Characterization of the Intermediates in the Reaction of Membrane-Bound Mixed-Valence-State Cytochrome Oxidase with Oxygen at Low Temperatures by Optical Spectroscopy in the Visible Region

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The 'pure' difference spectra of the three species, I_M , II_M and III_M , formed in the low-temperature reaction of membrane-bound mixed-valence-state cytochrome oxidase with O_2 relative to unliganded membrane-bound mixed-valence-state cytochrome oxidase were characterized by optical spectroscopy in the visible region. The difference spectrum of species I_M was characterized by a peak at 590 nm and a trough at 608 nm, that of species II_M by a peak at 606 nm, and that of species III_M by a peak at 610 nm. A comparison with the difference spectra of species II_M and III_M obtained with soluble cytochrome oxidase [Clare, Andréasson, Karlsson, Aasa & Malmström (1980) *Biochem. J.* **185**, 155–167] revealed small but significant differences in the peak positions and bandwidths of the 605–610 nm absorption band.

The minimum functioning unit of mammalian cytochrome oxidase (EC 1.9.3.1) is thought to consist of two A-type haems, cytochromes *a* and *a₃*, differing only in the nature of their axial ligands, and two copper atoms, Cu_A and Cu_B (Malmström, 1973).

The addition of excess ferricyanide to the fully reduced cytochrome oxidase–CO complex results in the formation of a mixed-valence-state cytochrome oxidase–CO complex in which cytochrome *a* and Cu_A are in the ferric and cupric states respectively, and cytochrome *a₃* and Cu_B in the ferrous and cuprous state respectively (Leigh *et al.*, 1974; Chance *et al.*, 1975, 1978, 1979). This complex has been well characterized by e.p.r. (Clare *et al.*, 1980b), visible and near-i.r. optical spectroscopy and X-ray absorption-edge spectroscopy (Powers *et al.*, 1979), and no evidence for electron redistribution to excess ferricyanide has been found.

Initial low-temperature optical studies (Chance *et al.*, 1975) on the reaction of mixed-valence-state membrane-bound cytochrome oxidase with O_2 in intact bovine heart mitochondria led to the discovery of two optically distinct species known as compounds A_2 and C. Further detailed kinetic studies (Clare & Chance, 1978) with intact bovine heart mitochondria revealed the existence of three distinct optical species, I_M , II_M and III_M . Species I_M and III_M were thought to be equivalent to compounds A_2 and C respectively. However, the spectra of compounds A_2 and C were recorded at only a

single time point in the reaction (Chance *et al.*, 1975) and the experimental conditions (in particular the temperature) were different from those used by Clare & Chance (1978), so that it is impossible to assess their spectral purity (namely, it is possible that the spectra of compounds A_2 and C represent a mixture of species I_M , II_M and III_M in various proportions). More recently, studies on soluble cytochrome oxidase have characterized the optical spectra in the visible region and the e.p.r. properties of species I_M , II_M and III_M . No spectra, however, of all three species for the membrane-bound oxidase in intact bovine heart mitochondria have been reported.

In the present paper, the visible spectra of species I_M , II_M and III_M formed in the mixed-valence-state cytochrome oxidase– O_2 reaction in intact bovine heart mitochondria are presented and compared with those obtained with the soluble oxidase. Such a comparison is of importance with respect to future studies on these species using other physical techniques, as the species are identified by their characteristic difference spectra in the visible region with respect to unliganded mixed-valence-state cytochrome oxidase. This is particularly important with regard to species II_M and III_M , whose difference spectra are qualitatively similar, differing only by a few nanometres in the position of their α -band absorption maximum.

Materials and Methods

The membrane-bound mixed-valence-state cyto-

Abbreviation used: f.w.h.h., full width at half height.

chrome oxidase-CO complex was prepared as described by Clore & Chance (1978).

The kinetics of the mixed-valence-state cytochrome oxidase- O_2 reaction were monitored with a multi-channel dual-wavelength Johnson Foundation spectrophotometer as described previously (Clore & Chance, 1978). The wavelength pairs used were 590–630, 604–630 and 608–630 nm, the choice being based on previous spectral (Chance *et al.*, 1975; Clore *et al.*, 1980b) and kinetic (Clore & Chance, 1978) studies.

