Oxygen-linked Equilibrium CuB-CO Species in Cytochrome ba3 Oxidase from Thermus thermophilus

IMPLICATIONS FOR AN OXYGEN CHANNEL AT THE CuB SITE

Received for publication, October 8, 2002, and in revised form, February 13, 2003
Published, JBC Papers in Press, February 19, 2003, DOI 10.1074/jbc.M210293200

Konstantinos Koutsoupakis‡, Stavros Stavrakis‡, Tewfik Soulimane§, and Constantinos Varotsis¶

From the ‡University of Crete, Department of Chemistry, 71409 Heraklion, Crete, Greece, and §Paul Scherrer Institut, Life Sciences, OSRA/008, CH-5232 Villigen PSI, Switzerland

We report the first study of O2 migration in the putative O2 channel of cytochrome ba3 and its effect to the properties of the binuclear heme a3-CuB center of cytochrome ba3 from Thermus thermophilus. The Fourier transform infrared spectra of the ba3-CO complex demonstrate that in the presence of 60–80 μM O2, the ν(C-O) of CuB1−-C-O at 2053 cm−1 (complex A) shifts to 2045 cm−1 and remains unchanged in H2O/D2O exchanges and in the pH 6.5–9.0 range. The frequencies but not the intensities of the C-O stretching modes of heme a3-CO (complex B), however, remain unchanged. The change in the ν(C-O) of complex A results in an increase of k−2, and thus in a higher affinity of CuB for exogenous ligands. The time-resolved step-scan Fourier transform infrared difference spectra indicate that the rate of decay of the transient CuB−-CO complex at pH 6.5 is 30.4 s−1 and 28.3 s−1 in the presence of O2. Similarly, the rebinding to heme a3 is slightly affected and occurs with k+2 = 26.3 s−1 and 24.6 s−1 in the presence of O2. These results provide solid evidence that in cytochrome ba3, the ligand delivery channel is located at the CuB site, which is the ligand entry to the heme a3 pocket. We suggest that the properties of the O2 channel are not limited to facilitating ligand diffusion to the active site but are extended in controlling the dynamics and reactivity of the reactions of ba3 with O2 and NO.

Cytochrome ba3 from Thermus thermophilus is a member of the large family of structurally related heme-copper oxidases (1, 2). It catalyzes both the four-electron reduction of O2 to H2O, converting the energy of this reaction to a transmembrane proton motive force, and the two-electron reduction of NO to N2O (1–4). Based on the crystal structure, the enzyme contains a homodinuclear copper (CuA), a low-spin heme b, and a heme a3-CuB binuclear center (1). The ba3-oxidase retains the electron transport chain functional under low oxygen tension and at high temperatures (2). However, no data exist in the literature to demonstrate the nature of the conformational changes that occur in the binuclear center in the presence of O2 in the channel. Because of the unusual ligand-binding and kinetic properties of the binuclear center, cytochrome ba3 oxidase is unique among the heme-copper oxidases in that it is susceptible to a detailed kinetic analysis of its ligand dynamics (4, 7). The binding of CO to the binuclear center of ba3 follows that found in all heme-copper oxidases and proceeds according to the Scheme 1 (7–12).

In our previous work (7), we identified the C-O stretching mode of the equilibrium CuB1−-CO species (complex A) at 2053 cm−1 and concluded that the environment in the binuclear center does not alter the protonation state of the CuB histidine ligands. Understanding the conformational transitions that are associated with protonation/deprotonation of labile residues is essential because ionizable groups whose pK values are near physiological pH are involved in proton uptake or release. A hydrogen-bonded connectivity between the propionates of heme a3, Asp-372, and H2O was also reported. Accordingly, plausible mechanisms of proton pathway(s) directly associated with the propionates of the heme a3 redox center and the proton-labile side chain of Asp-372 were suggested.

