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RESOLUTION OF TWO COMPOUND C-TYPE INTERMEDIATES IN THE REACTION WITH OXYGEN OF MIXED-VALENCE STATE MEMBRANE-BOUND CYTOCHROME OXIDASE

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Summary

The reaction of mixed-valence state membrane-bound cytochrome oxidase with oxygen has been studied by difference spectroscopy with reference to the unliganded state and by the low temperature technique of Chance and coworkers.

Three intermediates, compound A_2 and two compound C-type components denoted C_{606} and C_{610}, have been resolved in time and wavelength in the α region. Their optical properties are defined in the visible range. Compound A_2 disappearance and compound C_{606} formation exhibit first-order kinetics with identical rate constants: $2.4 \cdot 10^{-3}$ s^{-1} at —94°C. Compound A_2 has its α band maximum at 590 nm and shares an isosbestic point at 595 nm with the C_{606} species. The α band of this intermediate peaks at 606 nm. Compound C_{610} is the real end point of the reaction and its α band maximum appears at 610 nm.

Compound C_{606} is interpreted as resulting from the transfer of one electron from heme a_3 copper to oxygen and compound C_{610} as expressing a molecular reorganization due to the effect of the temperature. Structural requirements for the location of Cu_B in the active site are discussed.

It is concluded that the three observed compounds are the only intermediates formed in the reaction between oxygen and mixed-valence state membrane-bound cytochrome oxidase.

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Introduction

The low-temperature trapping technique introduced by Chance et al. [1] opened the way to the discovery of a series of intermediates in the oxygen reduction reaction by cytochrome oxidase [2,3]. From the beginning, two distinct initial conditions have been considered, the fully reduced state $a^{2+}Cu^{+}_{A} \cdot a_3^{2+}Cu^{+}_{B}$ and the mixed-valence state $a^{3+}Cu^{2+}_{A} \cdot a_3^{2+}Cu^{+}_{B}$ [4,5] of cytochrome oxidase in either the membrane-bound [2,3,6—9] or the isolated form [10].

The reaction with oxygen from the fully reduced state leads first to compound A_1 interpreted as the oxygen bound form [2,3]. The optical changes occurring after A_1 formation, called compound B by Chance et al. [2], are interpreted by others not as a single compound but as expressing a complex superposition of multiple redox states from both optical [11] and magnetic properties [12]. A kinetic analysis of compound B formation led Clore and Chance to propose initially a three-step sequential mechanism [13] which, from a recent optical and electronic paramagnetic resonance (EPR) study by Clore et al. [12] has been reinterpreted by a branch mechanism.

The reaction of oxygen with the mixed-valence state was characterized by the formation of compound A_2 (spectrally analogous to compound A_1) followed by the one of compound C [2]. Results from numerical analysis of corresponding kinetics have suggested the existence of a third intermediate from a three-step sequential mechanism [14]. The identification of these three intermediates (I_M, II_M, III_M) with the two optically resolved A_2 and C was unsatisfying [14].

By monitoring the reaction from redox levels in between the fully reduced state and the mixed-valence one, Chance et al. [9] have shown that as soon as heme *a* and presumably its associated copper, Cu_A, are oxidized, the reaction of the corresponding cytochrome oxidase molecule with oxygen goes to compound C (noted also C_2) formation while the complexes with reduced heme *a* react with O_2 to exhibit the compound B optical changes. Therefore the mixture of the above two initial conditions i.e. fully reduced and mixed-valence states, does not generate new process of intermediate formation.

We report here spectral evidence of the resolution in time and wavelength of two compound C-type intermediates after compound A_2 formation. These intermediates are denoted C_{606} and C_{610} according to the peak position of their α bands in our experimental conditions. The difference spectra referred to the unliganded mixed-valence state exhibit an isosbestic point at 595 nm between compounds A_2 and C_{606} which accumulates when the temperature is lower than —95°C. At higher temperature, compound C_{606} is rapidly converted into a second C-type species, C_{610}, characterized by an α peak position at 610 nm. Compound A_2 disappearance and compound C_{606} formation follow first-order kinetics with identical rate constants. The formation of the C_{606} species appears far less sensitive to the temperature than the one of the C_{610} species. This would suggest the involvement of different processes in their formation, for instance, electron transfer and molecular reorganization, respectively.

