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## A RE-EVALUATION OF THE LOW-TEMPERATURE KINETICS OF THE REACTION OF FULLY REDUCED MITOCHONDRIAL CYTOCHROME OXIDASE WITH CARBON MONOXIDE AND THE SPECTRAL CHARACTERIZATION OF SPECIES I<sub>C</sub> IN THE Soret AND VISIBLE REGIONS

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### Summary

The kinetics of the reaction of fully reduced membrane bound cytochrome oxidase with CO following photolysis of the fully reduced cytochrome oxidase-CO complex have been re-examined by re-analysing the data of Clore and Chance ((1978) *Biochem. J.* 175, 709–725) at six temperatures in the 178–203 K range simultaneously at only a single wavelength pair, 444–463 nm. The choice of the 444–463 nm wavelength pair was based on the fact that the absorbance change produced at 444–463 nm on photolysis of the CO complex is sufficiently large and the separation between monitoring and reference wavelengths sufficiently small to render the effects of any possible time dependent scattering changes insignificant. On the basis of our analysis only a two step mechanism (Model 1 of Clore and Chance (1978) *Biochem. J.* 175, 709–725) satisfies the triple requirement of a S.D. within the standard error of the data, a random distribution of residuals and good determination of the optimized parameters. The single step mechanism of De Fonseka and Chance ((1978) *Biochem. J.* 175, 1137–1138) fails to satisfy all three requirements. The pure difference spectra of species I<sub>C</sub> minus E, E minus II<sub>C</sub> and I<sub>C</sub> minus II<sub>C</sub> are calculated from the computed kinetics of the individual species and repetitive

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Abbreviations: Species E, unliganded fully reduced cytochrome oxidase; species II<sub>C</sub>, the fully reduced cytochrome oxidase-CO complex; S.D.<sub>ln</sub>, standard deviation of the natural logarithm of an optimized parameter; C, correlation index;  $E_{A_i}^0$ , zero-point activation energy.

slow wavelength scanning difference spectra (reaction sample minus the CO complex) taken during the course of the reaction of fully reduced cytochrome oxidase with CO at 176 K.

## 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,  $\text{Cu}_A$  and  $\text{Cu}_B$  [1]. The kinetics of the reaction of CO with cytochrome oxidase have received considerable attention [2–7] as they allow one to study the binding of a molecule of similar nature and size to molecular  $\text{O}_2$  to the iron atom of cytochrome  $a_3$  in the absence of electron transfer.

Clore and Chance [4,5] have analysed the low temperature kinetics and thermodynamics of the reaction of both fully reduced ( $\text{Cu}_A^+a^{2+} \cdot \text{Cu}_B^+a_3^{2+}$ ) and mixed valence state ( $\text{Cu}_A^{2+}a^{3+} \cdot \text{Cu}_B^+a_3^{2+}$ ) cytochrome oxidase with CO at six temperatures in the 178–203 K range and three wavelength pairs (444–463, 590–630 and 608–630 nm) simultaneously. Significant but small differences in the normalized absorbance changes were found at these three wavelength pairs, and, on the basis of Beer's law, a minimum three species hypothesis was proposed. Two reaction schemes involving three species (Models 1 and 2) and one reaction scheme involving two species (Model 3) were analysed by means of numerical integration and non-linear optimization techniques (see Table I

TABLE I

SCHEMES FOR THE REACTION OF FULLY REDUCED CYTOCHROME OXIDASE WITH CO AT LOW TEMPERATURES FOLLOWING FLASH PHOTOLYSIS OF THE FULLY REDUCED CYTOCHROME OXIDASE-CO COMPLEX, TOGETHER WITH THE OVERALL S.D. OF THEIR FITS TO THE DATA IN FIG. 3.

The initial conditions at  $t = 0$  s (i.e. immediately after photolysis) are given, as is the precise definition of the relative absorption coefficient of the  $i$ -th species at the  $i$ -th wavelength,  $\alpha_i(l)$ , for each model (the general definition of  $\alpha_i(l)$  is given by Eqn. 2). The overall standard error of the data in Fig. 3 is  $2.0 \pm 0.36\%$ .