The nature of heme-copper oxidases is to bring in O2 through ligand-entry channels to the binuclear center and then remove protons and H2O from the active site. Because it has been proposed that 1) CuB is a way-stop for ligand entry to heme a3 (8) and 2) the O2 channel is located at the CuB site, we sought to determine the properties of the binuclear center by applying our FTIR approach (7, 9, 11) to study the CO bound cytochrome ba3 at room temperature in the presence of low-oxygen concentrations in the medium (70 μM), preventing spontaneous replacement of CO by O2. In cytochrome ba3, the exceptionally high affinity for CO binding to CuB (K1 > 104) has allowed us to perform such experiments. We have also used time-resolved...
step-scan Fourier transform infrared spectroscopy (FTIR)\(^1\) to investigate the ligand dynamics subsequent to CO photolysis at room temperature in the presence of \(-70 \mu M\) O\(_2\) and compare the results with those obtained in the absence of O\(_2\), which is essential for elucidating the unique chemical mechanisms of the redox processes catalyzed by the enzyme and the dynamics of the binuclear center.

**EXPERIMENTAL PROCEDURES**

Cytochrome ba\(_3\) was isolated from *T. thermophilus* HB8 cells according to previously published procedures (3). The samples used for the FTIR measurements had an enzyme concentration of \(-1\) mM and were placed in a desired buffer (pD 5.5–6.5, MES; pD 7.5, HEPES; pD 8.5–9.5, CHES). The pH solutions prepared in D\(_2\)O buffers were measured by using a pH meter and assuming pD = pH (observed) + 0.4. Dithionite reduced samples were exposed to 1 atmosphere CO (1 mm) in an anaerobic cell to prepare the CO adduct and transferred to a tightly sealed FTIR cell under anaerobic conditions (pathlength \(1 = 15\) and \(25\) \(\mu m\) for the experiments in H\(_2\)O and D\(_2\)O, respectively). CO gas was obtained from Messer (Frankfurt, Germany) and isotopic CO (\(^{13}\)CO) was purchased from Isotec (Miamisburg, OH). The ba\(_3\) carbonononyx/O\(_2\) adduct was prepared by addition of aliquots of 5 \(\mu l\) of an oxygen-saturated buffer solution (1.2 mm O\(_2\)) to make the final O\(_2\) concentration of 60–90 \(\mu M\). The 532 nm pulse from a Continuum neodymium-yttrium-aluminum garnet (Nd-YAG) laser (7-ns width, 3 Hz) was used as a pump light (10 mJ/pulse) to photolyze the ba\(_3\)-CO complex. The time-resolved step-scan FTIR spectra were obtained with spectral resolution of 8 \(\text{cm}^{-1}\) and 100-\(\mu s\) time resolution for the 0–75 ms measurements. A total of 10 co-additions per retardation data point was collected. Changes in intensity were recorded with a mercury cadmium telluride detector, amplified (dc-coupled), and digitized with a 200-kHz, 16-bit, analog-to-digital converter. Blackman-Harris three-term apodization function with 32 \(\text{cm}^{-1}\) phase resolution and the Mertz phase correction algorithm were used. Difference spectra were calculated as \(\Delta A = -\log(\text{intensity of sample/intensity of reference})\). The detailed experimental set-up for the time-resolved step-scan FTIR has been described previously (7, 9, 11). The rate constants for each phase of the decay of the Cu\(_{ba}\)-(13)CO complex and for CO rebinding to heme \(a_1\) were calculated, assuming first-order kinetics, with three-parameter exponential fits to the experimental data. Optical absorbance spectra were recorded before and after FTIR measurements to assess sample stability with a PerkinElmer Lambda 20 UV-visible spectrometer.