On the basis of these data we discuss the possibility for the presence of other intermediates. We also suggest some structural requirements involving heme a_3 and Cu_B. A preliminary report of this work has been presented in the abstract form [15].

Materials and Methods

Biochemical methods

Beef heart mitochondria [16] (kindly provided by Dr. C.P. Lee) are suspended at 25°C in a medium comprising 0.1 M mannitol, 50 mM sodium phosphate buffer, pH 7.2, and 5 mM succinate, and left for 10 min (i.e., until anaerobiosis is established). The preparation is then cooled at 0°C and saturated with CO. Ethylene glycol is added (final concentration, 30% 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 mM; the concentration of mitochondria is 20 mg/ml. Aliquots of concentrated soluble type I cytochrome oxidase [17] (kindly provided by Dr. T.E. King), which had a ratio of $\epsilon_{445}(\text{red})/\epsilon_{424}(\text{red})$ of 2.3 or better [18], 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 [19]). 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 [9,19,20]. The mixed-valence state oxidase, CO-inhibited ($a^{3+}Cu_A^{2+} \cdot a_3^{2+}COCu_B^{+}$) [4,5] is prepared by adding potassium ferricyanide (at a final concentration of 3.4 mM) 30 s before oxygenation [9,19,21].

Spectrometric techniques

Dual wavelength scanning [22] 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 base line 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 thermoregulated nitrogen is flowing 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). 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 [9,23] 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 −85 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. In this temperature range the reaction is slow enough to be followed in a repetitive scanning mode at a rate of 45 s per scan with a 128 nm interval. The output is the difference spectrum between the reaction state and the initial mixed-valence state stored in the range −120 to −130°C after photolysis.

This spectra-recording procedure was our main adaptation of the low temperature trapping technique of Chance and coworkers [1,2,9]. The possibility for side effects due to the temperature increase initiating the reaction has been investigated and published previously [23]. The usual thermal effect on absorption bands, i.e. narrowing of the bandwidth and peak shift to the lower wavelengths when the temperature is decreased, is minimized in the reported experiments. Indeed, the contribution of reduced heme a_3 in the α region is admittedly small and the broad and weak α bands of the oxidized respiratory chain are less sensitive to the temperature step initiating the reaction than the reduced ones [23]. Whatever the possible extent of this effect, it would consist in a fixed distortion between the two corresponding baselines and could not be confused with the kinetics of the reaction.

Results

The above experimental conditions have allowed us to resolve in time and wavelength two compound C-type intermediates after compound A_2 formation. Their appearance and the amount of the formed species are very sensitive to the temperature.

In Fig. 1A, spectra are referred to the mixed valence state spectrum stored at −125°C after 100% CO photolysis. Bottom traces exhibit the formation of compound A_2 at −99°C. Compound A_2 is well characterized by its 590 nm α band and the 606 nm trough. A β band with its maximum at 546 nm is associated with compound A_2 formation. The origin on the graph has been displaced to avoid the confusion which would have resulted from the superposition of the complete sequence. After completion of the absorbance increase at 590 nm the sample has been gradually warmed up to −94°C to clarify the display of the formation of the following compound which can be accumulated when the temperature is low enough. It is characterized by the absorbance increase at 606 nm and therefore is called compound C_{606}. A further step, raising the temperature to −89°C resulted after 10 min in a significant bandshift of 4 nm towards the higher wavelengths along with a final increase in absorbance. This temperature increase which only amounts to 6°C could not account in any case for such a bandshift. The spectral form at 610 nm ferreted out in this experiment, provides evidence for a third intermediate, noted C_{610}, in the reaction of the mixed-valence state cytochrome oxidase with O_2 in beef heart mitochondria.