Optical spectra in the visible region were recorded with 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 transmitted light was monitored with 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, and from which corrective signals to the measuring wavelengths were read out, subtracting the stored baseline from the incoming data. The fixed reference wavelength employed was 630 nm. All optical spectra were recorded with a bandwidth of 5 nm, a scanning rate of 1.4 nm/s and a time constant of 3 s, at a temperature of 77 K.

Flat 2 mm-path-length optical cuvettes were used throughout for optical studies, and the temperature of the samples was maintained by a flow of cold N_2 and regulated by a copper/constantan thermocouple. Photolysis was carried out with a 200 J xenon flash lamp (pulse width 1 ms).

For the kinetic recordings, the reaction of mixed-

Fig. 1. Comparison of the experimental and computed normalized absorbance changes at 590–630, 604–630 and 608–630 nm (a) together with the computed kinetics of the individual intermediates (b) for the reaction of membrane-bound mixed-valence-state cytochrome oxidase with O_2 at 173 K

Symbols: Δ , 590–630 nm; $\bullet$, 604–630 nm; $\blacksquare$, 608–630 nm. The computed normalized absorbance changes are shown as continuous lines. The overall s.d. of the fit is 1.5% (compared with the standard error of the data of $1.5 \pm 0.2\%$) and the distribution of residuals is random. The optimized values of the parameters obtained by fitting the kinetic scheme given in eqn. (1) to the experimental data are given in Table 1. The experimental conditions are: 15 mg of bovine heart mitochondria/ml containing $5 \mu M$ -cytochrome oxidase (calculated from $\epsilon_{red.-ox.}^{605} = 24 \text{ mM}^{-1} \cdot \text{cm}^{-1}$; Van Gelder, 1963), 50 mM-mannitol, 50 mM-sodium phosphate buffer, pH 7.4, 30% (v/v) ethylene glycol, 5 mM-succinate, 1 mM- $K_3Fe(CN)_6$, 0.6 mM-CO and 0.5 mM- O_2 . (Note the change of time scale at 300 s.)

valence-state cytochrome oxidase with O₂ was initiated by flash photolysis of the mixed-valence-state cytochrome oxidase-CO complex at 173 K. The flash was approximately 99% saturating. CO did not recombine to any detectable extent in the presence of the relatively high O₂ concentration employed, as shown by control experiments where repeated flashes over the course of the reaction with O₂ only produced about 1% further photolysis of the CO complex, the intermediates formed in the reaction with O₂ not being susceptible to photolysis at the flash intensity used. The methods of kinetic analysis, numerical integration and non-linear optimization were as described previously (Clore & Chance, 1978).

For the optical spectra, the spectrum of the mixed-valence-state cytochrome oxidase-CO complex was initially stored in the digital memory. The CO complex was photolysed at 77 K, a temperature at which neither O₂ nor CO reacts with cytochrome oxidase (Chance *et al.*, 1975; Denis & Clore, 1979; Clore *et al.*, 1980a,b), and the difference spectrum of unliganded mixed-valence-state cytochrome oxidase minus the CO complex was recorded. The spectrum of the unliganded mixed-valence-state cytochrome oxidase was then stored in the digital memory and used as the reference spectrum for all further difference spectra. The reaction with O₂ was initiated by warming the photolysed samples to 173 ± 0.5 K in a liquid-N₂-cooled isopentane bath for a given time and stopped by cooling the sample to 77 K in liquid N₂, as described by Clore *et al.* (1980a,b). This procedure was repeated, and optical difference spectra of the reaction sample minus unliganded mixed-valence-state cytochrome oxidase were recorded after each warming and cooling cycle.

The temperature of 173 K was chosen so as to allow a direct comparison with the kinetic data and analysis given by Clore & Chance (1978).

Results and Discussion

In Fig. 1(a) the kinetics of the mixed-valence-state membrane-bound cytochrome oxidase-O₂ reaction at 173 K are shown at three wavelength pairs and clearly demonstrate the triphasic nature of the reaction. Looking at the 608–630 nm trace, we see that the first phase is characterized by a rapid decrease in absorbance; this is followed by a relatively rapid second phase and a slow third phase, during which the absorbance increases. The same changes are seen at 590–630 nm, except that the signs of the absorbance changes are reversed. With the 604–630 nm trace, however, no clearly distinct third phase is seen.

