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An engineered heme–copper center in myoglobin: CO migration and binding ☆

Karin Nienhaus [a], John S. Olson [b], G. Ulrich Nienhaus [a,c,d,*]

[a] Institute of Applied Physics and Center for Functional Nanostructures (CFN), Karlsruhe Institute of Technology (KIT), Wolfgang-Gaede-Str. 1, D-76131 Karlsruhe, Germany
[b] Department of Biochemistry and Cell Biology, Rice University, Houston, TX 77005-1892, USA
[c] Institute of Toxicology und Genetics, Karlsruhe Institute of Technology (KIT), Hermann-von-Helmholtz-Platz 1, 76344 Eggenstein-Leopoldshafen, Germany
[d] Department of Physics, University of Illinois at Urbana-Champaign, 1110 W. Green Street, Urbana, IL 61801, USA

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ABSTRACT

We have investigated CO migration and binding in Cu_BMb, a copper-binding myoglobin double mutant (L29H–F43H), by using Fourier transform infrared spectroscopy and flash photolysis over a wide temperature range. This mutant was originally engineered with the aim to mimic the catalytic site of heme–copper oxidases. Comparison of the wild-type protein Mb and Cu_BMb shows that the copper ion in the distal pocket gives rise to significant effects on ligand binding to the heme iron. In Mb and copper-free Cu_BMb, primary and secondary ligand docking sites are accessible upon photodissociation. In copper-bound Cu_BMb, ligands do not migrate to secondary docking sites but rather coordinate to the copper ion. Ligands entering the heme pocket from the outside normally would not be captured efficiently by the tight distal pocket housing the two additional large imidazole rings. Binding at the Cu ion, however, ensures efficient trapping in Cu_BMb. The Cu ion also restricts the motions of the His64 side chain, which is the entry/exit door for ligand movement into the active site, and this restriction results in enhanced geminate and slow bimolecular CO rebinding. These results support current mechanistic views of ligand binding in hemoglobins and the role of the Cu_B in the active of heme–copper oxidases. This article is part of a Special Issue entitled: Oxygen Binding and Sensing Proteins.

1. Introduction

Heme–copper oxidases (HCOs) are terminal oxidases in the respiratory chains of bacteria and mitochondria of eukaryotes [1–4]. These large, multi-subunit metalloenzymes catalyze the four-electron reduction of dioxygen (O_2) to water while suppressing the production of toxic, reactive oxygen species (ROS) such as superoxide or peroxide. This process contributes to the electrochemical gradient across the mitochondrial or cytoplasmic membranes that drives the synthesis of the cellular energy source, ATP [5]. O_2 reduction takes place at a high-spin heme a_3 copper binuclear center. The heme iron is coordinated by a proximal histidine, and the copper ion, Cu_B, is complexed by three other histidines ~5 Å away from the heme iron. Interestingly, one of the histidines is covalently cross-linked to a tyrosine residue [6,7]. Electrons are funneled into the active site via a two-copper site, Cu_A, which accepts electrons from cytochrome c, and a low-spin heme that passes the electrons into the active site heme a_3 Cu_B center.

More than 30 years ago, Alben and coworkers [8] pioneered the use of Fourier transform infrared (FTIR) photolysis difference spectroscopy to probe ligand binding to the heme iron and the Cu_B ion at the catalytic site of HCOs in order to elucidate the catalytic reaction. By using carbon monoxide (CO) as a model ligand, the FTIR spectra revealed that, after photolysis from the heme iron, the CO becomes transiently coordinated to Cu_B [8–10]. FTIR temperature derivative spectroscopy (TDS) experiments on cytochrome c oxidase from *Rhodobacter sphaeroides* showed that the CO ligand returns from Cu_B to the heme iron at 160–180 K [11]. Using time-resolved FTIR spectroscopy, Heberle and coworkers [12] recorded the dynamics of transient CO binding to Cu_B in bovine cytochrome *c* oxidase. Recently, a combined X-ray crystallography/infrared spectroscopy further confirmed that, after photodissociation from the heme iron, the CO ligand does coordinate to Cu_B of the cytochrome ba_3-oxidase of *Thermus thermophilus* [13]. The pathway of CO from the solution to its binding site at the heme a_3 iron may also involve transient binding at Cu_B [14]. Therefore, it has been proposed that Cu_B acts as a trap for facilitating the capture of CO and, by inference, other ligands such as NO and O_2 and their subsequent coordination with the reduced high-spin heme a_3 [15,16].