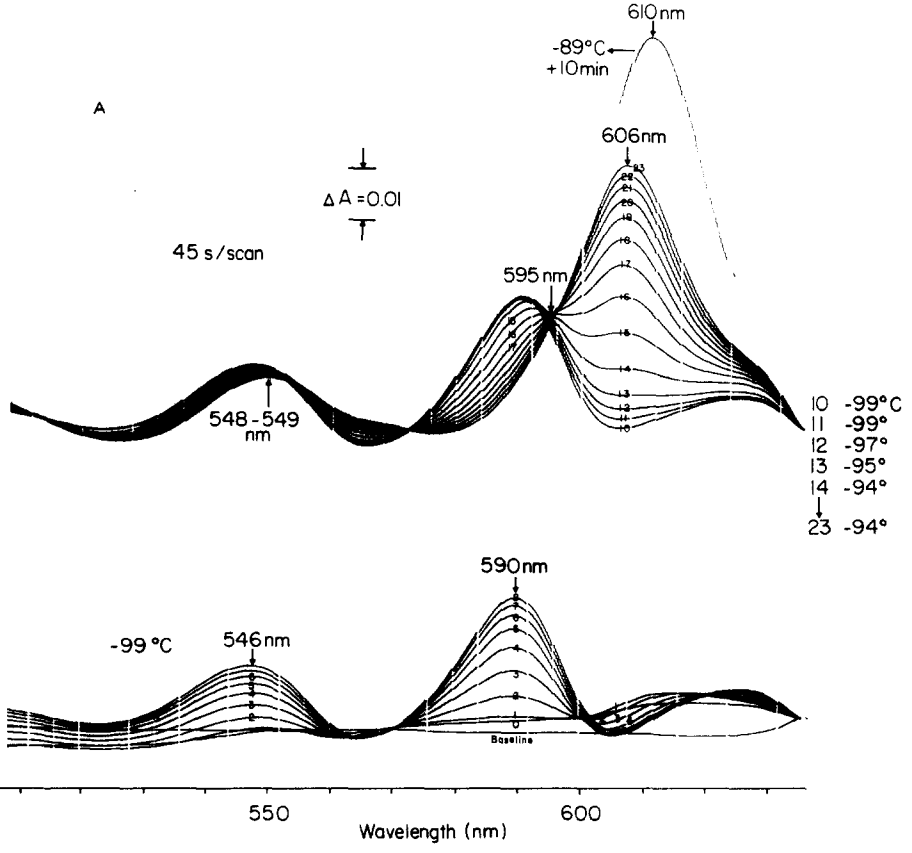
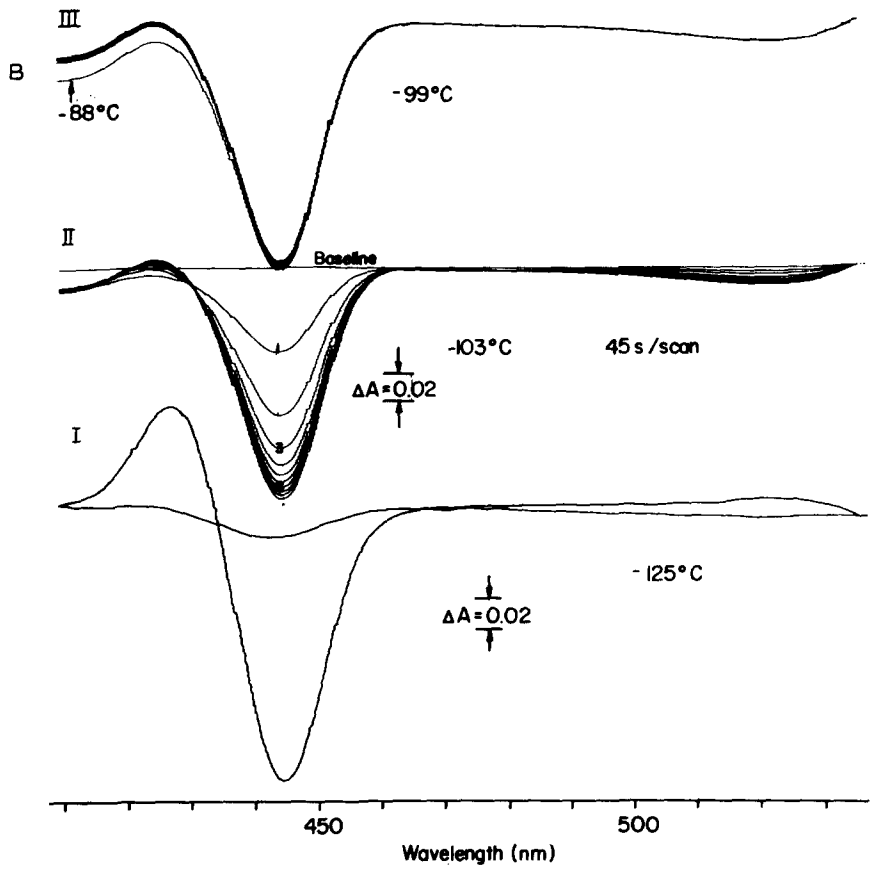