Model	Initial conditions at $t = 0$ s (i.e. immediately after photolysis)	Definition of $\alpha_i(l)$	Overall S.D. of the fit to the data in Fig. 3 (%)
$1. \begin{array}{c} \text{E} + \text{CO} \xrightleftharpoons[k_{-1}]{k_{+1}} \text{IC} \xrightleftharpoons[k_{-2}]{k_{+2}} \text{IIC} \\ \uparrow \text{flash photolysis} \end{array}$	$[\text{E}] = 7 \mu\text{M}$ $[\text{CO}] = 1.2 \text{ mM}$ $[\text{IC}] = [\text{IIC}] = 0$	$\alpha_i(l) = \frac{\Delta \epsilon_i^T (l - \text{IIC})}{\Delta \epsilon_i^T (\text{E} - \text{IIC})}$	1.70
$2. \begin{array}{c} \text{E} + \text{CO} \xrightleftharpoons[k_{-1}]{k_{+1}} \text{IC} \xrightleftharpoons[k_{-2}]{k_{+2}} \text{IIC} \\ \uparrow \text{flash photolysis} \end{array}$	$[\text{IC}] = 7 \mu\text{M}$ $[\text{CO}] = 1.2 \text{ mM}$ $[\text{E}] = [\text{IIC}] = 0$	$\alpha_i(l) = \frac{\Delta \epsilon_i^T (l - \text{IIC})}{\Delta \epsilon_i^T (\text{IC} - \text{IIC})}$	2.12
$3. \begin{array}{c} \text{E} + \text{CO} \xrightleftharpoons[k_{-1}]{k_{+1}} \text{IIC} \\ \uparrow \text{flash photolysis} \end{array}$	$[\text{E}] = 7 \mu\text{M}$ $[\text{CO}] = 1.2 \text{ mM}$ $[\text{IIC}] = 0$	$\alpha_i(l) = \frac{\Delta \epsilon_i^T (l - \text{IIC})}{\Delta \epsilon_i^T (\text{E} - \text{IIC})}$	3.35

for details of models). Only Model 1 was found to satisfy the triple requirement [8] of a S.D. within the standard error of the data, a random distribution of residuals and good determination of the optimized parameters. The single step reversible mechanism (Model 1) fails to fit the data for both reactions on the basis of a S.D. (>10%) significantly greater than the standard error of the data (2%).

In a later paper De Fonseca and Chance [6] analysed data at two temperatures separately and found that some of the parameters for Model 1 were poorly determined. Using Model 3, however, they found that the S.D. values of the fits at each temperature were within the standard errors of their data and the optimized parameters were well determined, but there were systematic errors in the distribution of residuals at both temperatures. In the belief that poorly determined parameters are a more important cause for rejection of a kinetic model than systematic errors in the distribution of residuals, they concluded that Model 3 was the best model representing the reaction of both fully reduced and mixed valence state cytochrome oxidase with CO. Their failure to determine all the parameters for Model 1, however, is not surprising and is simply due to the use of a small data-information base (three wavelength pairs and two temperatures analysed individually) compared to the much larger data-information base (three wavelength pairs and six temperatures analysed simultaneously) used by Clore and Chance [4,5].

In a further paper De Fonseca and Chance [7] examined the spectral changes in the Soret and visible regions occurring during the recombination of CO with fully reduced and mixed valence state cytochrome oxidase by slow wavelength scanning optical spectroscopy, and failed to detect a distinct optical species corresponding to species  $I_C$  of Model 1. Further, they suggested that the differences in the normalized absorbances changes observed by Clore and Chance [4,5] at 444–463, 590–630 and 608–630 nm were artefactual and due to time dependent scattering changes.

In view of the disagreement between De Fonseca and Chance [6,7] and Clore and Chance [4,5] in regard to the choice between Models 1 and 3, it is essential to re-examine in detail (1) the significance of time dependent scattering changes, and (2) the kinetics of the recombination of CO with fully reduced cytochrome oxidase. In this paper I demonstrate that the effects of any possible time dependent scattering changes are rendered insignificant when care is taken over the choice of reference wavelength such that the largest separation between the monitoring and reference wavelengths is not greater than 50 nm. The kinetics of the fully reduced cytochrome oxidase-CO reaction have been re-examined by analysing the data of Clore and Chance [4] at a single wavelength pair, 444–463 nm, and six temperatures in the 178–203 K range simultaneously. By choosing a wavelength pair at which the absorbance changes are reasonably large and the separation between monitoring and reference wavelengths small ( $\leq 20$  nm), the effects of any possible time dependent scattering changes are rendered negligible. By only analysing data at a single wavelength pair the spurious introduction of an intermediate on the basis of differences in the normalized absorbance changes at different wavelength pairs which may be artefactual, is excluded. By analysing the data at six temperatures simultaneously a large enough data-information base is provided with sufficient inherent

constraints to enable one to select on a quantitative basis the appropriate kinetic model from the three proposed models.