**RESULTS AND DISCUSSION**

The FTIR spectrum of the CO-bound cytochrome ba\(_3\) complex at neutral pH exhibits peaks at 1967, 1973, 1982, and 2053 \(\text{cm}^{-1}\) (Fig. 1, trace A). In the \(^{13}\)CO\(_2\) derivative, these peaks shift to 1923, 1928, 1937, and 2007 \(\text{cm}^{-1}\), respectively (Fig. 1, trace B). The peaks at 1967, 1973, and 1982 \(\text{cm}^{-1}\) have been assigned to the C-O stretching modes of heme \(a_1\)-CO (complex B), and the peak at 2053 \(\text{cm}^{-1}\) to the C-O stretching mode of Cu\(_{ba}\)-1(13)CO (complex A). As shown in Fig. 1, trace C, addition of 70 \(\mu M\) buffered O\(_2\) to the ba\(_3\)-CO complex leads to a shift of the CO mode of Cu\(_{ba}\)-1 to 2045 \(\text{cm}^{-1}\). Addition of oxygen-saturated buffer (\(>300 \mu M\)) causes the spontaneous replacement of CO by O\(_2\), as evidenced by the full disappearance of both the heme \(a_1\)-CO modes at 1967, 1973, and 1982 \(\text{cm}^{-1}\) and the Cu\(_{ba}\)-CO mode at 2053 \(\text{cm}^{-1}\). Confirmation that the newly developed 2045 \(\text{cm}^{-1}\) mode is CO-sensitive is shown in the \(^{13}\)CO spectrum (Fig. 1, trace D), where it shifts to 1999 \(\text{cm}^{-1}\). These data also demonstrate a significant growth of the 2045 \(\text{cm}^{-1}\) conforma-

---

\(^1\) The abbreviations used are: FTIR, Fourier transform infrared; CHES, 2-(cyclohexylamino)ethanesulfonic acid; MES, 4-morpholineethanesulfonic acid.

---

**Fig. 1.** FTIR spectra of the cytochrome ba\(_3\)-CO complex in the absence (A), (13)CO, B, and in the presence of O\(_2\) (C), (13)CO, D at pH 7.5 and 293 K. Inset, UV-visible spectra of the cytochrome ba\(_3\)-CO complex in the absence (a) and presence (b) of O\(_2\). Enzyme concentration was \(-1\) \(\mu M\) and the pathlength was 15 \(\mu M\). The spectral resolution was 2 \(\text{cm}^{-1}\).
Dynamics and reactivity of the reactions of diffusion to the active site but are extended in controlling the delivery channel generating a conformational change in the developed conformer is the result of a dynamic effect in the ligands, causing the weakening of the C-O bond, or the newly increased intensity of nitric oxide and other ligands, including cyanide. The increased behavior is extended to the physiological function of the enzyme.

We attribute the transition to an increased electron density in the CO antibonding orbitals that results in weakening the C-O bond. It remains to be determined whether the O$_2$ is hydrogen-bonded to one of the Cu$_B$-His ligands, causing the weakening of the C-O bond, or the newly developed conformer is the result of a dynamic effect in the delivery channel generating a conformational change in the Cu$_B$ environment. It becomes intriguing to speculate 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 nitric oxide and other ligands, including cyanide. The increased value of $k_{-2}$ indicates that Cu$_B$ undergoes structural changes to behave as an efficient trap for CO. We suggest that this behavior is extended to the physiological function of the enzyme. This way, conformational changes associated with Cu$_B$ occur, facilitating the entry and the later coordination of O$_2$ to the binuclear center.

Fig. 3A shows the time-resolved step-scan FTIR difference spectra ($t_d$ = 0–75 ms, 8 cm$^{-1}$ spectral resolution) of fully reduced ba$_3$-CO subsequent to CO photolysis by a nanosecond laser pulse (532 nm). Upon photolysis, CO is transferred from heme a$_3$ to Cu$_B$. It should be noted that the Cu$_B$-CO complex (complex A) is not photolabile and thus remains a spectator in the photodynamic events occurring to complex B. Under our 8 cm$^{-1}$ spectral resolution, the heme a$_3$-Fe-CO peaks at 1967, 1973, and 1982 cm$^{-1}$ are not resolved; thus, a single negative peak at 1976 cm$^{-1}$ indicates the photolyzed heme a$_3$-CO$_2$ complex. The positive peak that appears at 2053 cm$^{-1}$ is attributed to the C-O stretch ($v_{C-O}$) of the transient Cu$_B$-CO$_2$ complex, and its frequency is the same as that obtained at pD 8.5 (7) and that of the equilibrium Cu$_B$-CO$_2$. At early times (0–3000 ms), the intensity of the 1976 and 2053 cm$^{-1}$ modes in the transient difference spectra remains unchanged, suggesting that dissociation of CO from Cu$_B$ does not occur on this time scale. At later times (3–75 ms), there is a decrease in the intensity of the 2053 cm$^{-1}$ mode that is accompanied by an increased intensity of the 1976 cm$^{-1}$ mode. At 75 ms after CO photolysis, the intensities of both the 1976 and 2053 cm$^{-1}$ modes are almost diminished. The intensity ratio ($v_{C-O}$) of the Fe-CO/Cu$_B$-CO remains constant for all data points, indicating that no significant fraction of CO escapes the binuclear center.

