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Review

Time-resolved step-scan Fourier transform infrared investigation of heme-copper oxidases: implications for O_2 input and H_2O/H^+ output channels

Constantinos Koutsoupakis, Eftychia Pinakoulaki, Stavros Stavrakis, Vangelis Daskalakis, Constantinos Varotsis*

Department of Chemistry, University of Crete, 71409 Heraklion, Crete, Greece

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Abstract

We have applied FTIR and time-resolved step-scan Fourier transform infrared (TRS^2-FTIR) spectroscopy to investigate the dynamics of the heme-Cu_B binuclear center and the protein dynamics of mammalian aa_3, *Pseudomonas stutzeri* cbb_3, and caa_3 and ba_3 from *Thermus thermophilus* cytochrome oxidases. The implications of these results with respect to (1) the molecular motions that are general to the photodynamics of the binuclear center in heme-copper oxidases, and (2) the proton pathways located in the ring A propionate of heme a_3-Asp372-H_2O site that is conserved among all structurally known oxidases are discussed.

1. Introduction

Cytochrome oxidase sustains mitochondrial electron transport and linked ATP synthesis by catalysing the four-electron reduction of dioxygen to water [1]. Among oxygen-metabolizing heme enzymes, cytochrome oxidase is unique in being susceptible to high time resolution spectroscopic investigation of its reaction time course and of the intermediates, partially reduced oxygen structures that occur in the binuclear center during oxygen reduction [2–10]. Substantial progress has been made in gaining a molecular level of understanding of the structures for many intermediates in the O_2/enzyme reaction. The time-resolved resonance Raman (TR^3) approach used by Babcock's, Rousseau's, and Kitagawa's groups, although under different experimental conditions, allowed the identification of the heme a_3 iron-bound oxy, ferryl, and hydroxyl species that occur at various stages in the mechanism [2–10]. There appears now to be general consensus as to the occurrence of these species, although the precise details of the structure of the **P** intermediate and the relationship between the electron transfer events and its formation remain to be determined unambiguously.

The binding and release of small molecular ligands from the binuclear heme-Cu_B center in cytochrome oxidase is a complex process that involves ligand motion through the protein and extensive changes in the heme electronic structure and molecular conformation. Studies of CO adducts of cytochrome oxidase have been shown to be important probes of the active site, and photodissociation and recombination studies have revealed the dynamic processes that occur in the binuclear center [11–22]. In order to determine the molecular basis for the function of heme-copper oxidases, it is necessary to understand the interactions between the heme-bound ligands and the residues in their distal environment. Ultimately, this should lead to an understanding of the ligand kinetic behavior and the active site(s) properties. The binding and photodynamics of CO to heme-copper oxidases proceed according to Schemes 1 and 2, respectively. Where k_1 and k_{-1} represent the reversible binding of CO to Cu_B, and k_2 is the first-order transfer of CO from Cu_B to the heme Fe [23]. In Scheme 2, the Cu_B^{1+}-CO complex is fully developed within 1 ps subsequent to CO photolysis from heme a_3, demonstrating the absence of activation barriers to the CO transfer from heme a_3 to Cu_B [23]. The state denoted by an asterisk represents a non-

Abbreviations: CcO, cytochrome *c* oxidase; FTIR, Fourier transform infrared; TR-FTIR, time-resolved Fourier transform infrared; MCT, mercury cadmium telluride; RR, resonance Raman

* Corresponding author. Fax: +30-2810-393601.
E-mail address: varotsis@edu.uoc.gr (C. Varotsis).

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$$Fe^{2+}\,Cu_B^{1+} + CO \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} Fe^{2+}, Cu_B^{1+}\text{-}CO \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} Fe^{2+}\text{-}CO,\ Cu_B^{1+} \quad (1)$$

A B

Scheme 1.

equilibrium heme a_3 state characterized by an upshifted Fe–His stretching vibration which, in the mammalian aa_3 oxidase, relaxes to the equilibrium reduced species at times >10 μs [24].