## Experimental

### *Biochemical methods*

The fully reduced membrane bound cytochrome oxidase-CO complex of intact beef heart mitochondria was prepared exactly as described by Clore and Chance [4].

### *Biophysical methods*

Optical spectra were recorded using a DBS-2 Johnson Foundation dual wavelength spectrophotometer equipped with two 200 mm focal length Jobin-Yvon monochromators (Model H-20, 1200 lines/mm) which employ aberration corrected holographic gratings. The measuring beam was provided by a tungsten iodide lamp, the intensity of which was not sufficient to perturb the measured kinetics, measuring beam photolysis proceeding at a rate less than  $10^{-5} \text{ s}^{-1}$ . The transmitted light was monitored using a multi-alkali photomultiplier for the 400–700 nm range (EMI 9592b). The electrical output from the photomultiplier was coupled to an 8 bit 1024 address digital memory (Varian C-1024) in which the characteristics of the baseline were stored (in this case the spectrum of the fully reduced membrane bound cytochrome oxidase-CO complex) and from which corrective signals were read out, subtracting the stored baseline from the incoming data. All optical spectra were recorded with a bandwidth of 5 nm, a scanning rate of 2.8 nm/s and a time constant of 3 s.

Flat 2 mm optical path length cuvettes were used throughout and the temperature of the samples was maintained by a flow of cold  $\text{N}_2$  and regulated by a copper-constantan thermocouple. Photolysis was carried out with a 200 J xenon flash lamp (pulse width 1 ms) which was approx. 99% saturating.

### *Kinetic data and analysis*

The kinetic data analysed were those of Clore and Chance [4] at 444–463 nm and at six temperatures (178, 183, 188, 193, 198 and 203 K). The intensity of the measuring beam in these experiments was not sufficient to perturb the measured kinetics, measuring beam photolysis proceeding at a rate of less than  $10^{-5} \text{ s}^{-1}$ . The overall standard error of the data is  $2 \pm 0.36\%$ . All the data were analysed simultaneously using the numerical methods of integration and optimization described by Clore and Chance [4,5,8].

## Results and Discussion

### *Significance of time dependent scattering changes*

De Fonseka and Chance [7] demonstrated that if time dependent scattering changes (possibly due to devitrification) occur, they may cause an apparent increase in absorbance at shorter more scattering wavelengths and an apparent decrease in absorbance at longer wavelengths. Thus when optical spectra are recorded using the dual wavelength technique over a wide wavelength range with only a single reference wavelength, as in the spectra of De Fonseka and

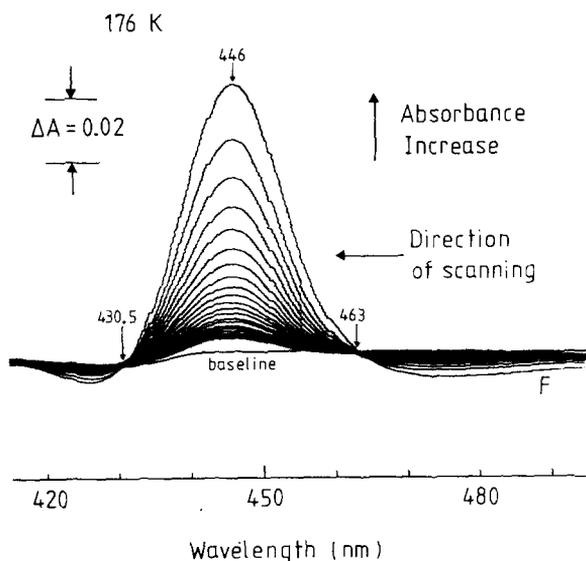


Fig. 1. Optical difference spectra (reaction sample minus the fully reduced cytochrome oxidase-CO complex) in the Soret region obtained by repetitive wavelength scanning illustrating the time course of the recombination of CO with fully reduced cytochrome oxidase following photolysis of fully reduced cytochrome oxidase-CO complex at 176 K. Experimental conditions were: 15 mg/ml beef heart mitochondria containing  $5 \mu\text{M}$  cytochrome oxidase (calculated using  $\epsilon_{\text{red-ox}}^{605} = 24.0 \text{ mM}^{-1} \cdot \text{cm}^{-1}$  [9]), 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. The difference spectra were recorded in a continuous repetitive scanning mode from right to left at a rate of 2.8 nm/s and a time interval between  $\lambda_i(\text{scan } j)$  and  $\lambda_i(\text{scan } j + 1)$  of 47 s. The reference wavelength is 463 nm. F is the first difference spectrum following photolysis. (For further experimental details see Experimental.)