Fig. 1. Optical identification of three intermediates. (A) Accumulation of the C_{606} species, first formed intermediate after compound A_2. The mixed-valence state was memorized as the reference spectrum at —125°C after 100% CO photolysis. The reference wavelength was 575 nm, scan speed 45 s/scan, absorbance increase in the upward direction. Compound A_2 formation was followed at —99°C, then the origin on the graph has been changed to avoid confusion from overlapping traces. The sample has been warmed up to —94°C for the same purpose as well. In these conditions, the disappearance of compound A_2 is accompanied by the accumulation of a C-type species characterized by an α band maximum at 606 nm. An additional temperature increase to —89°C resulted after 10 min in a final absorbance increase and in a 4 nm shift of the α band towards the higher wavelengths. Beef heart mitochondria (10 mg/ml) were suspended in 30% ethylene glycol, 25 mM phosphate buffer, pH 7.4, 2.5 mM succinate, 0.6 mM CO, 1.4 mM potassium ferricyanide and 1 mM O_2, these values corresponding to the final concentrations. (B) Optical properties of the intermediates in the Soret region. Reference wavelength was at 460 nm. Experimental conditions as in (A) Trace I is the CO compound difference spectrum ($a_3^{2+}Cu_B^+$-$a_3^{2+}COCu_B^+$) recorded at —125°C after 100% CO photolysis. Absorbance increase is in downward direction. Spectra of traces II and III are referred to the unliganded state spectrum memorized at —125°C. Absorbance increase is in the upward direction. Spectra recorded repetitively during the reaction in progress at —103°C and —99°C up to the end-point at —88°C after 10 min, did not exhibit any special feature related to the intermediate formation, in contrast with the α region.

The spectral changes in the Soret region did not provide any resolution in time and wavelength of these different species. This is illustrated in Fig. 1B. Trace I is the CO compound difference spectrum recorded at —125°C. It corresponds to the difference between the photolysed mixed-valence state and the *CO-bound state* (i.e. $a_3^{2+}Cu_B^+$-$a_3^{2+}COCu_B^+$). Absorbance increase being in the downward direction in trace I, the CO compound difference spectrum is



characterized in the Soret region by a peak at 444 nm and a trough at 427 nm. It is very similar to the CO compound difference spectrum related to the fully reduced state of cytochrome oxidase in beef heart mitochondria [9]. In traces II and III absorbance increase is in the upward direction. Spectra of traces II were taken during the course of the reaction activated at −103°C and correspond essentially to compound A_2 formation. Increasing further the temperature to −99°C, then −88°C (traces III) did not provide after 10 min any particular bandshift or subsequent absorbance decrease at 444 nm. From recordings involving both the Soret and the α region (not shown) we can say that 80% of the total absorbance decrease at 444 nm is related to compound A_2 formation, the remaining 20% occurring during the formation of the compound C species.

A remarkable feature in Fig. 1A is the isosbestic point at 595 nm supporting the direct conversion of compound A_2 into the C_{606} species. Compound A_2 disappearance as well as compound C_{606} formation followed first-order kinetics with identical rate constants. Fig. 2 illustrates in semilog coordinates the kinetic of absorbance changes at 606 nm from spectra of Fig. 1A. The corresponding rate constant at −94°C is $2.4 \cdot 10^{-3}$ s^{-1}.

