxidase-CO complex during redox titrations [17] and the large increase in entropy for the E-to-I_C transition ($\Delta S_{E \leftrightarrow I_C}$ = —286 J · mol^{-1} · K^{-1}) could possibly be accounted for by the formation of a CO bridge structure between the iron of cytochrome a^{2+}_3 and Cu^{+}_B in species I_C; and that the observed infrared C—O stretching frequency at 1963.5 cm^{-1} for the CO complex at room temperature [30] which is typical for a terminal-end-on metal carbonyl [31], and the increase in entropy for the I_C-to-II_C transition

($\Delta S_{I_C \leftrightarrow II_C} = +126\ J \cdot mol^{-1} \cdot K^{-1}$) could be accounted for by the breaking of the bond between CO and Cu_B^+ resulting in the formation of a terminal-end-on CO bond to the iron atom of cytochrome a_3^{2+}.

The entropy changes reported in this paper for the mixed valence state cytochrome oxidase-CO reaction ($\Delta S_{E_M \leftrightarrow I_{MC}} = -328\ J \cdot mol^{-1} \cdot K^{-1}$ and $\Delta S_{I_{MC} \leftrightarrow II_{MC}} = +450\ J \cdot mol^{-1} \cdot K^{-1}$) are certainly consistent with the above proposals. It should be emphasized, however, that the correlation of entropy changes with structural changes is frought with danger. Further, several alternative models for species I_{MC} and I_C could account equally well for the observed data. For example, CO could be bound to the iron and an amino acid residue, to Cu_B^+ and to an amino acid residue, to Cu_B^+ or to an amino acid residue alone in species I_{MC} and I_C. One should also bear in mind the possibility that CO may be bound only to the iron atom in species I_{MC} and I_C with the iron in an intermediate S = 1 state.

Unfortunately, optical spectroscopy in the Soret and visible regions is of no use in differentiating between these various alternatives. Further studies aimed at elucidating the chemical nature of species I_{MC} and I_C will therefore require a careful kinetic analysis of the changes in the magnetic susceptibility of cytochrome oxidase and the infrared C—O stretching frequency during the course of CO recombination at low temperatures.

Concluding remarks

The mixed valence state cytochrome oxidase-CO complex ($Cu_A^{2+}a^{3+} \cdot Cu_B^+a_3^{2+} \cdot$ CO) formed by the addition of excess ferricyanide to the fully reduced cytochrome oxidase-CO complex is well characterized by EPR [4], near-infrared optical spectroscopy and X-ray absorption edge spectroscopy [22]. No evidence has been found for electron redistribution from the $a_3^{2+}Cu_B^+$ couple to excess ferricyanide.

Although, at the present time, we are not able to relate the kinetic and thermodynamic differences between the fully reduced and mixed valence state membrane-bound cytochrome oxidase-CO reactions in the frozen state to differences in the geometric and electronic properties of cytochrome a_3^{2+} and Cu_B^+ in fully reduced and mixed valence state cytochrome oxidase, the results presented in this paper demonstrate quantitatively and unambiguously that there is significant interaction between the cytochrome a_3 (and possible Cu_B) unit and the cytochrome a (and possibly Cu_A) unit. Given that there appears to be no magnetic interaction between these two units on the basis of EPR [32], MCD [6,33—36] and magnetic susceptibility studies [37,38], it seems likely that the interaction between these two units is mediated by structural changes in the protein moiety of cytochrome oxidase.

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