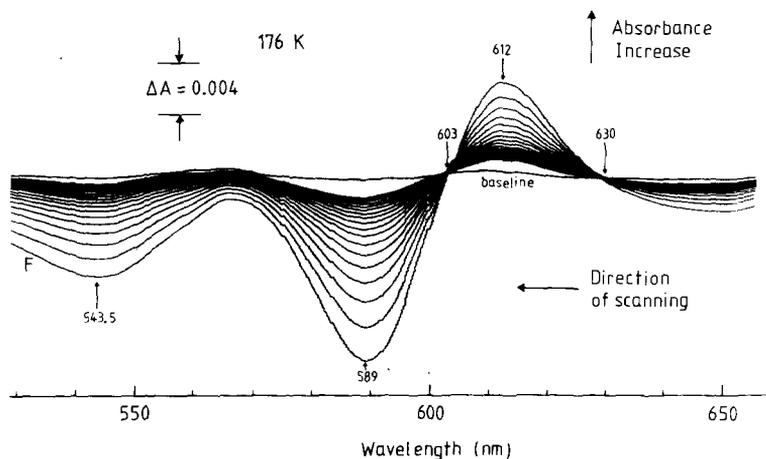


Fig. 2. Optical difference spectra (reaction sample minus the fully reduced cytochrome oxidase-CO complex) in the visible region obtained by repetitive wavelength scanning illustrating the time course of the recombination of CO with fully reduced cytochrome oxidase following photolysis of the fully reduced cytochrome oxidase-CO complex at 176 K. Experimental conditions as in Fig. 1. The difference spectra were recorded in a continuous repetitive scanning mode from right to left at a rate of 2.8 nm/s and a time interval between  $\lambda_i(\text{scan } j)$  and  $\lambda_i(\text{scan } j + 1)$  of 47 s. The reference wavelength is 603 nm. F is the first difference spectrum following photolysis.