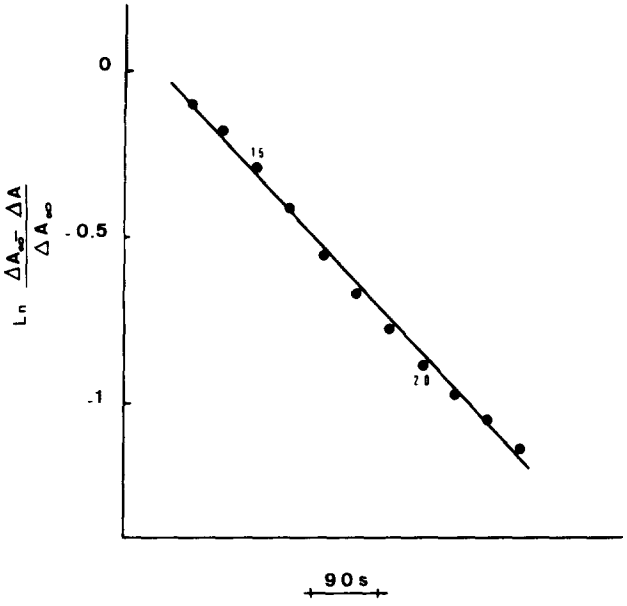


Fig. 2. Kinetics of species C_{606} formation in semilog coordinates. Data were collected from Fig. 1A at 606 nm. The data-point numbers are the ones of the corresponding spectra.

In order to better characterize the C_{610} species, the reaction has been monitored at a slightly higher temperature, namely —90°C as illustrated by Fig. 3. Trace I of Fig. 3A is the CO compound difference spectrum ($a_3^{2+}Cu_B^+$-a_3^{2+}CO-Cu_B^+) recorded at —120°C. It is characterized by a negative α band at 587 nm and a positive contribution at 607 nm. A negative β band is formed at 545 nm. The temperature being higher than in the case of Fig. 1A, the reaction proceeds more rapidly as shown by traces II of Fig. 3A. Spectrum number 4, the first one recorded after the maximum of compound A_2 formation, provides evidence of the formation of the C_{606} species with the beginning of compound A_2 disappearance. Due to the higher temperature, the C_{606} species does not accumulate as in Fig. 1A but is continuously converted into the C_{610} species, the real end-point of the reaction. This is demonstrated by the continuous bandshift from 606 to 610 nm where it stabilizes. We must point out that no temperature effect could be proposed to account for this bandshift, since the reaction proceeds at a fixed temperature, —90° C. As illustrated by Fig. 3B, this bandshift is linear in time.

Discussion

One must be careful when the difference spectral changes observed in the reaction of cytochrome oxidase with oxygen are discussed. The CO-bound state as reference makes data difficult to interpret because of the succession of two processes involving CO dissociation and O_2 reduction. The unliganded state is obviously the best reference to use when possible. The procedure described in Materials and Methods raises more errors in the initial time of the reaction