Recently, we and others have applied time-resolved step-scan Fourier transform infrared spectroscopy (TRS^2-FTIR) to investigate the protein dynamics of heme-copper oxidases at room temperature [14,17,19–22]. It has been demonstrated that the dynamics of a large protein complex such as cytochrome oxidase can be resolved on the single vibrational level with TRS^2-FTIR spectroscopy. In this work, we present a comprehensive TRS^2-FTIR study of bovine cytochrome aa_3, cytochrome cbb_3 from *Pseudomonas stutzeri*, and ba_3 and caa_3 from *Thermus thermophilus*. The implications of these results with respect to the input O_2 and output H^+/H_2O channels are discussed.

2. Materials and methods

Mammalian CcO was isolated from beef hearts [7]. The purification of cbb_3, ba_3, and caa_3 oxidase was according to Refs. [19–21]. The pD solutions prepared in D_2O buffers were measured by using a pH meter and assuming pD = pH (observed) + 0.4. Dithionite reduced samples were exposed to 1 atm CO (1 mM) in an anaerobic cell to prepare the carbonmonoxy adduct and loaded anaerobically into a cell with CaF_2 windows and a 0.025-mm spacer. CO gas was obtained from Messer (Germany) and isotopic CO (^{13}CO) was purchased from Isotec. FTIR spectra were obtained from 400–500 μM samples with a Bruker Equinox 55 FTIR spectrometer equipped with a liquid nitrogen cooled mercury cadmium telluride detector. The 532-nm pulse from a Continuum Nd-YAG laser (7-ns width, 3 Hz) was used as a pump light (3–4 mJ/pulse) to photolyze the heme a_3-CO complex. The time-resolved FTIR spectra were obtained with spectral resolution of 8 cm^{-1} and 5-μs time resolution for the 5 μs–4 ms measurements or 100 μs for the 100 μs–80 ms measurements. Total number of slices was 800, with 40 slices before and 760 after the laser firing. A total of 10 co-additions per retardation data point was collected. Changes in intensity were recorded with an MCT detector, amplified (dc-coupled) and digitized with a 200-kHz, 16-bit, analog-to-digital converter. Blackman–Harris three-term apodization function with 32-cm^{-1} phase resolution and the Mertz phase correction algorithm were used. Difference spectra were calculated as $\Delta A = -\log(I_S/I_R)$. Optical absorbance spectra were recorded before and after FTIR measurements in order to assess sample stability with a Perkin-Elmer Lamda 20 UV–VIS spectrometer.

3. Results and discussion

Fourier transform infrared difference spectroscopy (FTIR) is a powerful structure-specific technique for exploring changes that occur to individual amino acid residues in a protein as a result of changes to redox and ligation states [13–22]. The FTIR difference approach has also been used to investigate the CO-photoproduct and the electrochemical oxidized-minus-reduced difference spectra of heme-copper oxidases. In the latter case, the perturbation is the redox state of the metal centers, whereas in the former is the photodissociation of CO from the heme. With the time-resolved step-scan approach, recently it was demonstrated that although the exogenous ligand vibrations (CO) were essentially identical between the room- and low-temperature spectra of photodissociated CO-cytochromes aa_3 and bo_3, significant differences exist in the protein bands between these temperatures. It was suggested that these differences originate from the fact that at room temperature, CO has dissociated from Cu_B, whereas in the low-temperature (80 K) the final state has CO coordinated to Cu_B [14].

3.1. Mammalian cytochrome aa_3 dynamics

Infrared and UV–VIS experiments of CO-bound aa_3 have shown that CO dissociates from Cu_B on a microsecond time scale ($k_{-1} = 5 \times 10^5$ s^{-1}), and a thermal equilibrium between Cu_B-bound and "free" CO in solution (K = 87 M^{-1}) is established [23]. The CO, however, does not return to heme a_3 for milliseconds ($k_2 = 1030$ s^{-1}). Fig. 1 shows the TRS^2 spectra ($t_d = 5$ μs–4 ms, 8 cm^{-1} spectral resolution) of mammalian fully reduced aa_3-CO subsequent to CO photolysis in D_2O. We detect no intensity changes of the 1965 cm^{-1} peak from $t_d = 5$–750 μs, which implies the lack

$$His\text{-}Fe^{2+}\text{-}CO,\ Cu_B^{1+} \xrightarrow{h\nu} \{His\text{-}Fe^{2+}\}^*,\ Cu_B^{1+}\text{-}CO \rightarrow CO\ (sol.),\ Cu_B,\ His\text{-}Fe^{2+} \rightarrow Fe^{2+}\text{-}CO,\ Cu_B^{1+} \quad (2)$$

Scheme 2.

