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A TEMPERATURE-INDUCED ABSORPTION BAND CENTRED IN THE REGION OF 666 nm RELATED TO THE CONFIGURATION OF THE ACTIVE SITE IN FROZEN CYTOCHROME OXIDASE

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Summary

The existence of a temperature-induced absorption band centred in the region of 666 nm is demonstrated for both membrane-bound and soluble cytochrome oxidase in the frozen state.

The 666 nm band is generated solely by an increase in temperature of both fully reduced and mixed valence state cytochrome oxidase in the presence of CO or O₂ within the 'pocket' containing the active site; it is not formed in the absence of both CO and O₂ from the sample.

The formation of the 666 nm band is entirely reversible when the temperature is decreased again and its formation is not dependent on the presence of liganded CO at the sixth coordination site of haem a_3 in the low temperature range (below -120°C) prior to photolysis.

The shape and intensity of the 666 nm band are not affected by the extent of CO recombination following flash and photolysis and temperature increase and are not affected by changes in the valence states of the four metal centres when the O₂ reaction is in progress.

Introduction

Cytochrome oxidase (EC 1.9.3.1), the terminal oxidase in the respiratory chain of all higher organisms, has been the focus of considerable interest due to

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Abbreviation: TMPD, *N,N,N',N'*-tetramethyl *p*-phenylene diamine.

its central role in biological oxidation and energy transduction. The minimum functioning unit of cytochrome oxidase is thought to contain two haems, a_3 and a , and two copper atoms [1–3]. One copper atom, termed Cu_A is EPR detectable [4] and thought to be magnetically isolated [5,6]; the other copper atom, termed Cu_B , is undetectable by EPR [4] and antiferromagnetically coupled to high-spin haem a_3^+ when in the cupric state [5–8].

The observation that the kinetics of the reactions of cytochrome oxidase with both O_2 [9] and CO [10,11] in the frozen state are pseudo-first-order, together with the demonstration of gas exchanges in the active site at low temperatures [12], suggests that a 'pocket' [13] for several ligand molecules exists near the active site that allows 'collisions' to occur at temperatures as low as -115°C . The linearity of Arrhenius plots from very low temperatures towards room temperature [9] would normally imply that the concentration of ligand within the 'pocket' is identical to that in the external solvent. At an O_2 concentration of $500\ \mu\text{M}$ in an aqueous solution, the average distance between O_2 molecules will be between 50 and $100\ \text{Å}$, suggesting that, on average, no more than one or two molecules could be in the 'pocket' at any one time. Such a conclusion, however, fails to take account of the observed kinetics and ignores factors such as the ten-fold increase in the solubility of both O_2 and CO in lipid, which would more than double the number of ligand molecules in the 'pocket'. The finding that the single C—O stretch band at $1963.5\ \text{cm}^{-1}$ for the fully reduced cytochrome oxidase-CO complex has a very narrow bandwidth ($\Delta\nu_{1/2}$, $6\ \text{cm}^{-1}$) indicates that the bound CO ligand must be isolated from the environment of the external solvent and experience uniform and stable solvation interactions [14,15]. This is consistent with the proposal of a ligand environment of a very non-polar hydrophobic character.

In the present study we have examined the effects of temperature changes on the spectra of fully reduced and mixed valence state cytochrome oxidase, both membrane-bound and soluble, in the presence and absence of CO and O_2 in order to explore the properties of the 'pocket' of cytochrome oxidase containing the active site, and the existence of a temperature-induced absorption band centred around $666\ \text{nm}$, specifically related to the presence of ligand molecules within the 'pocket', is demonstrated.

Materials and Methods

Biochemical methods

Beef heart mitochondria [16] (kindly provided by C.P. Lee) are suspended at 25°C in a medium comprising $0.1\ \text{M}$ mannitol, $50\ \text{mM}$ sodium phosphate buffer, $\text{pH}\ 7.2$, and $5\ \text{mM}$ succinate, and left for $10\ \text{min}$ (i.e., until anaerobiosis is established). The preparation is then cooled to 0°C and saturated with CO. Ethylene glycol is added (final concentration $30\%\ \text{v/v}$) and the preparation is resaturated with CO in order to ensure full anaerobiosis and CO saturation. The concentration of CO in the CO saturated preparation is $1.2\ \text{mM}$; the concentration of mitochondria is $20\ \text{mg/ml}$ which contains $6.67\ \mu\text{M}$ cytochrome oxidase (calculated from $\epsilon_{\text{red/ox}}$ ($605\text{--}630\ \text{nm}$) = $24.0\ \text{mM}^{-1} \cdot \text{cm}^{-1}$; [17,18]). Aliquots of concentrated soluble type I cytochrome oxidase [19] (kindly provided by T.E. King), which had a ratio of $\epsilon_{445}(\text{red})/\epsilon_{424}(\text{red})$ of 2.3 or better [20], are

reduced in a medium comprising 50 mM sodium phosphate buffer, pH 7.2, 8.3 mM ascorbate, 70 μM *N,N,N',N'*-tetramethyl *p*-phenylene diamine (TMPD), 2 μM cytochrome *c* (Sigma type III) and 1.2 mM CO. 30 min at room temperature are allowed for complete reduction of the soluble oxidase as checked by optical spectra taken at room temperature. Both preparations are stored in an airtight syringe at -21°C until further use, when they are transferred into 1-mm optical path cuvettes previously deaerated with CO for optical studies.