This is also consistent with both the low-temperature experiments (21 K) and those obtained at pD 8.5 at room temperature (7, 8). The high signal-to-noise ratio in the time-resolved FTIR difference spectra has allowed us to monitor the decay and reappearance of the 2053 and 1976 cm$^{-1}$ modes, respectively. Fig. 3A, inset, compares the decay of the Cu$_B$-CO complex, as measured by $\Delta A$ of the 2053 cm$^{-1}$ mode shown in Fig. 3A, with the formation of the heme a$_3$-CO$_2$ complex by measuring $\Delta A$ of the 1976 cm$^{-1}$ modes. The rate of decay of the transient Cu$_B$-CO complex is 30.4 s$^{-1}$, and the observed rate of rebinding...
ing to heme \(a_3\) is 26.3 s\(^{-1}\). Both rates are 10% lower than those observed at pH 8.5 (7). The pH/pD dependence of the rates of decay of the CuB complex and the rebinding to heme \(a_3\) will be presented elsewhere.\(^2\)

Fig. 3B shows the time-resolved step-scan FTIR difference spectra (\(t_\text{delay} = 0–75\) ms, 8 cm\(^{-1}\) spectral resolution) of fully reduced \(b_{\alpha_3}\)-CO in the presence of 70 \(\mu\)l \(O_2\) after CO photolysis by a nanosecond laser pulse (532 nm). The results are very similar to those obtained in the absence of \(O_2\), including the intensity ratio (2) of the Fe-CO/CuB-CO modes. This observation indicates that \(O_2\) is not coordinated to CuB in complex B, before photolysis. Importantly, the observed rates for the decay of the transient CuB\(^{1+}\)-CO complex (28.3 s\(^{-1}\)) and the heme \(a_3\)^2\(^+\) recombination (\(k_2 = 24.6\) s\(^{-1}\)) that we have determined, shown in Fig. 3B, inset, indicate that the presence of \(O_2\) in the delivery channel has no direct control in the CO ligation/dissociation dynamics. The observation of the C-O stretch (\(v_{\text{C}-\text{O}}\)) of the transient CuB\(^{1+}\)-CO complex at 2053 cm\(^{-1}\) and not at 2045 cm\(^{-1}\), as observed in the equilibrium CuB\(^{1+}\)-CO complex in the presence of \(O_2\), indicates the absence of an equilibrium between the transient CuB\(^{1+}\)-CO complex and \(O_2\) at the time-scale of our observations (0–75 ms).

This work presents a very peculiar and unexpected observation. However, the \(b_{\alpha_3}\) oxidase is expressed under limited amounts of oxygen; thus, an \(O_2\)-concentration-dependent behavior, such that presented in this work, is feasible. Several control experiments with Mb-CO (data not shown) indicate that under the same experimental conditions (\(O_2\) concentration, pH-range, temperature), \(O_2\) spontaneously replaces CO. The experiments reported here have been repeated with different enzyme preparations to avoid some sort of artifact and clearly demonstrate \(O_2\) migration in the delivery channel that is located at the CuB site. The presence, but not coordination, of \(O_2\) at the CuB site results in a structural reorientation of the CuB environment and concomitantly to an increase of \(k_2\). This effect can be either steric or electrostatic involving either one of the CuB-His ligands (polar) or the CuB atom directly (electrostatic), producing an increased electron density in the CO antibonding orbitals that results in weakening the C-O bond strength (lower \(v_{\text{C}-\text{O}}\)). Our data also support the developing consensus that CuB is a way stop for \(O_2\) en route to its heme binding site (8). Whether the presence of Glu-278 in other heme-copper oxidases (5, 6) interrupts the putative oxygen channel provided by Ile-235 in \(b_{\alpha_3}\), and thus the efficient diffusion of low \(O_2\) concentrations to the binuclear center, remains to be determined. Moreover, it is important to establish whether the conformational changes induced to CuB environment by \(O_2\), which is present in low concentrations at physiological conditions because of the reduced gas solubility at higher temperatures, in the putative channel of \(b_{\alpha_3}\), are extended to the superfamily of cytochrome oxidases. Experiments toward this goal are in progress in our laboratory.