Abbreviations: FTIR, Fourier transform infrared; TDS, temperature derivative spectroscopy; Mb, myoglobin; HCO, heme–copper oxidase; Cu_BMb, copper-binding Mb mutant L29H–F43H

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* Corresponding author at: Institute of Applied Physics and Center for Functional Nanostructures (CFN), Karlsruhe Institute of Technology (KIT), Wolfgang-Gaede-Str. 1, D-76131 Karlsruhe, Germany. Tel.: +49 721 608 43401; fax: +49 721 608 48480.
E-mail address: uli@illinois.edu (G.U. Nienhaus).

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2. Cu_BMb, a myoglobin-based heme–copper oxidase

Because of the size and complexity of HCOs, there have been considerable efforts to synthesize small model systems that are well suited to elucidate the molecular details of the enzymatic reaction, in particular, to understand the role of Cu_B and to identify reaction intermediates. Different synthetic, porphyrin-based Fe–Cu complexes were engineered [17–19], some of which were able to mimic the mechanism of CO binding [20]. In 2000, a minimum HCO model system based on myoglobin (Mb) was created by C. Lu and coworkers [21]. A Cu binding site was engineered into the protein by replacing Leu29 and Phe43 by histidines. In this Cu_BMb, the Cu ion is complexed by His29, His43 and His64. Recently, even more refined model systems have been presented. In Gly65Tyr–Cu_BMb and Phe33Tyr–Cu_BMb, an additional tyrosine was introduced close to the Cu binding site to improve the catalytic capabilities of the Mb model system [22]. In imiTyrCu_BMb, the unnatural amino acid imiTyr mimics the cross-linked Tyr-His moiety in native cytochrome c oxidase [23].

Over the past ten years, Cu_BMb was employed to probe the role of Cu ions and protons in HCOs [24], to monitor the effects of various metal ions on the redox properties of the heme [25,26] and also to elucidate the redox-dependent structural changes upon chloride binding [27]. Resonance Raman studies on Cu_BMb indicated that the presence of copper affects the CO and NO binding kinetics [28], and stopped-flow experiments confirmed that a CO ligand first binds to Cu_B and then transfers to the heme iron [28].

Here we have employed FTIR spectroscopy in combination with TDS at cryogenic temperatures to investigate the effect of the Cu center on ligand migration and binding within Cu_BMb. Using the CO ligand as an internal probe, we have mapped out its migration pathway within the protein matrix, both with and without Cu present. These results are compared with detailed data on protein–ligand interaction in wt MbCO. Additional flash photolysis experiments at ambient temperature complement the FTIR/TDS data and reveal the effect of the Cu ion on ligand association and dissociation from the protein.

3. CO migration in Cu_BMb

3.1. Sample preparation and data collection

The gene of recombinant sperm whale Mb was taken as a template to introduce mutations Leu29His and Phe43His. The Mb sequence differs from that of the native protein by the addition of an N-terminal methionine to allow translation in *Escherichia coli* and an originally incorrect D122N substitution. The double mutant, termed Cu_BMb, was purified as described [29,30].

To prepare IR samples, a concentrated protein solution in 75%/25% (vol./vol.) glycerol/buffer (1 M potassium phosphate) cryosolvent was equilibrated with 1 atm CO for 1 h. A two-fold molar excess of sodium dithionite was added anaerobically before stirring for another 15 min under a CO atmosphere. The sample solution (final pH 6.6 and 7.4, respectively) was centrifuged at 5000 rpm (Eppendorf table top centrifuge) for 15 min prior to loading it into the sample cell to remove any precipitated protein. To load the Cu_BMbCO mutant protein with Cu ions, increasing amounts of $CuSO_4$ were added to the sample solution. A few microliters of the protein solution were placed between two CaF_2 windows (diameter 25.4 mm) separated by a 75 μm thick mylar washer. The windows were sandwiched inside a block of oxygen-free high-conductivity copper mounted on the cold-finger of a closed-cycle helium refrigerator (model SRDK-205AW, Sumitomo, Tokyo, Japan). The sample temperature was measured with a silicon temperature sensor diode and regulated by a digital temperature controller (model 330, Lake Shore Cryotronics, Westerville, OH). Samples were photolyzed by a continuous wave, frequency-doubled Nd–YAG laser (model Forte 530-300, Laser Quantum, Manchester, UK), emitting 300 mW output power at 532 nm. The laser beam was split and focused with lenses on the sample from both sides. We collected FTIR transmission spectra in the mid-infrared between 1800 and 2400 cm^{-1} at a resolution of 2 cm^{-1} (IFS 66v/S, Bruker, Karlsruhe, Germany).