Oxygenation of the samples is carried out in the dark by the addition of O_2 saturated 30% v/v ethylene glycol solution (2 mM O_2 at -23°C ; Chance, B., personal communication). The sample is then further cooled to -78.5°C in an ethanol/solid CO_2 bath and stirred vigorously in the dark until the viscosity increases and freezing occurs. This procedure prevents ligand exchange between O_2 and the CO inhibited system [9,21–24,26]. The mixed valence state oxidase ($a_3^+ \text{COCu}_B^+ \cdot a^{3+} \text{Cu}_A^{2+}$) is prepared by adding potassium ferricyanide (at a final concentration of 3.4 mM) 30 s before oxygenation [9,22–24]. It should be noted that Lanne et al. [25] have tentatively suggested on the basis of optical and EPR studies that ferricyanide reacts slowly at room temperature with purified soluble cytochrome oxidase over a period of approximately 50 min to form a complex, the redox properties of which differ from those of the free soluble cytochrome oxidase. However, at the low temperatures employed in this study any possible complex formation with ferricyanide over the duration of the experiment will be negligible [24].

Under the conditions employed in these experiments, the pH of the sample remains constant over a wide range of temperatures. Experiments using phenol red as a pH indicator have demonstrated that no significant change in pH occurs on lowering the temperature of the mitochondrial preparation down to -130°C owing to the large buffering capacity of the high concentration of protein present in the preparation [26,27].

The choice of solvent in low temperature experiments with haem proteins is important if artefacts due to freezing are to be avoided. On cooling, the solvent should not crystallize, crack or precipitate the solute, but should form a glass with good light transmission. The solute should remain a monomer and not aggregate or denature. Ethylene glycol/water mixtures have these desired properties [28,29]. Because light transmission is lower in the glass than in the liquid state, a thin cell with a light path length of 1 mm is used. Extensive kinetic studies on the reaction of CO with myoglobin [28], protohaem [30] and cytochrome oxidase [10,11] over a very wide range of temperatures have shown that the nature of the solvent affects the outer barrier of these proteins governing the movement of ligand molecules from the external solvent into the 'pocket' of the protein, but only disturbs slightly the intramolecular processes. At the low temperatures of the experiments described in this paper, ligand exchange between the external solvent and the 'pocket' does not occur, due to the marked temperature dependence of diffusion limited processes [10,11, 28,30].

Spectrometric techniques

Dual wavelength scanning [31] is carried out with a Johnson Foundation spectrometer built from two J.Y. 20 monochromators (Instruments S.A. Jobin

Yvon, France) which employ aberration-corrected holographic gratings. The transmitted light is monitored using a multi-alkali photomultiplier for the 400–700 nm range (EMI 9592 b). The electrical output from the photomultiplier is coupled to a dual 1024-bit digital spectral memory (Varian C-1204) in which the characteristics of the baseline are stored and from which corrective signals to the measuring wavelengths are read out, subtracting the stored baseline from the incoming data. The fixed reference wavelength employed here was 630 nm.

Spectra recordings

The CO inhibited system is placed in the Dewar flask of the spectrometer through which is flowing thermoregulated nitrogen at a temperature in the range -120 to -130°C , and equilibrated for 10 min in the dark. The temperature of the measuring chamber is controlled by a copper-constantan thermocouple calibrated at 0°C (water/ice) and -78.5°C (between two pieces of solid CO_2). In the photolysis experiments, the sample is photolysed with a 200 J xenon flash lamp (pulse width, 1 ms), up to seven flashes being used in order to ensure 100% CO dissociation. In this temperature range, the rates of the reactions of both CO and O_2 with cytochrome oxidase are insignificant [9,24,26,38], and the spectrum of the photolysed cytochrome oxidase is recorded and stored in the digital memory for use as the baseline in the next part of the experiment. The thermoregulator is then set in the range -95 to -110°C resulting in the warming of the sample to the desired temperature in about 3 min and consequent activation of the reaction of cytochrome oxidase with O_2 or CO. In this temperature range, both reactions are slow enough to be followed in a repetitive scanning mode at a rate of 90 s per scan with a 256 nm interval. The output is the difference spectrum between the reaction state and the initial fully reduced or mixed valence state stored in the range -120 to -130°C after photolysis.

Results

Fig. 1 shows that as the temperature is increased from -120 to -100°C , an absorption band centered in the region of 666 nm appears in fully reduced CO-inhibited mitochondria. At the same time the absorption bands of the cytochromes shift to longer wavelengths. But in the absence of either CO or O_2 no such 666-nm band appears (Fig. 2).

The shape of the -100°C trace in Fig. 2 results essentially from thermal effects on the reduced cytochrome α and β bands. The same results are obtained with mixed valence state membrane bound cytochrome oxidase and fully reduced and mixed valence state soluble cytochrome oxidase.

As the 666-nm band is obtained with both membrane bound and soluble cytochrome oxidase, its formation cannot be due to (a) thermal effects on the mitochondrial membranes and (b) heterogeneities in the soluble cytochrome oxidase preparation [32]. We therefore conclude, on the basis of these observations, that the 666-nm band, generated by an increase in temperature, is related to the presence of CO within the pocket of cytochrome oxidase containing the active site. In order to determine whether the 666-nm band results from