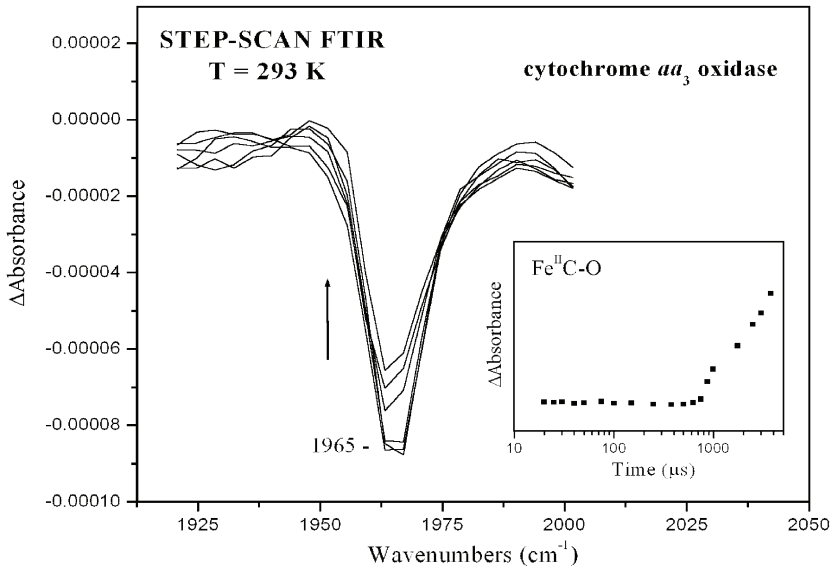

Fig. 1. Time-resolved step-scan FTIR difference spectra of the photolyzed CcO-CO complex at 25, 100, 500, 1000, 2000, and 3500 μs. Inset: Plot of the 1965 cm^{-1} ($\nu_{FeC\text{-}O}$, squares) mode vs. time subsequent to CO photolysis.

of CO rebinding to heme a_3. At times longer than 750 μs, the intensity increase of the 1965-cm^{-1} peak signals the onset of ligand rebinding to heme a_3. This behavior is analogous to that observed by Bailey et al. [14] and Findsen et al. [24]. The former authors proposed that on the time scale CO leaves Cu_B ($t_{1/2}=1.5$ μs), there are no intrinsic heme barriers to recombination with heme a_3 because typical geminate recombination rates are nanoseconds. Thus, another barrier must form which prevents CO recombination prior to 1 ms, and suggested as possible candidates either a local protein conformational change or the binding of an endogenous ligand which blocks the distal ligation site. Time-resolved ps RR experiments [25] have demonstrated that heme a_3^{2+} is five-coordinate, high spin, with proximal histidine ligation to the heme at the earliest times following CO photodissociation, and thus, no endogenous ligand blocks the distal binding site of heme a_3. Thus, it is highly unlikely that the barrier is the result of an endogenous ligand binding that blocks the distal ligation site, and a local protein conformational change is the most probable cause of its formation. Obviously, the heme a_3-Cu_B pocket is constructed in such a manner that results in rapid migration of the photodissociated CO from heme a_3 to Cu_B and slow recombination of CO to heme a_3.

3.2. Cytochrome cbb_3 dynamics

The microaerobic cbb_3-type cytochrome *c* oxidase isolated from *P. stutzeri* is an isoenzyme in the family of cytochrome *c* oxidases (CcO) [19]. It contains three *c*-type low-spin hemes, one low-spin *b*-type heme, and a heme b_3-Cu_B binuclear center. The structural characteristics of the binuclear center in cbb_3 are unique when compared to those of aa_3 type oxidases; it lacks the hydroxyethylfarnesyl side chain and the highly conserved among the heme-copper oxidases tyrosine 244, both of which have been proposed to play a crucial role in the properties of the binuclear center. Data on the photodynamics of the CO-bound adduct of cytochrome cbb_3 in conjunction with those of the aa_3-type oxidase can be interpreted to yield specific information concerning electronic and heme/Cu_B geometric properties, and heme/Cu_B−axial ligand bonding interactions. The time-resolved FTIR data revealed that in cbb_3-type oxidase the decay of the transient Cu_B-CO complex is concurrent with the formation of the heme b_3-CO complex, and the ν(CO) of Cu_B at 2065 cm^{-1}, despite the lack of the cross-link tyrosine 244 is similar to that observed in cytochrome aa_3 [23] and cytochrome bo_3 [14]. The former observation contrasts sharply with the well-known behaviour of CO recombination to the heme Fe of cytochrome aa_3 and bo_3-type oxidases. The cbb_3 is the first heme-copper oxidase reported in which the decay of the Cu_B-CO complex is accompanied by the formation of the Fe-CO complex at room temperature. It is also the only oxidase with an open heme-copper structure lacking the α-form of the heme, thus the distal interactions due to Cu_B and its local environment, and also lacking the hydrogen bonding between the farnesyl hydroxyl and the tyrosine hydroxyl which couples together the heme a_3 and Cu_B.