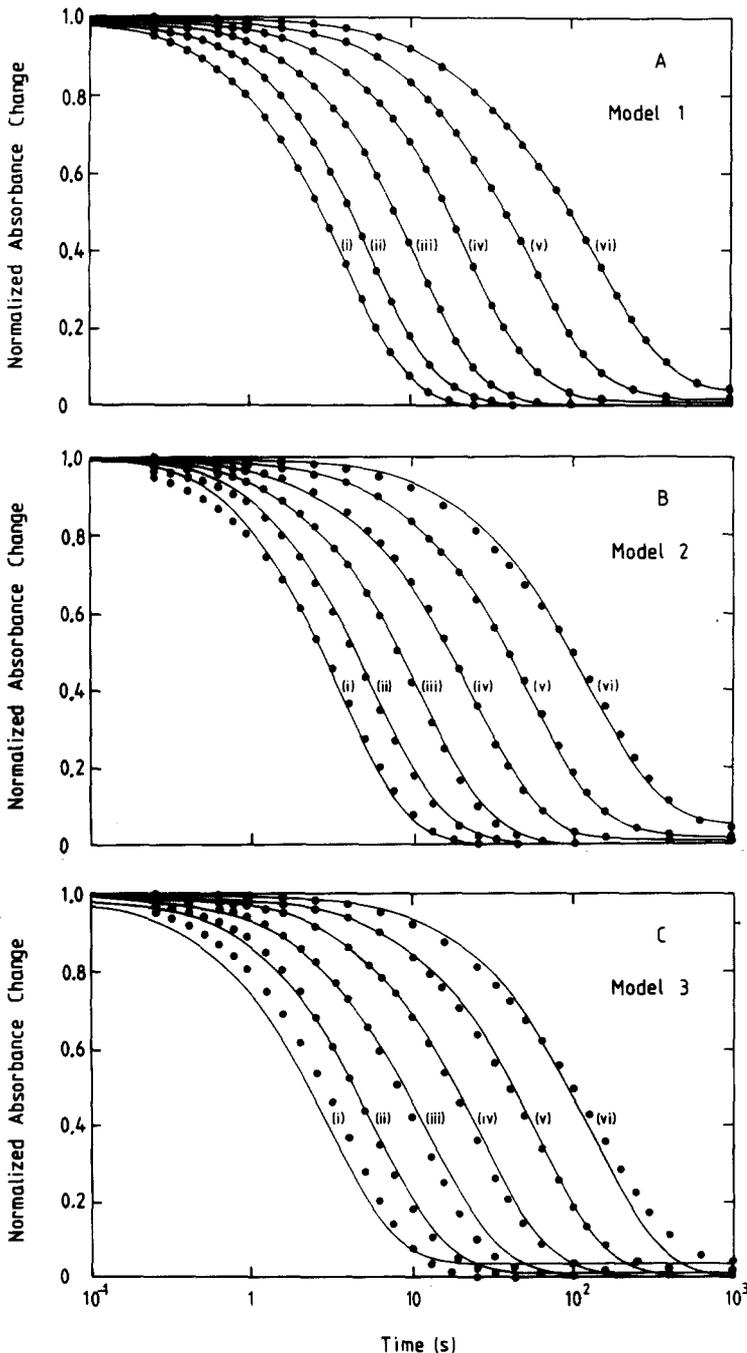


Fig. 3. Comparison of the normalized observed kinetics of the reaction of fully reduced cytochrome oxidase with CO at 444–463 nm in the 178–203 K range with the best fit computed kinetics obtained for Model 1 (A), Model 2 (B) and Model 3 (C). The experimental points are shown as ●, and the computed best fit curves as solid lines. The curves are normalized relative to the absorbance change produced on photolysis, i.e. relative to  $\Delta A_{444}(E - II_C)$ . The experimental conditions are: 21 mg/ml beef heart mitochondria containing 7  $\mu$ M cytochrome oxidase (calculated from  $\epsilon_{\text{red-ox}}^{605} = 24.0 \text{ mM}^{-1} \cdot \text{cm}^{-1}$  [9]), 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. The six temperatures are: (i) 203 K; (ii) 198 K; (iii) 193 K; (iv) 188 K; (v) 183 K; (vi) 178 K. The data at all six temperatures are fitted simultaneously to each kinetic scheme (see Results for further details of the fitting procedure).

Chance [7] which extended from 400 to 650 nm with the reference wavelength at 575 nm, a clockwise rotation of the entire spectrum may occur. This effect can be reduced by placing a diffuser plate between the sample and the photomultiplier [7]. On the basis of these observations De Fonseka and Chance [7] concluded that the differences in the normalized absorbance changes at 444–463 nm, 590–630 nm and 608–630 nm, seen by Clore and Chance [4,5] in both the reactions of fully reduced and mixed valence state membrane bound cytochrome oxidase with CO at low temperatures, could possibly be attributed to time dependent scattering changes. However, all distortions due to any possible time dependent scattering changes can be entirely avoided in kinetic recordings obtained using the dual wavelength technique by ensuring that the monitoring and reference wavelengths are not separated by more than 50 nm. This may be demonstrated by recording optical spectra over a small wavelength range, say 120 nm, with the reference wavelength at approximately the mid-point of the wavelength range, without using a diffuser plate between the sample and photomultiplier. Such spectra illustrating the recombination of CO with fully reduced cytochrome oxidase following photolysis of the fully reduced cytochrome oxidase-CO complex at 176 K in both the Soret and visible regions are shown in Figs. 1 and 2, respectively. In Fig. 1 the spectra extend from 415 nm to 495 nm with the reference wavelength at 463 nm, and in Fig. 2 from 530 nm to 655 nm with the reference wavelength at 603 nm. No clockwise rotation or other distortions of the spectra are seen. It should also be noted that if significant time dependent scattering changes were to take place and distort the observed kinetics, the kinetics of these changes would be zero order and therefore could not be accounted for by the introduction of an intermediate in a kinetic scheme which would follow first or higher order kinetics.

*Re-analysis of the data of Clore and Chance [4] at 444–463 nm and six temperatures in the 178–203 K range*

The experimental normalized absorbance changes (normalized with respect to the total absorbance change produced on flash photolysis, i.e. with respect to the states before and immediately after photolysis) versus  $\log t$  at 444–463 nm over the 178–203 K range for the reaction of fully reduced membrane bound cytochrome oxidase with CO obtained by Clore and Chance [4] are shown in Fig. 3. The computed normalized absorbance change at 444–463 nm,  $N_{444}(t)$  is given by:

$$N_{444}(t) = \sum_l \alpha_{444}(l) \cdot F_l(t)/B \quad (1)$$

where  $B$  is the total concentration of cytochrome oxidase;  $F_l(t)$  the concentration of the  $l$ -th species at time  $t$  obtained by numerical integration of the coupled simultaneous ordinary differential equations derived for each kinetic model with the initial conditions given in Table I; and  $\alpha_{444}(l)$  the relative absorption coefficient of the  $l$ -th species at 444–463 nm defined by

$$\alpha_{444}(l) = \Delta\epsilon_{444}^T(l-z)/\Delta\epsilon_{444}^T(x-z) \quad (2)$$

where  $\Delta\epsilon_{444}^T(l-z)$  and  $\Delta\epsilon_{444}^T(x-z)$  are the molar difference absorption coefficients between species  $l$  and  $z$ , and species  $x$  and  $z$ , respectively, at 444–463