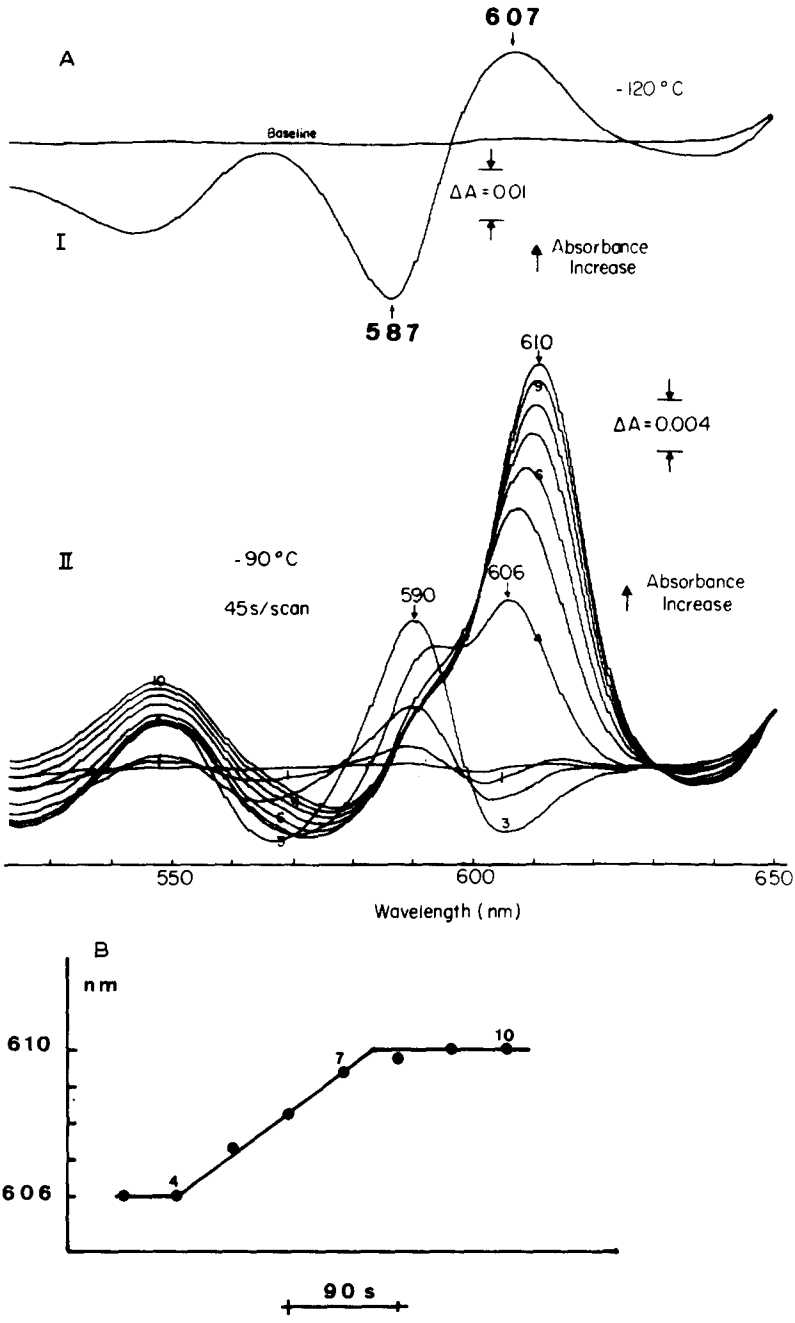


Fig. 3. Characterization of the C_{610} species at —90°C. (A) Experimental conditions are those of Fig. 1 except ethylene glycol, 15%, and potassium ferricyanide, 0.3 mM. The reference wavelength was at 630 nm. The upper trace I is the CO compound difference spectrum ($a_3^{2+}Cu_B^+$-$a_3^{2+}COCu_B^+$) recorded at —102°C after 100% CO photolysis. It is characterized by negative α and β bands at 587 and 545 nm, respectively, and a positive contribution at 607 nm. Spectra of traces II are referred to the unliganded state spectrum memorized at —120°C. They exhibit a continuous shift of the α band maximum during the C_{606} species formation and its simultaneous conversion into the C_{610} species. (B) Maximum α bandshift during the conversion of the C_{606} species into the C_{610} one as observed in traces II of Fig. 3A. The data-point numbers are the ones of the corresponding spectra. The bandshift appears linear in time.

(initiated by raising the temperature) than the single flash technique at constant temperature [2]. However, the resulting spectral changes are far more clear than those referred to the CO bound state. It is important to note that no spectral event can be missed in our procedure, since the reference spectrum is recorded at a temperature at which no reaction proceeds. We have discussed elsewhere a temperature effect revealed by our technique [23].

As previously reported [15,23], our procedure reveals the existence of β bands associated with the intermediates. They were not apparent in the spectra of the earlier works [2,3].

Spectra of Fig. 1A clearly identify the direct conversion of compound A_2 into the C_{606} species. This is evidenced by the presence of the isosbestic point at 595 nm and reinforced by the fact that compound A_2 disappearance and compound C_{606} formation are first-order processes with identical rate constants. The value of $2.4 \cdot 10^{-3}$ s^{-1} at $-94°C$ for the rate constant of compound C_{606} formation (see Fig. 2) should be compared to the value reported by Chance et al. [2] 0.5 s^{-1} at $-81°C$. Taking at face value the results in Ref. 2, i.e. the first-order velocity constant of 0.5 s^{-1} at $-81°C$ and the apparent energy of activation for compound C formation of 11.5 kcal/mol, our rate constant value should correspond to a temperature of $-110°C$ instead of $-94°C$. Experimental deviations and differences in calibrating the thermocouple [1,2,23] reasonably account for this apparent discrepancy, along with the fact that the compared values are related to the unresolved system.