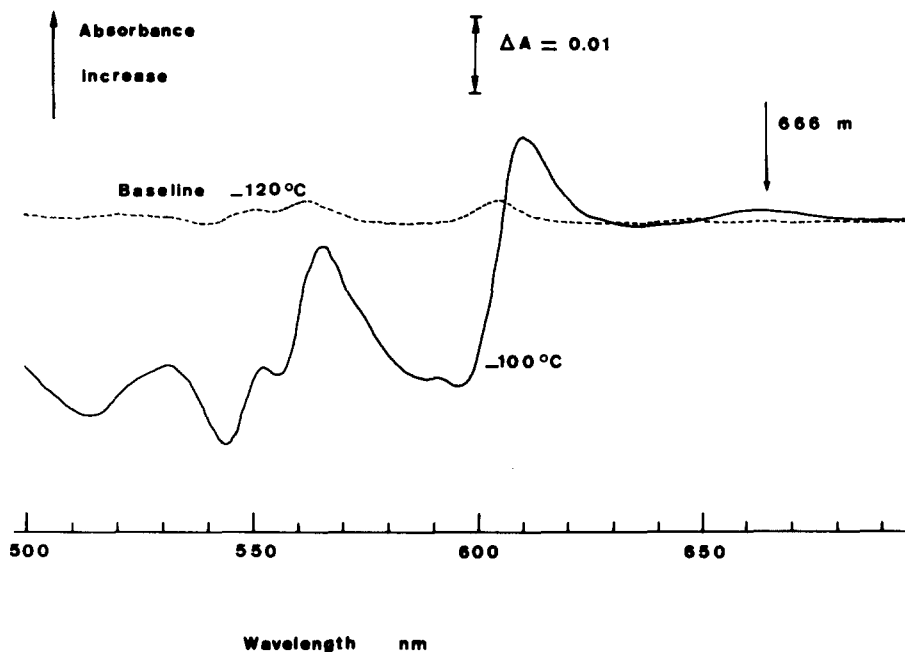


Fig. 1. Formation of the 666-nm band following an increase in temperature of the fully reduced membrane bound cytochrome oxidase-CO complex from -120 to -100°C . The spectrum stored at -120°C is used as the reference for the spectrum recorded at -100°C . Experimental conditions: 30 mg/ml beef heart mitochondria containing $10\ \mu\text{M}$ cytochrome oxidase, 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.

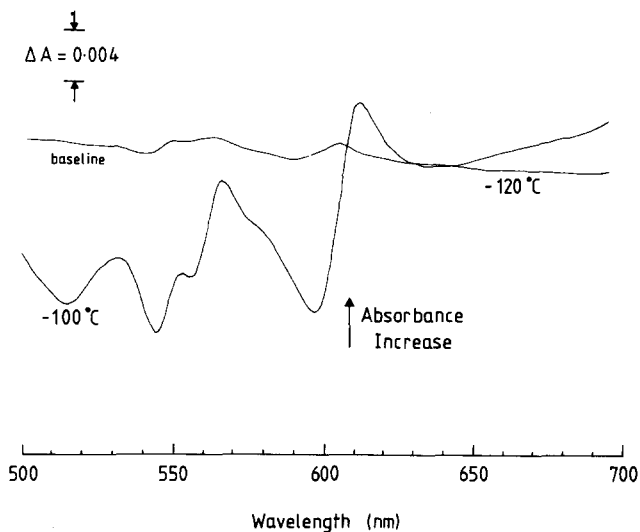


Fig. 2. Effect of temperature increase on the spectrum of fully reduced membrane bound cytochrome oxidase in the absence of both CO and O_2 . The spectrum stored at -120°C is used as the reference of the spectrum recorded when temperature is raised up to -100°C . Increasing the temperature to -100°C produces a spectrum whose shape is determined by thermal effects on the reduced cytochrome α and β bands. No 666-nm band, however, is formed. Experimental conditions as in Fig. 1 except that there is no CO and the concentration of beef heart mitochondria is 20 mg/ml containing $6.67\ \mu\text{M}$ cytochrome oxidase.

a relaxation between temperature-dependent configurations of the 'pocket' or between temperature-dependent configurations of the liganded sixth coordination site of haem a_3 , we have conducted a series of flash photolysis experiments.