3.3. Cytochrome caa_3 dynamics

Fourier transform infrared and step-scan time-resolved FTIR difference spectra have been reported for the carbonmonoxy cytochrome caa_3 from *T. thermophilus* [21]. A major C−O mode of heme a_3 at 1958 cm^{-1} and two minor modes at 1967 and 1975 cm^{-1} (7:1:1) have been identified at room temperature, and remained unchanged in H_2O/D_2O exchange. The time-resolved FTIR data indicate that the

Table 1
Kinetic parameters for CO binding to mammalian aa_3, cbb_3, caa_3 and ba_3 from *T. thermophilus*

	k_2 (s^{-1})	k_{-1} (s^{-1})	Reference
aa_3	1030	7×10^5	[23]
cbb_3	4.8×10^3	5.8×10^3	[19]
caa_3	34.1	2.3×10^4 (35%) 36.3 (65%)	[21]
ba_3	28.6	34.5	[20]

transient Cu_B^{1+}-CO complex is formed at room temperature as revealed by the CO stretching mode at 2062 cm^{-1}. Therefore, the caa_3 enzyme is the only documented member of the heme-copper superfamily whose binuclear center consists of an a_3-type heme of a β-form and a Cu_B atom of an α-form. These results illustrate that the properties of the binuclear center in other oxidases resulting in the α-form are not required for enzymatic activity. Dissociation of the transient Cu_B^{1+}-CO complex is biphasic. The rate of decay is 2.3×10^4 s^{-1} (fast phase, 35%) and 36.3 s^{-1} (slow phase, 65%). The observed rate of rebinding to heme a_3 is 34.1 s^{-1}. Table 1 summarizes the CO kinetic properties in heme-copper oxidases.

3.4. Cytochrome ba_3 dynamics

We have reported the first evidence for the existence of the equilibrium Cu_B^{1+}-CO species of CO-bound reduced cytochrome ba_3 from *T. thermophilus* at room temperature [20]. The frequency of the C–O stretching mode of Cu_B^{1+}-CO is located at 2053 cm^{-1} and remains unchanged in H_2O/D_2O exchanges and between pH/pD 5.5 and 9.7, indicating that the chemical environment does not alter the protonation state of the Cu_B histidine ligands. The time-resolved step-scan FTIR difference spectra indicate that the rate of decay of the transient Cu_B^{1+}-CO complex is 34.5 s^{-1} and rebinding to heme a_3 occurs with $k_2 = 28.6$ s^{-1}. The rate of decay of the transient Cu_B^{1+}-CO complex displays similar time constant as the absorption changes at 1694(+)/1706(−), attributed to perturbation of the heme a_3 propionates (COOH). The ν(C–O) of the transient Cu_B^{1+}-CO species is the same as that of the equilibrium Cu_B^{1+}-CO species and remains unchanged in the pD range 5.5–9.7, indicating that no structural change takes place at Cu_B between these states. The transient binding of CO to Cu_B is dynamically linked to structural changes at the ring A propionate of heme a_3 (1694/1706 cm^{-1}) and concomitantly to a change in the local environment of Asp372 (1726 cm^{-1}).