The identification of compound A_2 has been extensively developed by Chance et al. [2,3,9,10]. The first-formed compound would result essentially from oxygen binding. This interpretation is also supported by the work of Babcock and Chang [24] on oxygen binding to ferrous heme *a* and its synthetic analog. Therefore, the first electron transfer to occur would be the one leading to the C_{606} species. We consider it as a one electron step, heme a_3 remaining reduced as we suggested it previously on the basis of the 655 nm band [11]. This interpretation differs from that of Erecinska and Wilson [25] who suggest a two electron step for the compound C formation. As the appearance of the C_{610} species can be prevented by a small difference in temperature (5°C in Fig. 1A), we suggest that the C_{610} species reflects some structural changes rather than an additional electron transfer.

Although a bandshift has been reported recently by Clore et al. [26] with a solubilized cytochrome oxidase preparation, we have never detected the C_{606} species when using this material. The α band of the observed compound C species peaked at 609 nm from the beginning of compound A_2 disappearance and for the lowest possible temperature (unpublished results; see also Ref. 10). The result of Clore et al. is very likely a side-effect of their procedure. Indeed changing the temperature up and down a number of times between 77 K and 173 K or 193 K would be sufficient to yield some structural perturbations of the crystalline state, thereby inducing spectral changes. It must be mentioned that their bandshift is only 2 nm and occurs in a time course equivalent to about 50 min at 193 K. It cannot be compared to the data of Fig. 3. The lack of the C_{606} species when using solubilized cytochrome oxidase could be due to a higher rate of conversion of the C_{606} compound into the C_{610} one. This difference would probably be induced by structural modifications related to the

preparation or the removal of the membrane itself.

The following points, (i) the first step of the reaction is oxygen binding with no electron transfer, (ii) the α bands of compounds A_2 and C_{606} share an isosbestic point at 595 nm, (iii) compound A_2 disappearance and compound C_{606} formation have first-order kinetics, (iv) compound C_{610} is a real end-point of the reaction (Refs. 4, 9 and this paper), lead us to conclude that there is no possibility for additional intermediates such as compound C_1 and C_3 [6] in the reaction of oxygen with the mixed-valence state cytochrome oxidase.

Our initial interpretation of the redox level of compound C, i.e. $a^{3+}Cu_A^{2+} \cdot a_3^{2+}O_2^-Cu_B^{2+}$ [11,15] has been supported by other works [6,9,10]. It remains valid in the present situation where two compound C-type species are resolved, C_{606} and C_{610}. Indeed, in our interpretation, only the C_{606} species involves an electron transfer, the C_{610} species, due to its sharp dependence on temperature, being supposed to result from a molecular reorganization rather than an additional electron transfer. In these conditions Cu_B would be the first electron donor to oxygen. This implies a specific position for the copper in the active site of cytochrome oxidase, i.e. a close proximity between Cu_B and O_2. This is not fulfilled by the imidazolate bridge model [20,21,27] which assigns the copper in a buried position in the protein, behind the heme. The μ-oxo bridging model proposed by Chance et al. [3] however is supported by Reed and Landrum [28]. On the basis of antiferromagnetic coupling interaction, they pointed out the compatibility of the μ-oxo model with very fast kinetic data. But in this model, the assumed compound C, Cu^{2+}-O-O-Fe^{4+} [3], would require the transfer of three electrons which is not supported by the present work. More recently, Chance and coworkers [9,29] suggested a binuclear model for the iron and copper of heme a_3 to account for the properties of compound C, though the nature of Cu_B as being the type I(blue) copper is controversial [30]. However the position of Cu_B being at the sixth coordination side of the heme seems strongly favorable.

As stressed by Chance et al. [9] more experiments are still to be done before one can propose an appropriate configuration for the active site of cytochrome *c* oxidase.

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