In Fig. 3 the formation of the 666-nm band following an increase in

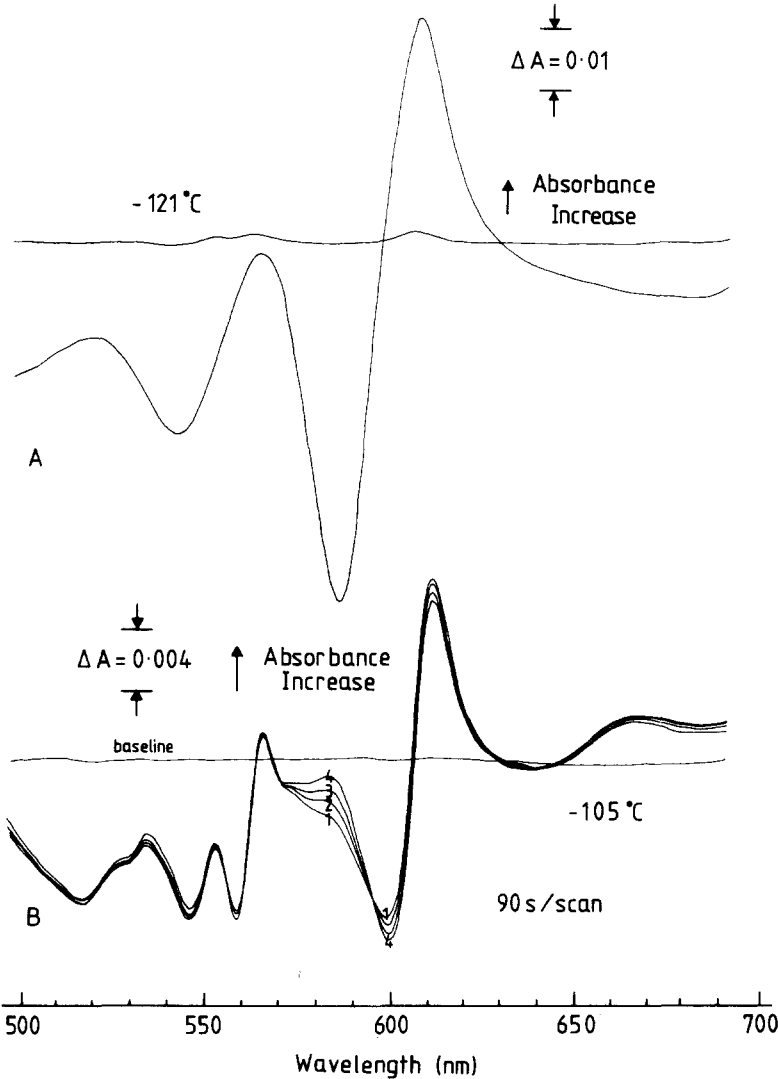


Fig. 3. Effect of temperature increase after CO photolysis of the fully reduced membrane bound cytochrome oxidase-CO complex in the absence of O_2 . A. Fully reduced membrane bound cytochrome oxidase minus CO compound difference spectrum recorded at -121°C after 100% CO photolysis. B. Successive spectra (recorded in repetitive scanning mode at 90 s per scan) of fully reduced membrane bound oxidase at -105°C minus the spectrum of fully reduced oxidase at -121°C following CO photolysis. These spectra show the 666-nm band, the thermal effects on the reduced cytochrome α and β bands similar to those observed in the absence of both CO and O_2 (Fig. 2), and a slow recombination of CO. Isosbestic points indicative of CO recombination are seen at 598, 577 and 560 and 602 nm, and the extent of CO recombination is only about 10% during the period of observation. The intensity and shape of the 666-nm band remains unchanged during the course of the reaction. The spectrum recorded at -121°C following 100% CO photolysis is used as the reference. Experimental conditions as in Fig. 2.

temperature of the photolysed fully reduced membrane bound cytochrome oxidase-CO complex is shown. The upper trace (Fig. 3A) is the fully reduced cytochrome oxidase (state after photolysis) minus CO compound (state before photolysis) difference spectrum, recorded at -121°C . The lower traces (Fig. 3B) represent difference spectra between samples at -105°C and at -120°C , following photolysis of the carbon monoxide complex. The 666-nm band, as well as the thermal effects on the reduced cytochrome α and β bands, is also seen in these spectra, although the haem a_3 is presumably unliganded (unlike the situation in Fig. 1). As explained in Materials and Methods, Figs. 3A and 3B do not use the same stored reference spectrum. In Fig. 3B, the spectrum of the state after photolysis, memorized at -121°C , is used as reference for the spectral changes after photolysis and followed at -105°C in a repetitive scanning mode (90 s per scan). Isosbestic points indicative of CO recombination are seen at 598, 577 and 560 nm [15]. The extent of CO recombination, monitored by the absorption changes around 587 and 602 nm, is seen to be minimal during the period of observation (about 10%). Similar results are obtained by flash photolysis of the mixed valence state membrane bound cytochrome oxidase-CO complex followed by similar temperature changes (Fig. 4). We note that, although spectral distortions on the red side are superimposed on the 666-nm band, the intensity of the 666-nm band is probably not directly related to the extent of CO recombination (see ref. 33 for an empirical method of correcting such spectral distortions). These results indicate that the 666-nm band is not dependent on the presence of CO liganded at the

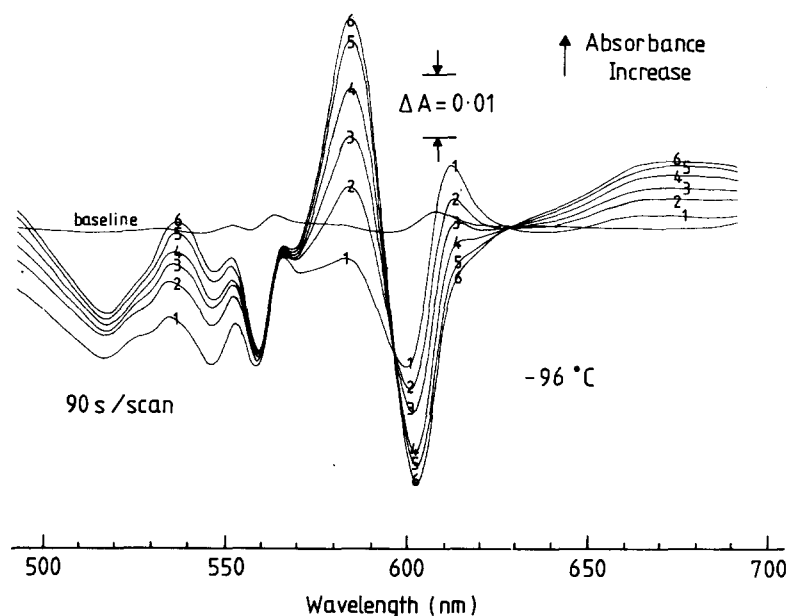


Fig. 4. Successive spectra of CO recombination at -96°C after 100% CO photolysis of the mixed valence state membrane bound cytochrome oxidase-CO complex at -121°C . The spectrum recorded at -121°C following 100% CO photolysis is taken as the reference for the spectra recorded in repetitive scanning mode (90 s per scan) at -96°C . Spectral distortion of the red side of the spectra is due to nonspecific thermal effect and has been attributed to ethylene glycol. Experimental conditions as in Fig. 2 plus 3.4 mM potassium ferricyanide.

sixth coordination site of haem a_3 at the higher temperature but is dependent solely on the presence of CO within the pocket of cytochrome oxidase containing the active site.

In all these experiments the initial state prior to photolysis is the cytochrome oxidase-CO complex. There therefore exists the possibility that the configuration of the pocket at the low temperature (i.e., below -120°C) is determined by the presence of CO liganded at the sixth coordination site of haem a_3 ; as conformational relaxation is insignificant at temperatures below -120°C [28], CO photolysis would leave the configuration of the pocket essentially unchanged. This possibility can be excluded by examining the formation and disappearance of the 666-nm band as a function of temperature following flash photolysis of the CO complex (Fig. 5). The baseline at -128°C is recorded after photolysis of the fully reduced soluble cytochrome oxidase-CO complex. As the temperature is increased to -104 and -102°C (traces 1 and 2 respectively), the 666-nm band is formed. When the temperature is lowered again to -130°C , the 666-nm band disappears (trace 3). The extent of CO recombination during the period of observation, as measured by the absorption change at 587 nm, is less than 10%. Identical results are obtained with mixed valence state soluble cytochrome oxidase and fully reduced and mixed valence state membrane bound cytochrome oxidase. We therefore conclude that the 666-nm band is not dependent on the presence of liganded CO at the sixth coordination site of haem a_3 in the low temperature range (i.e., below -120°C) prior to photolysis. It therefore seems likely that the temperature induced 666-nm band arises from a relaxation between temperature dependent configurations of the pocket of cytochrome oxidase containing the active site and is dependent on the presence of CO within the pocket.