A kinetic analysis of a similar set of data has

already been presented, and the only mechanism that satisfied the triple requirement of an S.D. within the standard error of the data, a random distribution of residuals and good determination of the optimized parameters was a three-species sequential mechanism stated as (Clore & Chance, 1978):

Table 1. *Optimized values of the rate constants, relative absorption coefficients, scale factors and offsets obtained by fitting the kinetic scheme given in eqn. (1) to the experimental data in Fig. 1*

The normalized curves (digitized so that the maximum of each curve is given a value of 1.0 and the minimum a value of 0) are fitted by using the equation:

$$N_i(t) = \left[\sum_l \alpha_l(l) \cdot F_l(t) \cdot S_l \right] - D_i$$

where $N_i(t)$ is the normalized absorbance change at the i th wavelength pair, $F_l(t)$ the concentration of the l th species at time t obtained by numerical integration of the differential equations derived for the kinetic scheme given by eqn. (1), S_l is a scale factor and D_i an offset. $\alpha_l(l)$ is the relative absorption coefficient of the l th species at the i th wavelength pair defined by $\Delta\epsilon_l(l-z)/\Delta\epsilon_l(x-z)$, where $\Delta\epsilon_l(l-z)$ and $\Delta\epsilon_l(x-z)$ are the molar difference absorption coefficients between species l and x and between species x and z respectively at the i th wavelength pair; the reference species corresponding to x is species I_M for the 590–630 nm curve, and species III_M for the 604–630 nm and 608–630 nm curves, so that $\alpha_{590}(I_M) = \alpha_{604}(III_M) = \alpha_{608}(III_M) = 1.0$; the reference species corresponding to z is species III_M for the 590–630 nm curve, and species I_M for the 604–630 nm and 608–630 nm curves so that $\alpha_{590}(III_M) = \alpha_{604}(I_M) = \alpha_{608}(I_M) = 0$. The relative errors ($\Delta p/p$) of the optimized parameters are within ±0.10.

Parameter	Dimensions	Value
k_{+1}	M ⁻¹ ·s ⁻¹	60.0
k_{-1}	s ⁻¹	0.0191
k_{+2}	s ⁻¹	0.0164
k_{-2}	s ⁻¹	0.000904
k_{+3}	s ⁻¹	0.00141
$\alpha_{590}(E_M)$		0.409
$\alpha_{590}(II_M)$		0.167
$\alpha_{604}(E_M)$		0.398
$\alpha_{604}(II_M)$		0.997
$\alpha_{608}(E_M)$		0.591
$\alpha_{608}(II_M)$		0.732
S_{590}	M ⁻¹	3.36×10^5
S_{604}	M ⁻¹	2.89×10^5
D_{604}		0.445
S_{608}	M ⁻¹	3.29×10^5
D_{608}		0.657

where E_M is unliganded mixed-valence-state cytochrome oxidase. A comparison of the experimental data and computed best fit curves, obtained by the methods described by Clore & Chance (1978), is shown in Fig. 1(a). The overall s.d. of the fit is 1.5% (compared with the standard error of the data of $1.5 \pm 0.2\%$) and the distribution of residuals is random. The optimized values of the rate constants and relative absorption coefficients of the intermediates at the three wavelength pairs are given in Table 1, and are the same as those found by Clore &

Fig. 2. Difference spectra of the membrane-bound mixed-valence-state cytochrome oxidase-CO complex and of the reaction sample at various times after the initiation of the membrane-bound mixed-valence-state cytochrome oxidase- O_2 reaction at 173 K minus unliganded membrane-bound mixed-valence-state cytochrome oxidase.

All spectra were recorded at 77 K with a bandwidth of 5 nm, a scanning rate of 1.4 nm/s and a time constant of 3 s. The fixed reference wavelength was 630 nm. The difference spectrum of the mixed-valence-state cytochrome oxidase-CO complex (E_M -CO) minus unliganded mixed-valence-state cytochrome oxidase was recorded after photolysis of the CO complex at 77 K. The reaction with O_2 was initiated by warming the photolysed sample to 173 ± 0.5 K and stopped by cooling the sample back to 77 K in liquid N_2 . This procedure was repeated and optical difference spectra of the reaction sample minus unliganded mixed-valence-state cytochrome oxidase were recorded after each warming and cooling cycle. Experimental conditions were as in Fig. 1.