TABLE II

VALUES OF THE CORRELATION INDICES FOR THE BEST FITS OF EACH COMPUTED CURVE WITH ITS CORRESPONDING EXPERIMENTAL CURVE SHOWN IN FIG. 3 FOR MODELS 1, 2 AND 3

The correlation index,  $C$ , is a measure of the distribution of residuals and is given by  $C = \Sigma R_i / (\Sigma R_i^2)^{1/2}$  where  $R_i$  is the residual at time point  $i$ . ( $R_i$  is given by  $R_i = (v_i - u_i)/\sigma$  where  $v_i$  is the observed value,  $u_i$  the calculated value at each time point  $i$ , and  $\sigma$  the standard error of the curve.) For  $|C| \lesssim 1$ , the distribution of residuals is random; for  $|C| \gg 1$ , the deviations between calculated and observed values are systematic (Clore and Chance [8]).

Curve	Correlation index		
	Model 1	Model 2	Model 3
203 K	-0.762	2.29	1.56
198 K	-1.03	-2.89	-2.62
193 K	1.18	0.576	-2.85
188 K	-1.29	-0.652	-1.82
183 K	1.02	1.18	1.29
178 K	-1.04	-2.26	1.50

nm and at a temperature  $T$ . The species corresponding to  $x$  and  $z$  (which from Eqn. 2 have relative absorption coefficients of 1 and 0 respectively) for Models 1, 2 and 3 are given in Table I.

All the experimental data at the six temperatures were fitted simultaneously using Eqn. 1 by optimizing the rate constants at a reference temperature  $T_D$  (chosen as 188 K), the corresponding zero-point activation energies ( $E_{A_i}^0$ ), and in the case of Model 1  $\alpha_{444}(I_C)$ , and in the case of Model 2  $\alpha_{444}(E)$ .

The S.D. values of the fits for each model are given in Table I; the correlation index ( $C$ , a measure of the distribution of residuals) for each best fit computed curve with its corresponding experimental curve for the three models is given in Table II; and the values and S.D.<sub>1n</sub> of the optimized parameters for the three models are given in Table III.

TABLE III

VALUES OF THE OPTIMIZED PARAMETERS OBTAINED BY FITTING MODELS 1, 2 AND 3 TO THE DATA IN FIG. 3

The reference temperature  $T_D$ , is 188 K. The S.D.<sub>1n</sub> of the optimized parameters are shown in parentheses.

Parameter	Model 1	Model 2	Model 3
$k_{+1}(T_D)$ ( $M^{-1} \cdot s^{-1}$ )	$1.23 \cdot 10^2$ (0.25)	$1.47 \cdot 10^2$ (0.97)	$3.17 \cdot 10^1$ (0.018)
$k_{-1}(T_D)$ ( $s^{-1}$ )	$3.61 \cdot 10^{-3}$ (0.41)	$2.27 \cdot 10^{-2}$ (0.94)	$1.35 \cdot 10^{-4}$ (1.3)
$k_{+2}(T_D)$ ( $s^{-1}$ )	$4.47 \cdot 10^{-2}$ (0.045)	$4.77 \cdot 10^{-2}$ (0.069)	—
$k_{-2}(T_D)$ ( $s^{-1}$ )	$9.22 \cdot 10^{-4}$ (0.24)	$6.46 \cdot 10^{-4}$ (0.36)	—
$E_{A_1}^0$ ( $kJ \cdot mol^{-1}$ )	$3.77 \cdot 10^1$ (0.052)	$1.11 \cdot 10^2$ (0.071)	$4.53 \cdot 10^1$ (0.012)
$E_{A_1}^0$ ( $kJ \cdot mol^{-1}$ )	$1.16 \cdot 10^2$ (0.037)	$1.53 \cdot 10^2$ (0.019)	$9.45 \cdot 10^1$ (0.19)
$E_{A_2}^0$ ( $kJ \cdot mol^{-1}$ )	$5.63 \cdot 10^1$ (0.041)	$5.19 \cdot 10^1$ (0.041)	—
$E_{A_2}^0$ ( $kJ \cdot mol^{-1}$ )	$2.37 \cdot 10^1$ (0.48)	$9.11 \cdot 10^1$ (1.4)	—
$\alpha_{444}(I_C)$	$7.43 \cdot 10^{-1}$ (0.078)	—	—
$\alpha_{444}(E)$	—	$1.16 \cdot 10^1$ (0.052)	—