In order to determine whether the 666-nm band can be generated in the

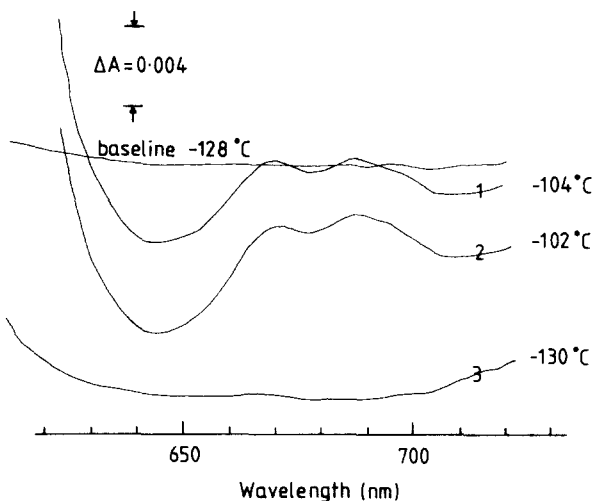


Fig. 5. Formation and disappearance of the 666 nm band as a function of temperature following CO photolysis of the fully reduced soluble cytochrome oxidase-CO complex at -128°C in the absence of O_2 . Experimental conditions, $8 \mu\text{M}$ soluble cytochrome oxidase, $2 \mu\text{M}$ cytochrome c , 30% v/v ethylene glycol, 8.3 mM ascorbate, $70 \mu\text{M}$ TMPD, 50 mM sodium phosphate buffer (pH 7.2) and 1.2 mM CO.

presence of other ligands in the pocket containing the active site and whether the shape and intensity of the 666-nm band is affected by alterations in the valence state of the four metal centres following the increase in temperature, we have conducted a series of photolysis experiments in the presence of O₂. During the oxygenation procedure at -21°C, O₂ displaces CO from the pocket; ligand exchange, however, at the sixth coordination site of haem a₃ does not take place as the rate of dissociation of CO from haem a₃ is insignificant at temperatures below -20°C in the dark [9,22-24,26].

Photolysis of the fully reduced membrane-bound cytochrome oxidase-CO complex in the presence of O₂ at -121°C followed by an increase in temperature to -105°C generates the 666-nm band and activates the reaction of fully reduced cytochrome oxidase with O₂ (Fig. 6). The upper trace (Fig. 6A) is the difference spectrum of fully reduced membrane-bound cytochrome oxidase minus CO compound recorded at -121°C. The lower traces (Fig. 6B) illustrate the reaction between O₂ and fully reduced cytochrome oxidase. The reaction is characterized by the initial formation of an absorption band centred in the region of 590 nm (traces 1 to 3) associated with the formation of compound A₁* [9,22,26]; this is followed by the disappearance of the 590-nm band and a further decrease in absorbance around 606 nm (traces 4 to 14) leading to the formation of compound B* [9,22,26] which illustrates the progression towards the fully oxidized state [34]. The intensity of the 666-nm band remains unchanged during the course of the reaction. In Fig. 7, the generation of the 666-nm band following CO photolysis of the fully reduced soluble cytochrome oxidase-CO complex in the presence of O₂ is shown in greater detail. The reference spectrum is the one of fully reduced state after photolysis at -128°C.

In Fig. 8 we show that photolysis of the mixed valence state membrane bound cytochrome oxidase-CO complex in the presence of O₂ at -121°C followed by an increase in temperature to -105°C also generates the 666-nm band and initiates the reaction of mixed valence state cytochrome oxidase with O₂. The reaction is characterized by the initial formation of a 590-nm band and a 606-nm trough (traces 1 and 2) associated with the formation of compound A₂** [9,22,26]; this is followed by the formation of an absorption band at 606 nm (traces 3 to 22) leading to the formation of compound C**. An isosbestic point at 595 nm characterizes the transformation of compound A₂** into compound C** [35]. As in the case of the temperature initiated activation of the reaction of fully reduced cytochrome oxidase with O₂, the intensity of the 666-nm band remains almost unchanged during the reactions provided that one takes into account the spectral distortions [33] on the red side of the spectra attributed to a side effect of ethylene glycol on the light scattering response to temperature change.

Comparing the 666-nm band obtained with fully reduced (Figs. 3 and 6) and mixed valence state (Figs. 4 and 8) cytochrome oxidase, we observe that the shape of the 666-nm band is affected by the initial valence state of the four

* Compound A₁ and B in the notation of Chance et al. [22] correspond to intermediates I and III in the notation of Clore and Chance [26].

** Compounds A₂ and C in the notation of Chance et al. [22] correspond to intermediates I_M and III_M in the notation of Clore and Chance [24].