Chance (1978) within the errors specified. The relative errors of the optimized parameters ($\Delta p/p$) are within ± 0.10 . The computed time courses of the intermediates are shown in Fig. 1(b).

In Fig. 2 difference spectra of the reaction sample minus unliganded mixed-valence-state cytochrome oxidase at various time points during the membrane-bound mixed-valence-state cytochrome oxidase- O_2 reaction at 173 K are shown. A difference spectrum of the mixed-valence-state cytochrome oxidase-CO complex minus unliganded mixed-valence-state cytochrome oxidase is also shown for comparison in Fig. 2. The spectrum taken at 30 s after the initiation of the mixed-valence-state cytochrome oxidase- O_2 reaction at 173 K is representative of species I_M . At 30 s species I_M is at its maximum concentration (39% of the total oxidase concentration) and the concentrations of species II_M and III_M are negligible (12 and 0.5% respectively of the total oxidase concentration; see Fig. 1). The spectrum of species I_M is characterized by a peak at 590 nm and a trough at 608 nm. This is similar to the spectrum of compound A_2 reported by Chance *et al.* (1975) and Denis & Clore (1979), except that the former had a trough at 612 nm and the latter one at 606 nm. The spectra taken at 100, 200 and 300 s illustrate the conversion of species I_M into species II_M . At 300 s species II_M is at its maximum concentration (66% of the total oxidase concentration) and the concentrations of species I_M and III_M are relatively small (10 and 16% respectively of the total oxidase concentration; see Fig. 1). The spectrum of species II_M is characterized by a peak at 606 nm and corresponds closely to the spectra of compound C reported by Chance *et al.* (1975) and Denis & Clore (1979), which also had a peak at 606 nm. The spectra taken at 800 and 3000 s illustrate the slow conversion of species II_M into species III_M , characterized by a 4 nm red-shift in the absorption maximum of the α -band from 606 to 610 nm.

In Fig. 3 the computed 'pure' difference spectra of species I_M , II_M and III_M (i.e. in 100% concentration) are shown. These are obtained by the solution of a set of linear simultaneous equations of the form:

$$\Delta A_i(t) = \sum_{l=1}^m F_l(t) \cdot \Delta \epsilon_l(l-r) \quad (2)$$

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 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 the kinetic scheme given by eqn. (1) by using the optimized values of the rate constants given in Table 1, and $\Delta \epsilon_l(l-r)$ is the molar difference absorbance coefficient at the i th wave-

Fig. 3. Computed 'pure' difference spectra of species I_M , II_M and III_M minus unliganded membrane-bound mixed-valence-state cytochrome oxidase at 77 K

The difference spectrum of the mixed-valence-state cytochrome oxidase-CO complex (E_M -CO) minus unliganded membrane-bound mixed-valence-state cytochrome oxidase is also shown for comparison. The 'pure' difference spectra of the species (i.e. in 100% concentration) are calculated from the difference spectra in Fig. 2 by using eqn. (2) as described in the text. The concentration of each species is $5 \mu M$.

length between the l th species and the reference species r (in this case unliganded membrane-bound mixed-valence-state cytochrome oxidase) obtained by solving eqn. (2). It is noteworthy (i) that the intensities of the 590 nm peak and 608 nm trough in intermediate I_M are considerably greater (by approximately 2-fold) than those of the corresponding 586 nm peak and 606 nm trough of the mixed-valence-state cytochrome oxidase-CO complex, and (ii) that the intensity of the 606 nm peak in intermediate II_M is approximately the same as that of the 610 nm peak in intermediate III_M .

The spectral characteristics of the difference spectra of species I_M , II_M and III_M and of the mixed-valence-state cytochrome oxidase-CO complex minus unliganded membrane-bound mixed-valence-state cytochrome oxidase are collected in Table 2, and compared with those obtained by Clore *et al.* (1980b) with the soluble oxidase. It should be noted that, although the spectra of species I_M , II_M and III_M obtained with the membrane-bound oxidase in intact bovine heart mitochondria are qualitatively similar to those obtained with the soluble oxidase, there are small but significant differences in the positions of the peaks and troughs.