Only Model 1 satisfies the triple requirement of a S.D. (1.70%) within the standard error of the data ( $2 \pm 0.36\%$ ), a random distribution of residuals for each curve ( $C \lesssim 1$ ) and good determination of the optimized parameters. Model 2 fails on two counts: the distribution of residuals is not random for the curves at 203, 198 and 178 K ( $C \gg 1$ ), and three of parameters,  $k_{+1}$ ,  $k_{-1}$  and  $E_{A-2}^0$ , are poorly determined. Model 3 fails on all three counts: the S.D. (3.5%) is greater than the standard error of the data, the distribution of residuals is not random for the curves at 203, 198, 192, 188 and 178 K ( $C \gg 1$ ), and one of the parameters,  $k_{-1}$ , is poorly determined.

Although more complex kinetic models are feasible, the corresponding increase in the number of parameters which would have to be optimized would result in an under-determined system. I therefore conclude, on the basis of the above data and analysis, that, over the 178–203 K range, Model 1 is the only model that satisfies the triple statistical requirement of Clore and Chance [8].

### *Spectral characterization of species $I_C$ in the Soret and visible regions*

The pure difference spectra (i.e. in 100% concentration) of species E and  $I_C$  minus  $II_C$  and of species  $I_C$  minus E may be obtained from the repetitive wavelength scanning difference spectra of Figs. 1 and 2, taken during the course of the reaction of fully reduced cytochrome oxidase with CO at 176 K, by the solution of a set of linear simultaneous equations of the form

$$\Delta A_i(t) = \sum_{l=1}^m F_l(t) \cdot \Delta \epsilon_i(l - II_C) \quad (3)$$

for each wavelength  $i$ , where  $\Delta A_i(t)$  is the observed difference in absorbance at the  $i$ -th wavelength between the reaction and reference samples (the latter being species  $II_C$ ) at time  $t$ ;  $F_l(t)$  is the computed concentration of the  $l$ -th species determined by numerical integration of the differential equations derived for Model 1 using the rate constants at 176 K calculated from the optimized values of the rate constants at 188 K and the corresponding zero-point activation energies given in Table III; and  $\Delta \epsilon_i(l - II_C)$  is the molar difference absorption coefficient at the  $i$ -th wavelength between the  $l$ -th species and species  $II_C$  obtained by solving Eqn. 3. The computed pure difference spectra of species E and  $I_C$  minus  $II_C$  and of species  $I_C$  minus E are shown in Fig. 4.

Comparing the difference spectrum of species  $I_C$  minus  $II_C$  with that of species E minus  $II_C$  (Fig. 4A), it can be seen that, although there are no differences in the wavelengths of the peaks and troughs (with the exception of a 3 nm red shift in the 542 nm trough of the species E minus  $II_C$  difference spectrum to 545 nm in the species  $I_C$  minus  $II_C$  difference spectrum), there are small differences in the intensities of the peaks and troughs and in the wavelengths of the isosbestic points. These differences, however, are too small to be detected in the repetitive wavelength scanning spectra of Figs. 1 and 2 taken during the course of the fully reduced cytochrome oxidase-CO reaction so that only a single set of isosbestic points are observed with no shifts in the wavelengths of the peaks and troughs, giving the apparent impression that there are only two species, E and  $II_C$ , involved in the reaction. In point of fact, the difference spectra in Figs. 1 and 2 provide a better reflection of the species  $I_C$  minus  $II_C$  difference spectrum than the species E minus  $II_C$  difference spectrum