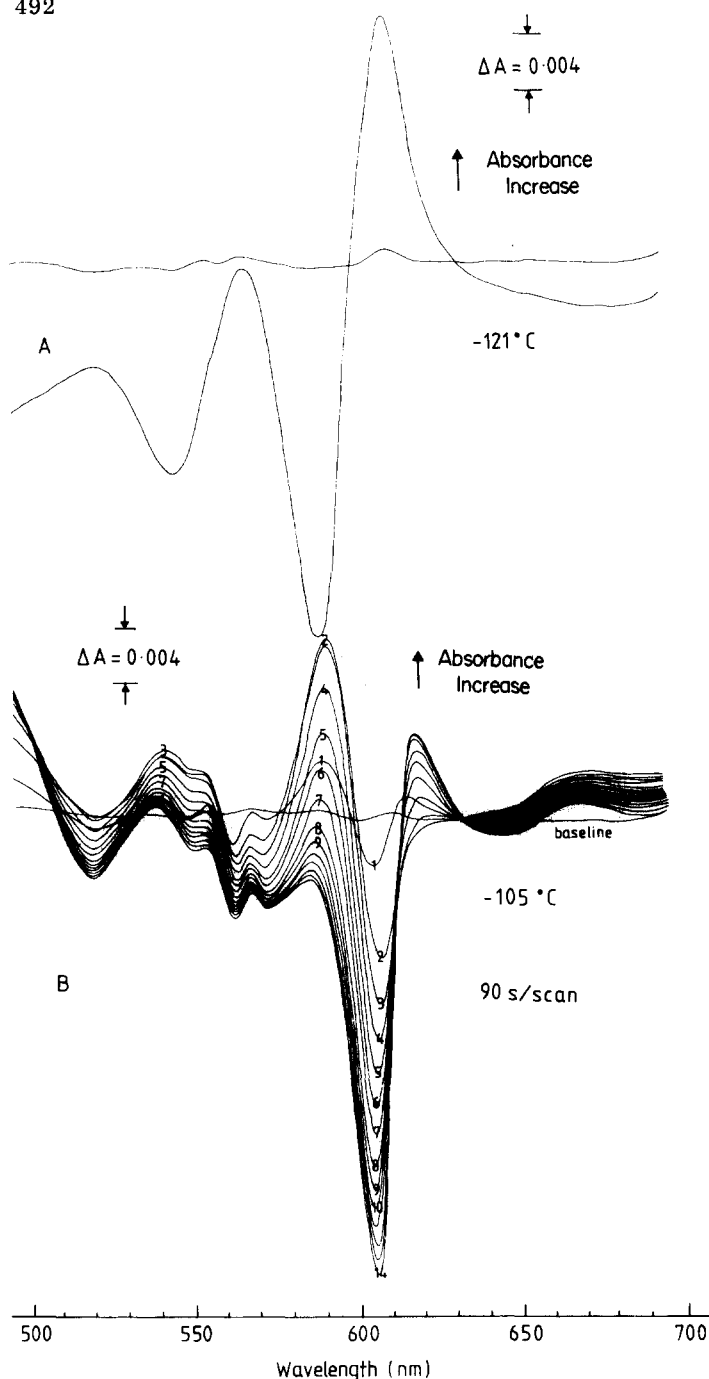


Fig. 6. Temperature activated reaction between O_2 and fully reduced membrane-bound cytochrome oxidase. A. Fully reduced membrane bound cytochrome oxidase minus CO compound difference spectrum recorded at -121°C after 100% CO photolysis. B. Successive spectra illustrating the course of the reaction of fully reduced cytochrome oxidase with O_2 . The reaction is characterized by the initial formation of an absorption band centered around 590 nm (traces 1 to 3) corresponding to the formation of compound A_1 . This is followed by the disappearance of the 590 nm band and a further decrease in absorbance around 606 nm (traces 4 to 14) leading to the formation of compound B. The spectrum of the fully reduced state recorded at -121°C after 100% CO photolysis is taken as the reference for the spectra recorded in repetitive scanning mode (90 s per scan) at -105°C . Experimental conditions: 10 mg/ml beef heart mitochondria containing $3.3 \mu\text{M}$ cytochrome oxidase, 30% v/v ethylene glycol, 0.05 M mannitol, 25 mM sodium phosphate buffer (pH 7.2), 2.5 mM succinate, 0.6 mM CO and 1.0 mM O_2 .

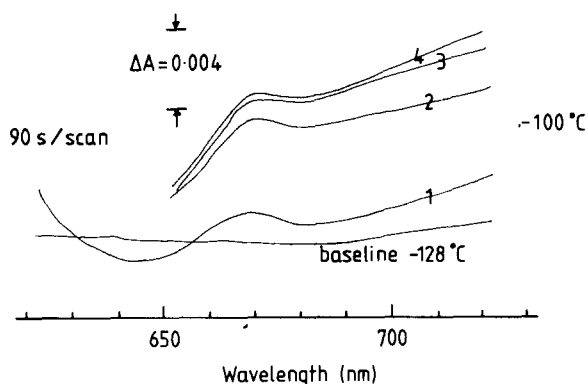


Fig. 7. Formation of the 666-nm band following an increase in temperature of fully reduced soluble cytochrome oxidase in the presence of O_2 . The spectrum of the fully reduced state recorded at $-128^\circ C$ after 100% CO photolysis is taken as the reference. The spectra are recorded at 90-s intervals. Experimental conditions: $4 \mu M$ soluble cytochrome oxidase, $1 \mu M$ cytochrome c, 30% v/v ethylene glycol, 4.15 mM ascorbate, $35 \mu M$ TMPD, 25 mM sodium phosphate buffer (pH 7.2), 0.6 mM CO and 1.0 mM O_2 .

