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Structural changes that occur upon photolysis of the $Fe(II)_{a3}$–CO complex in the cytochrome ba_3-oxidase of *Thermus thermophilus*: A combined X-ray crystallographic and infrared spectral study demonstrates CO binding to Cu_B ☆

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ABSTRACT

The purpose of the work was to provide a crystallographic demonstration of the venerable idea that CO photolyzed from ferrous heme-a_3 moves to the nearby cuprous ion in the cytochrome *c* oxidases. Crystal structures of CO-bound cytochrome ba_3-oxidase from *Thermus thermophilus*, determined at ~2.8–3.2 Å resolution, reveal a Fe–C distance of ~2.0 Å, a Cu–O distance of 2.4 Å and a Fe–C–O angle of ~126°. Upon photodissociation at 100 K, X-ray structures indicate loss of Fe_{a3}–CO and appearance of Cu_B–CO having a Cu–C distance of ~1.9 Å and an O–Fe distance of ~2.3 Å. Absolute FTIR spectra recorded from single crystals of reduced ba_3–CO that had not been exposed to X-ray radiation, showed several peaks around 1975 cm^{-1}; after photolysis at 100 K, the absolute FTIR spectra also showed a significant peak at 2050 cm^{-1}. Analysis of the 'light' *minus* 'dark' difference spectra showed four very sharp CO stretching bands at 1970 cm^{-1}, 1977 cm^{-1}, 1981 cm^{-1}, and 1985 cm^{-1}, previously assigned to the Fe_{a3}–CO complex, and a significantly broader CO stretching band centered at ~2050 cm^{-1}, previously assigned to the CO stretching frequency of Cu_B bound CO. As expected for light propagating along the tetragonal axis of the $P4_32_12$ space group, the single crystal spectra exhibit negligible dichroism. Absolute FTIR spectrometry of a CO-laden ba_3 crystal, exposed to an amount of X-ray radiation required to obtain structural data sets before FTIR characterization, showed a significant signal due to photogenerated CO_2 at 2337 cm^{-1} and one from traces of CO at 2133 cm^{-1}; while bands associated with CO bound to either Fe_{a3} or to Cu_B in "light" *minus* "dark" FTIR difference spectra shifted and broadened in response to X-ray exposure. In spite of considerable radiation damage to the crystals, both X-ray analysis at 2.8 and 3.2 Å and FTIR spectra support the long-held position that photolysis of Fe_{a3}–CO in cytochrome *c* oxidases leads to significant trapping of the CO on the Cu_B atom; Fe_{a3} and Cu_B ligation, at the resolutions reported here, are otherwise unaltered. This article is part of a Special Issue entitled: Respiratory Oxidases.

1. Introduction

1.1. Cytochrome ba_3

Cytochrome ba_3 first described in 1988 by Zimmermann et al. [1] and characterized in detail by Keightley et al. [2], is now considered a B-type cytochrome *c* oxidase [3], and is one of two terminal heme–copper oxidases [4] in the Gram-negative eubacterium *Thermus thermophilus*, which couple the reduction of dioxygen to proton translocation across the inner bacterial membrane. The enzyme contains a homo-dinuclear copper center, Cu_A, a low-spin heme-*b*, and a binuclear center including a high spin heme-a_3 in close proximity to the three-coordinate Cu_B atom [1,5–7]. This binuclear center serves as the site where dioxygen is reduced to H_2O (see Ref. [8]). While time-resolved structural studies of metal bound oxygen forms of the enzyme may be the most desirable way to understand the molecular mechanism of the catalytic reaction, the diffusion limited rate of dioxygen binding [9,10] followed by its rapid reduction by the binuclear site, recommends the use of generally redox inactive carbon monoxide (CO) as an alternative to study ligand binding and coordination changes at the binuclear site, free of the complications resulting from dioxygen reduction chemistry. By exploiting the 100% quantum yield for photodissociation of heme–CO complexes [11], CO has been used to probe both Fe

Abbreviations: FTIR, Fourier transform infrared; MGy, 1×10^6 Gray; FWHM, full width at half maximum.

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and Cu in the catalytic site of heme–Cu oxidases [12,13]. Indeed, CO photodissociation is a powerful tool for studying dynamic features of the active site and how it adjusts to "photo freed" CO [13–15].

The purpose of this work was to provide a crystallographic demonstration of the long-held assertion that CO bound to heme-a_3 of the cytochrome *c* oxidases moves, upon illumination, from Fe_{a3}(II) to Cu_B(I). This assignment was originally evidenced by shifts of the IR spectral line associated with the CO stretch from ~1975 cm^{-1}, arising from the CO-stretch mode of Fe-bound CO, to ~2050 cm^{-1}, thought to arise from the CO-stretch of Cu_B-bound CO [14,16,17] as suggested in Scheme 1. Recent work by Varotsis and co-workers [18] indicates, however, that the stretching frequency of non-metal bound CO may show similar values for CO bound in polar protein cavities. If proven to occur in the cytochrome *c* oxidases, such a finding would complicate the now venerable interpretation that the observed ~2050 cm^{-1} band represents the CO stretching frequency of the Cu_B–CO complex.