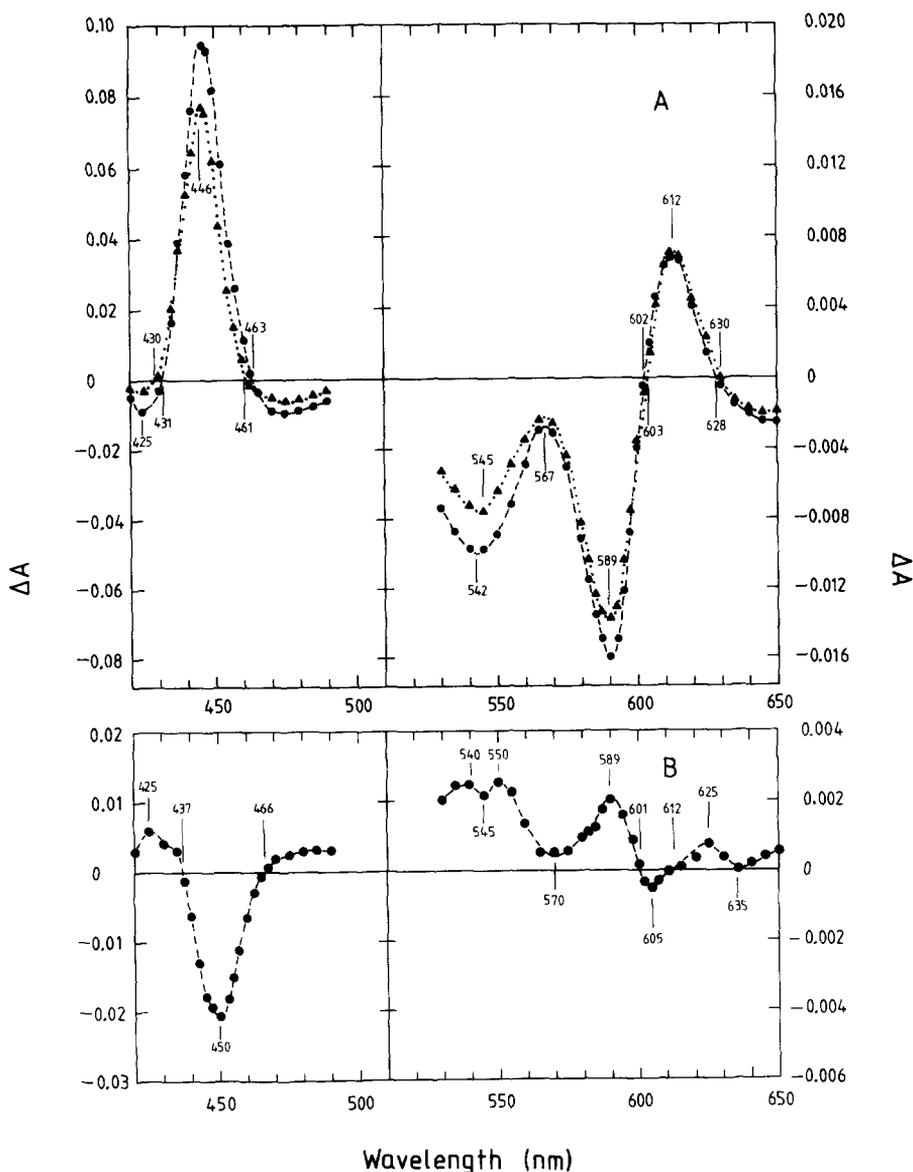


Fig. 4. Computed pure difference spectra of species E (●- - - -●) and  $I_C$  (▲- - - -▲) minus species  $II_C$  (A) and species  $I_C$  minus E (B) at 176 K. The pure difference spectra of the species are calculated from the repetitive wavelength scanning spectra in Figs. 1 and 2 taken during the course of the fully reduced cytochrome oxidase-CO reaction at 176 K using Eqn. 3 as described in the text. The concentration of each species is 5  $\mu M$ .

as the concentration of species E decreases rapidly, such that at the beginning of the third scan (94 s after photolysis) the concentration of species E is calculated to be less than 10% of the total cytochrome oxidase concentration.

Comparing the difference spectrum of species  $I_C$  minus E (Fig. 4B) with that of species  $II_C$  minus E (Fig. 4A), it can be seen that the intensities of the peaks and troughs in the former vary between 5 and 25% of those in the latter, there

are marked differences in the positions of the isosbestic points in the Soret region, and some prominent shifts in the wavelengths of the peaks and troughs.

### Concluding remarks

The present study confirms the finding of Clore and Chance [4] that the fully reduced cytochrome oxidase-CO reaction in the 178–203 K range proceeds by the kinetic mechanism given by Model 1 (see Table I) which involves an intermediate species  $I_C$ . Unfortunately, however, the spectral characterization of species  $I_C$  in the Soret and visible regions does not provide any definite information as to the chemical nature of species  $I_C$ , the spectra being consistent with any suggestion involving a perturbation of the iron atom of cytochrome  $a_3$ . The full characterization of species  $I_C$  will require a careful kinetic analysis of the changes in the infra-red C—O stretching frequency and in the magnetic susceptibility of cytochrome oxidase occurring during the course of the fully reduced cytochrome oxidase-CO reaction at low temperatures.

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