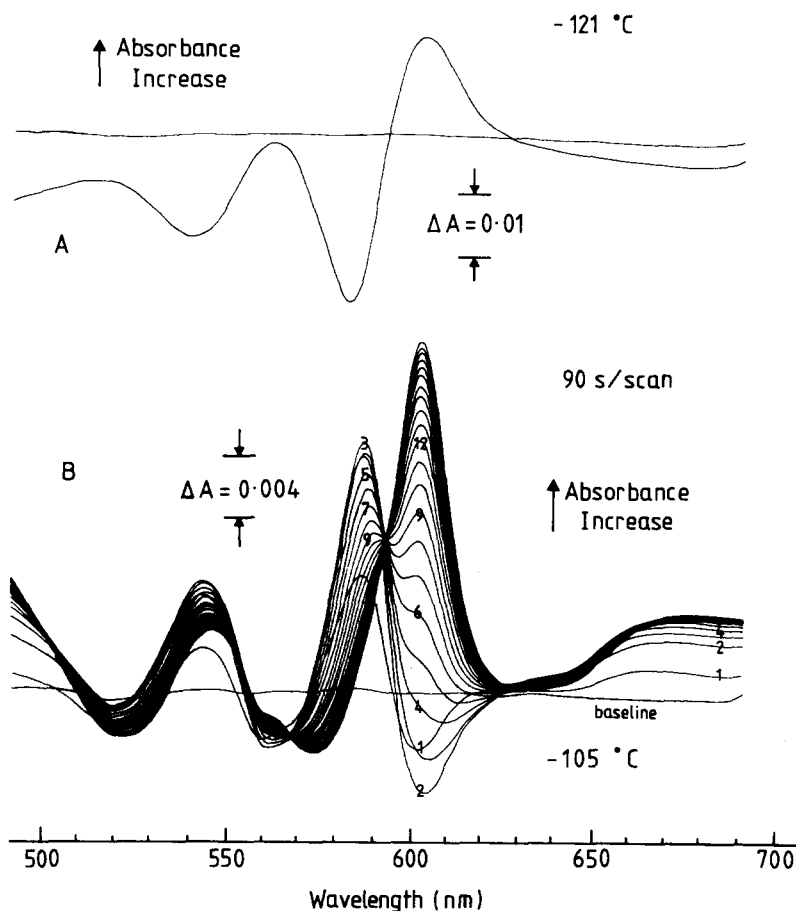


Fig. 8. Temperature activated reaction between O_2 and mixed valence state membrane bound cytochrome oxidase. A. Mixed valence state membrane-bound cytochrome oxidase minus CO compound difference spectrum recorded at $-121^\circ C$ after 100% CO photolysis. B. Successive spectra illustrating the course of the reaction of mixed valence state cytochrome oxidase with O_2 . The reaction is characterized by the initial formation of a 590-nm band and a 606-nm trough (traces 1 to 2) corresponding to the formation of compound A_2 . This is followed by the formation of a 606-nm band (traces 3 to 22) leading to the formation of compound C. An isosbestic point at 595 nm characterizes the conversion of compound A_2 into compound C. The spectrum of the mixed valence state recorded at $-121^\circ C$ after 100% CO photolysis is taken as the reference for the spectra recorded in repetitive scanning mode (90 s per scan) at $-105^\circ C$. Experimental conditions as in Fig. 6 plus 3.4 mM potassium ferricyanide.

metal centres prior to photolysis, being slightly distorted at the red side with consequent flattening of the 666-nm band in the case of mixed valence state cytochrome oxidase. However, no significant change in the shape of the 666-nm band is induced by the O₂ reaction with both fully reduced and mixed valence state cytochrome oxidase in the above conditions, in comparison with the temperature effect on the CO complexes in absence of oxygen. Further, the shape of the 666-nm band is not affected by the nature of the gas molecule in the pocket (CO or O₂; compare the shape of the 666-nm band in Fig. 3 with that in Fig. 6, and the band in Fig. 4 with that in Fig. 8).

Discussion

From the results obtained in the last section, the properties of the 666-nm band may be summarized as follows.

(i) It is generated solely by an increase in temperature of both fully reduced and mixed valence state cytochrome oxidase in the presence of CO and/or O₂ within the 'pocket' containing the active site; it is not formed in the absence of both CO and/or O₂ from the sample.

(ii) Its formation is entirely reversible when the temperature is decreased again.

(iii) Its formation is not dependent on the presence of liganded CO at the sixth coordination site of haem *a*₃ in the low temperature range (below -120°C) prior to photolysis.

(iv) It is formed when both membrane-bound and soluble cytochrome oxidases are used.

(v) Its shape and intensity are not affected by the extent of CO recombination following flash photolysis and temperature increases.

(vi) Its shape and intensity are not affected by changes in the valence states of the four metal centres following photolysis and increases in temperature (viz. the O₂ reactions).

The above properties indicate that the temperature induced 666-nm band is solely related to the presence or absence of ligand molecules (O₂ or CO) within the 'pocket' of cytochrome oxidase containing the active site. We suggest the following explanation for the formation of the 666-nm band. On warming the sample a new crystallization equilibrium is established inducing a new configuration of the pocket. The relaxation between these two temperature-dependent configurations would then be responsible for the formation of the 666-nm band. In the absence of both O₂ and CO from the pocket, temperature-induced configurational changes in the pocket do not occur, and the 666-nm band is not formed.

We must emphasize that the temperature induced 666-nm band described in this paper is totally unrelated to the 655-nm band described by Beinert et al. [36] and attributed to anti-ferromagnetic coupling between high-spin haem *a*₃³⁺ and Cu_B²⁺ [5]. The 655-nm band is observed at fixed temperature [34,37], the 666-nm band requires a change in temperature, the 655-nm band is observed in the reaction of fully reduced cytochrome oxidase with O₂ during the progression to the fully oxidized enzyme and occurs only at temperatures above -80°C with times of similar length [34,36]; the 666-nm band is independent of the cytochrome oxidase-O₂ reaction and is formed at much lower temperatures; the 655-nm band is not observed in the reaction of mixed valence state cytochrome oxidase with O₂ [34,35], the 666-nm band is seen when both fully

reduced and mixed valence state cytochrome oxidases are employed. Further, the 666-nm band is unrelated to the gas-dependent EPR shifts in the low spin ferric haem $g = 3$ signal described by Hartzell and Beinert [12] since the latter are observed at fixed temperature whereas the former is not.