Acknowledgment—We thank E. Pinakoulaki for helpful discussions.

REFERENCES

1. Soulmaine, T., Buse, G., Bourenkov, G. P., Bartunik, H. D., Huber, R., and Than, M. E. (2000) EMBO (Eur. Mol. Biol. Organ.) J. 19, 1766–1776
2. Than, M. E., and Soulmaine, T. (2001) in Handbook of Metalloproteins (Messerschmidt, A., Huber, R., Poulos, T., and Wieghardt, K., eds) pp. 363–378, John Wiley and Sons, Ltd., Chichester, UK.
3. Giuffrè, A., Stubsauer, G., Sart, P., Brunori, M., Zamft, W. G., Buse, G., and Soulmaine, T. (1999) Proc. Natl. Acad. Sci. U. S. A. 96, 14718–14723
4. Giuffrè, A., Forte, E., Antonini, G., D’Itri, E., Brunori, M., Soulmaine, T., and Buse, G. (1999) Biochemistry 38, 1057–1065
5. Iwata, S., Ostermeier, C., Ludwig, B., and Michl, H. (1995) Nature 376, 660–669
6. Yoshikawa, S., Shizawa-Itoh, K., Nakashima, R., Yasno, K., Yamashita, E., Inoue, N., Yao, M., Fei, M. J., Iheu, C. P., Mizushima, T., Yamaguchi, H., Tomizaki, T., and Tsukihara, T. (1998) Science 280, 1723–1729
7. Koutsoupakis, K., Stavrakis, S., Pinakoulaki, E., Soulmaine, T., and Varotsis, C. (2002) J. Biol. Chem. 277, 32860–32866
8. Woodruff, W. H. (1993) J. Bioenerg. Biomembr. 25, 177–188
9. Stavrakis, S., Koutsoupakis, K., Pinakoulaki, E., Urbani, A., Saraste, M., and Varotsis, C. (2002) J. Am. Chem. Soc. 124, 3814–3815
10. Varotsis, C., Kreszwinski, D. H., and Babcock, G. T. (1996) Bio spectroscopy 2, 331–338
11. Pinakoulaki, E., Soulmaine, T., and Varotsis, C. (2002) J. Biol. Chem. 277, 32867–32874
12. Pinakoulaki, E., Pfitzner, U., Ludwig, B., and Varotsis, C. (2002) J. Biol. Chem. 277, 13563–13568

\(^2\)K. Koutsoupakis, T. Soulmaine, and C. Varotsis, unpublished observations.
Oxygen-linked Equilibrium Cu₆CO Species in Cytochrome \(ba₃\) Oxidase from \(Thermus\ thermophilus\): IMPLICATIONS FOR AN OXYGEN CHANNEL AT THE CuBSITE

Konstantinos Koutsoupakis, Stavros Stavrakis, Tewfik Soulimane and Constantinos Varotsis

*J. Biol. Chem.* 2003, 278:14893-14896.  
doi: 10.1074/jbc.M210293200 originally published online February 19, 2003

Access the most updated version of this article at doi: 10.1074/jbc.M210293200

Alerts:
- When this article is cited  
- When a correction for this article is posted

*Click here* to choose from all of JBC's e-mail alerts

This article cites 11 references, 6 of which can be accessed free at [http://www.jbc.org/content/278/17/14893.full.html#ref-list-1](http://www.jbc.org/content/278/17/14893.full.html#ref-list-1)