We have also reported the first study of O_2 migration in the putative O_2 channel of cytochrome ba_3 [26,27], and its effect to the properties of the binuclear heme a_3-Cu_B center of cytochrome ba_3 from *T. thermophilus* [22]. The FTIR spectra of the ba_3-CO complex demonstrate that in the presence of 60–80 μM O_2, the ν(C–O) of Cu_B^{1+}-CO at 2053 cm^{-1} (Complex **A**) shifts to 2045 cm^{-1}, and remains

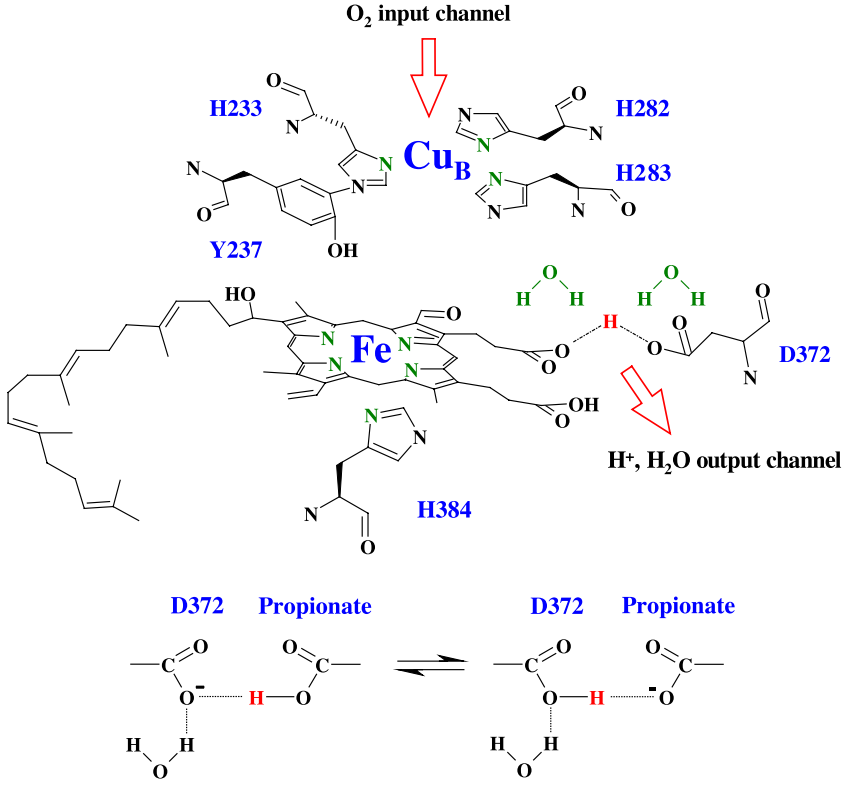

Fig. 2. Model for input/output channels in heme-copper oxidases. The residues are numbered following the sequence of cytochrome ba_3 oxidase from *T. thermophilus* (For color see online version).

unchanged in H_2O/D_2O exchanges and in the pH 6.5–9.0 range. The frequencies, but not the intensities, of the C–O stretching modes of heme a_3-CO (complex **B**), however, remain unchanged. The change in the ν(CO) of complex **A** results in an increase of k_{-2}, and thus to a higher affinity of Cu_B for exogenous ligands. The step-scan time-resolved FTIR difference spectra indicate that the rate of decay of the transient Cu_B^{1+}-CO complex at pH 6.5 is 30.4 s^{-1}, and 28.3 s^{-1} in the presence of O_2. The rebinding to heme a_3 occurs with $k_2 = 26.3\ s^{-1}$, and 24.6 s^{-1} in the presence of O_2. The results provide solid evidence that in cytochrome ba_3 the ligand delivery channel is located at the Cu_B site, which is the ligand entry to the heme a_3 pocket. We suggest that the properties of the O_2 channel are not limited to facilitate ligand diffusion to the active site, but are extended in controlling the dynamics and reactivity of the reactions of ba_3 with O_2 and NO.

3.5. Model for input/output channels in heme-copper oxidases

The determination of the structures in the binuclear center upon CO binding and the photodissociation/rebinding process remains an important problem that is central to elucidating the dynamics of heme-copper oxidases. The data presented here allow analysis of the general structural perturbations that are responsible for the unique conformations of heme-copper oxidases. Based on the existing data, we present a model (Fig. 2) for the O_2 input channel located at the Cu_B site and an H^+/H_2O output channel located at the ring A propionate of heme a_3-Asp372-H_2O site (*T. thermophilus* numbering), which is conserved among all structurally known oxidases. TRS^2-FTIR experiments are in progress to address these issues.

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