Although, as discussed by Clore *et al.* (1980b), the optical spectra of the species formed during the reaction of mixed-valence-state cytochrome oxidase with O₂ do not provide any conclusive information about the valence states of the metal centres, some useful information on the electronic properties of the species may still be extracted. More importantly, these difference spectra provide an easy means of identification for these species, and this is an essential prerequisite for further studies involving more elaborate physical techniques such as e.p.r., i.r., magnetic circular dichroism, laser Raman, Mössbauer and X-ray absorption-edge spectroscopy, all of which will be required for the full characterization of these species.

As discussed on previous occasions (Clore &

Table 2. Absorption maxima and minima of the difference spectra of species I_M , II_M and III_M and of the mixed-valence-state cytochrome oxidase-CO complex minus unliganded mixed-valence-state cytochrome oxidase in the visible region for the membrane-bound oxidase

The absorption maxima and minima for intermediates I_M , II_M and III_M may be obtained either from the spectra at which their respective computed concentrations are at a maximum, namely from the spectra trapped 30, 300 and 3000 s respectively after initiation of the membrane-bound mixed-valence-state cytochrome oxidase-O₂ reaction at 173 K shown in Fig. 2, or from the computed 'pure' difference spectra of the intermediates shown in Fig. 3. The corresponding absorption maxima and minima for the soluble oxidase are shown for comparison in parentheses (these are from the data of Clore *et al.*, 1980b). Abbreviations: p, peak; t, trough.

Species	β -Region			α -Region	
	521 (525) t	547 (547) p	567 (566) t	586 (586) p	605 (610) t
Mixed-valence-state cytochrome oxidase-CO complex					
I_M	524 (527) t	549 (549) p	568 (570) t	590 (592) p	608 (614) t
II_M	528 (529) t	550 (550) p	579 (575) t		606 (605) p
III_M	529 (530) t	551 (552) p	582 (582) t		610 (607) p

Chance, 1978, 1979; Clore *et al.*, 1980*a,b*), the spectrum of species I_M can be entirely accounted for by the formation of a cytochrome a_3 -O₂ bond best represented by the configuration $a_3^{2+\delta}$ -O₂^{-δ}, in which the charge localized on the iron of cytochrome a_3 is greater than +2.5 ($\delta > 0.5$).

The most unusual and in many ways the most interesting of the species formed in the low-temperature reactions of cytochrome oxidase with O₂ are species II_M and III_M, which are characterized by a high-intensity absorption band in the 605–610 nm region. This band is symmetric, indicating that it probably represents a single electronic transition. The bandwidths (defined as full width at half height, f.w.h.h.) of the 605–610 nm absorption band of species II_M and III_M are 490 cm⁻¹ and 540 cm⁻¹ respectively for the membrane-bound form compared with values from Clore *et al.* (1980*b*) of 355 and 410 cm⁻¹ respectively for the soluble form of cytochrome oxidase at 77 K. (All bandwidths are accurate to ± 5 cm⁻¹.)

A comparison of the spectral features of the 605–610 nm absorption band of species II_M and III_M for the membrane-bound and soluble oxidase shows that for the membrane-bound oxidase the peaks are red-shifted (Table 2) and the f.w.h.h. broader relative to those of the soluble oxidase. This is not, however, the case for the β -band around 550 nm, where there is no significant difference in peak position (Table 2) or f.w.h.h. between the membrane-bound and soluble oxidase for either species II_M or III_M (the f.w.h.h. of the band around 550 nm is 765 ± 5 and 855 ± 5 cm⁻¹ for species II_M and III_M respectively). This strongly suggests a significant interaction between the lipid membrane and the electronic transition responsible for the 605–610 nm band. This would be expected if the 605–610 nm band were due to a charge-transfer transition involving O₂, as the environment of the bound O₂ molecule, namely the 'pocket' containing the O₂-binding site (Chance,

1977; Sharrock & Yonetani, 1977; Denis & Clore, 1979), would be expected to be sensitive to the presence of a lipid membrane.

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