We therefore conclude that the temperature-induced absorption band centred around 666 nm provides a sensitive probe with which to examine the configurational state of the pocket of cytochrome oxidase containing the active site.

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References

- 1 Lemberg, M.R. (1969) *Physiol. Rev.* **49**, 48–121
- 2 Muijsers, A.O., Tiesjema, R.H., Henderson, R.W. and Van Gelder, B.F. (1972) *Biochim. Biophys. Acta* **267**, 216–221
- 3 Caughey, W.S., Wallace, W.J., Volpe, J.A. and Yoshikawa, S. (1976) in *The Enzymes*, 3rd edn. (Boyer, P.D., ed.), Vol. 13, pp. 299–344, Academic Press, New York
- 4 Aasa, R., Albracht, S.P.J., Falk, K.-E., Lanne, B. and Vanngard, T. (1976) *Biochim. Biophys. Acta* **422**, 260–272
- 5 Palmer, G., Babcock, G.T. and Vickery, L.E. (1976) *Proc. Natl. Acad. Sci. U.S.A.* **73**, 2206–2210
- 6 Falk, K.-E., Vanngard, T. and Angstrom, J. (1977) *FEBS Lett.* **75**, 23–27
- 7 Thomson, A.J., Brittain, T., Greenwood, C. and Springall, J.P. (1976) *FEBS Lett.* **67**, 94–98
- 8 Thomson, A.J., Brittain, T., Greenwood, C. and Springall, J.P. (1977) *Biochem. J.* **165**, 327–336
- 9 Chance, B., Saronio, C. and Leigh, J.S. (1975) *J. Biol. Chem.* **250**, 9226–9237
- 10 Sharrock, M. and Yonetani, T. (1976) *Biochim. Biophys. Acta* **434**, 333–344
- 11 Sharrock, M. and Yonetani, T. (1977) *Biochim. Biophys. Acta* **462**, 718–730
- 12 Hartzell, C.R. and Beinert, H. (1974) *Biochim. Biophys. Acta* **368**, 318–338
- 13 Chance, B. (1977) *Biophys. J.* **17**, 241a
- 14 Volpe, J.A., O'Toole, M.C. and Caughey, W.S. (1975) *Biochem. Biophys. Res. Commun.* **62**, 48–53
- 15 Yoshikawa, S., Choc, M.G., O'Toole, M.C. and Caughey, W.S. (1977) *J. Biol. Chem.* **252**, 5498–5508
- 16 Low, H. and Vallin, I. (1963) *Biochim. Biophys. Acta* **69**, 361–374
- 17 Van Gelder, B.F. (1963) *Biochim. Biophys. Acta* **73**, 663–665
- 18 Van Gelder, B.F. (1966) *Biochim. Biophys. Acta* **118**, 36–46
- 19 Kuboyama, M., Yong, F.C. and King, T.E. (1972) *J. Biol. Chem.* **247**, 6375–6383
- 20 Gibson, Q.H., Palmer, G. and Wharton, D.C. (1965) *J. Biol. Chem.* **240**, 915–920
- 21 Chance, B., Graham, N. and Legallais, V. (1975) *Anal. Biochem.* **67**, 552–579
- 22 Chance, B., Saronio, C. and Leigh, J.S. (1975) *Proc. Natl. Acad. Sci. U.S.A.* **74**, 4777–4780
- 23 Chance, B., Saronio, C., Leigh, J.S., Ingledew, W.J. and King, T.E. (1978) *Biochem. J.* **171**, 787–798
- 24 Clore, G.M. and Chance, E.M. (1978) *Biochem. J.*, **173**, 811–820
- 25 Lanne, B., Malmstrom, B. and Vanngard, T. (1977) *FEBS Lett.* **77**, 146–150
- 26 Clore, G.M. and Chance, E.M. (1978) *Biochem. J.*, **173**, 799–810
- 27 Williams-Smith, D.L., Bray, R.C., Barter, M.J., Tsoponakis, A.D. and Vincent, S.P. (1977) *Biochem. J.* **167**, 593–600
- 28 Austin, R.H., Beeson, K.W., Eisenstein, L., Frauenfelder, H. and Gunsalus, I.C. (1975) *Biochemistry* **14**, 5355–5373
- 29 Douzou, P., Sireix, R. and Travers, F. (1970) *Proc. Natl. Acad. Sci. U.S.A.* **66**, 787–792
- 30 Alberding, N., Austin, R.H., Chan, S.S., Eisenstein, L., Frauenfelder, H., Gunsalus, I.C. and Nordlund, T.M. (1976) *J. Chem. Phys.* **65**, 4701–4711
- 31 Chance, B. and Graham, N. (1971) *Revs. Sci. Instrum.* **42**, 941–945
- 32 Gibson, Q.H. and Greenwood, C. (1965) *J. Biol. Chem.* **240**, 2694–2698
- 33 Denis, M. and Ducet, G. (1975) *Physiol. Veg.* **13**, 709–720
- 34 Denis, M. (1977) *FEBS Lett.* **84**, 296–298
- 35 Denis, M. and Chance, B. (1977) 11th BEBS Meet., Abstr. A4-11-716
- 36 Beinert, H., Hansen, R.E. and Hartzell, C.R. (1976) *Biochim. Biophys. Acta* **423**, 339–355
- 37 Chance, B., Leigh, J.S. and Waring, A. (1977) in *Structure and Function of Energy Transducing membranes* (van Dam, K. and van Gelder, B.F., eds.), BBA Library Vol. 14, pp. 1–10, Elsevier/North-Holland, Amsterdam
- 38 Clore, G.M. and Chance, E.M. (1978) *Biochem. J.* **175**, 209–225