The heme–CO complex exhibits unusual vibrational properties in comparison with other heme proteins. The deviation of the C–O and Fe–CO frequencies from the linear correlation established for other histidine-ligated heme proteins has been attributed to compression of the C – O bond or distortion of the FeCO unit from the nearly linear geometry observed for other heme proteins [19]. The linewidths of the C–O stretching band are also unusually narrow for heme–Cu oxidases [12,13]. The frequency and oscillator strength of the photolyzed C–O have values intermediate between those of heme–CO and CO gas but are similar to those observed for CO binding to Cu proteins [20]. The high barrier for geminate rebinding of photolyzed CO to the heme (40–60 kJ/mol) in comparison with typical heme proteins (10 kJ/mol) has also been associated with the presence of Cu [12,13]. In this work, X-ray crystallography provides evidence for photo induced transfer of CO from Fe_{a3}–CO to Cu_B–CO with no other changes in metal ligation, while infrared spectroscopy shows that CO binding within a single crystal of ba_3 is highly similar to that which occurs in solution. Parallel characterization of the structure and vibrational properties of the Cu–CO complex in a heme–Cu oxidase, as presented here, serves as a springboard for structurally calibrated spectroscopic investigations.

2. Materials and methods

2.1. Protein expression, purification, and crystallization

Recombinant wild type and mutant (II-E4Q/I-K258R) cytochrome ba_3 oxidase genes were expressed in *T. thermophilus* HB8, and the enzyme was purified as described [21,22]. (Numerous observations in the Fee laboratory indicate these mutations are benign, and the results reported here support this conclusion.) Final protein samples were dissolved in 10 mM Bis–Tris pH 7.0 with 0.1 M KCl and 13 mM n-nonyl-β-D-glucopyranoside (2 times its critical micelle concentration) after performing the detergent exchange using a small DEAE resin (GE Science) in an Econo-Column (BioRad), the sample was concentrated to ~20 mg/mL using an Ultra YM-10 concentrator (Microcon). Spectral characterization was determined using an SLM/AMINCO model DB3500 dual beam spectrophotometer. The purity index, A_{413}/A_{280}, of the enzyme used in this work ranged from ≥0.7 to 0.9. Optical spectra are shown in Fig. S1. The concentration of fully constituted protein was obtained using $\Delta\varepsilon^{red}_{560-590} = 26\ mM^{-1}\ cm^{-1}$ from a reduced *minus* oxidized difference spectrum using Na-dithionite as the reductant. The protein was crystallized in air at room temperature as described (Ref. [22]); trays containing crystals so prepared were transferred into a glove box (COY Laboratory Products Inc, USA) filled with a gas mixture of 10% (v/v) hydrogen and 90% (v/v) nitrogen.

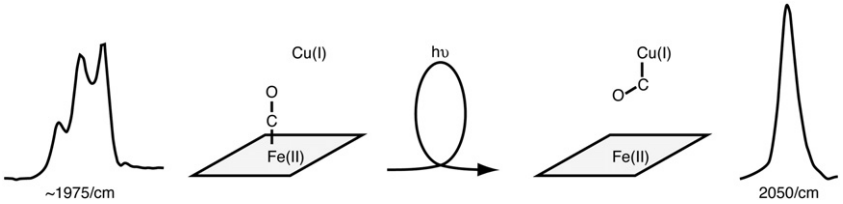
2.2. Carbon monoxide binding to ba_3 in crystals and in solution

In order to realize the CO bound state, a cryo-protectant solution [50% (±)-2-methyl-2,4-pentanediol, 14% Polyethylene Glycol 2000, 0.1 M KCl, 13 mM n-nonyl-β-D-glucopyranoside in 0.1 M Bis–Tris (pH 7.0), and 0.1 mM methyl viologen] was sparged vigorously with carbon monoxide in a fume hood for 5–10 min, sealed with Parafilm, and moved into the glove box. Several small vials containing weighed amounts of dry Na dithionite were also brought into the anaerobic chamber. After the increased O_2-levels within the chamber, caused by these operations, had returned to <1 ppm, crystals were reduced in their crystallization droplet by adding several microliters of CO saturated cryoprotectant being 10 mM in Na dithionite, and 0.1 mM methyl viologen. Droplets containing crystals were re-covered and allowed to stand for 1 to 2 min before a crystal was loaded onto a nylon loop and flash frozen in liquid nitrogen. Total time of this operation starting with reduction of the crystals was approximately 2 to 5 min. The intense blue color of the methylviologen free radical, visible in the droplet, provided continuous evidence that the droplet was reducing and oxygen free, thereby making possible the binding of CO to the Fe^{2+} form of the high-spin heme [13]. At this point in the experiment, the only assay available for Fe_{a3}–CO formation in the frozen crystal is X-ray structural determination.

2.3. X-ray data-collection, structure determination and refinement

Data sets were collected at 100 K using beam line 11-1 at the Stanford Synchrotron Radiation Lightsource at 0.979 Å and using a MAR325 detector (plus other beam lines as indicated in Table 1). Diffraction image data were processed with the programs MOSFLM [23] and SCALA [24]. The wild type recombinant structure, (PDB ID: 1XME) was utilized as the initial model to obtain phase information using molecular replacement with PHASER [25].

Initially, the structures were refined by rigid body refinement followed by restrained refinement with REFMAC 5.0 [26]. Without including carbon monoxide in the refinements, the $|F_o–F_c|$ electron density maps clearly indicated the likely presence of a CO molecule between Fe_{a3}- and Cu_B that was best modeled by a CO molecule bound to the Fe_{a3} or Cu_B and in a configuration somewhat off perpendicular to the plane of heme-a_3. CO was further treated as a free molecule having a bond length of 1.128 Å in subsequent refinements with an allowed deviation of ±0.005 Å. A better fit to the $|F_o–F_c|$ map was always obtained when the C-atom was involved in bonding to either to Fe or Cu. COOT [27] was utilized to display structure models and electron density maps. Details of data collection statistics and final refinement statistics are given in Table 1. Structural figures were created



Scheme 1. Scheme depicting protoinduced CO transfer from Fe(II)-CO to form Cu(I)-CO. Typical difference spectra are also shown.