Flash photolysis experiments are performed on dilute samples, prepared by dissolving the protein in 100 mM sodium phosphate buffer, pH 8, to a final concentration of 10 μM, followed by reduction with excess sodium dithionite under a CO atmosphere. For loading Cu_BMb with Cu ions, a concentrated, de-aerated $CuSO_4$ solution was added anaerobically. Samples were photolyzed using a 6-ns (full width at half maximum) pulse from a frequency doubled, Q-switched Nd:YAG laser (model Surelite II, Continuum, Santa Clara, CA). The ensuing absorbance changes were monitored at 436 nm using light from a tungsten source passed through a monochromator and the sample. The light intensity was measured with a photomultiplier tube (model R5600U, Hamamatsu, Middlesex, NJ) and recorded with a digital storage oscilloscope (model TDS 520, Tektronix) from 10 ns to 50 μs and a home-built logarithmic time-base digitizer (Wondertoy II) from 2 μs to 100 s.

3.2. Fourier transform infrared spectroscopy

The CO stretching vibration in the infrared region of the spectrum is fine-tuned by the local electric field and extremely sensitive to small structural changes in the vicinity of the CO that may escape X-ray crystallography [31–34]. FTIR studies comparing many Mb distal pocket mutants and theoretical studies revealed that electrostatic interactions (vibrational Stark effect) between the heme-bound CO and residues in its immediate environment are responsible for the dispersion of the CO stretching vibration [31,32,35–38]. In an apolar distal pocket, the heme-bound CO typically exhibits a stretching band at ~1960 cm^{-1}. P positive partial charges near the CO oxygen shift the stretching vibration to lower wavenumbers, whereas negative electrostatic fields near the bound ligand have the opposite effect. Upon photolysis of the CO from the heme iron at cryogenic temperatures, the photolyzed CO molecules migrate through the protein and become trapped at well-defined transient docking sites [39–41], where they give rise to characteristic photoproduct ν_{C-O} bands in the IR spectra [42–44]. Ligands in these docking sites are also seen by time-resolved X-ray crystallography and are, in some cases, physiologically relevant [45–49].

Photodissociated CO molecules experience only weak interactions with the protein because their infrared bands appear near the CO gas-phase frequency of 2143 cm^{-1}. Typically, narrow band shapes are observed, indicative of a well-defined orientation at the photoproduct site. Often, a doublet of photoproduct bands arises from CO binding in opposite orientations at these sites [50,51]. In general, the integrated absorbance of IR bands is ~20× smaller for photodissociated CO in the protein matrix than for heme-bound CO [8,52].

Below ~180 K, solvent dynamics are arrested [53]; concomitantly, large scale conformational motions of proteins no longer occur [54–56]. As a consequence, ligand rebinding from a particular docking site is governed by a static distribution of rebinding enthalpy barriers, $g(H)$ [57,58]. FTIR-TDS is a useful technique for the investigation of thermally activated rate processes with distributed enthalpy barriers [52,59]. In our heme-protein studies, a non-equilibrium state is created by photolysis at the lowest temperature that can be attained. Afterwards, the sample temperature, T, is increased linearly in time, t, and one FTIR transmission spectrum is taken for every 1-K temperature increase. The integrated absorbance, A, of a spectral band taken at the lowest temperature represents the total photolyzed population, N. In the simplest analysis, we assume that any change in integrated absorbance is proportional to a population change, ΔN, during acquisition of two successive spectra, e.g., a rebinding fraction. This assumption may not always be valid, however [60,61].

For a quantitative analysis of TDS data, absorbance difference spectra are computed from transmission spectra at successive temperatures, thereby approximating the temperature derivative of the population, dN/dT. These data are typically presented as two-dimensional contour