Chemical Physics 396 (2012) 10–16

Contents lists available at ScienceDirect

Chemical Physics

journal homepage: www.elsevier.com/locate/chemphys

Ultrafast heme–ligand recombination in truncated hemoglobin HbO from *Mycobacterium tuberculosis*: A ligand cage

Audrius Jasaitis [a,b,1], Hugues Ouellet [c,2], Jean-Christophe Lambry [a,b], Jean-Louis Martin [a,b], Joel M. Friedman [d], Michel Guertin [c], Marten H. Vos [a,b,*]

[a] Laboratory for Optical Biosciences, CNRS, Ecole Polytechnique, 91128 Palaiseau, France
[b] INSERM, U696, 91128 Palaiseau, France
[c] Department of Biochemistry, Microbiology and Bioinformatics, Faculty of Sciences and Engineering, Laval University, Quebec, Canada G1K 7P4
[d] Department of Physiology and Biophysics, Albert Einstein College of Medicine, Bronx, NY 10461, USA

ARTICLE INFO

Article history:
Available online 8 April 2011

Keywords:
Heme proteins
Dynamics
Ultrafast spectroscopy
Truncated hemoglobin

ABSTRACT

Truncated hemoglobin HbO from *Mycobacterium tuberculosis* displays very slow exchange of diatomic ligands with its environment. Using femtosecond spectroscopy, we show that upon photoexcitation, ligands rebind with unusual speed and efficiency. Only ~1% O_2 can escape from the heme pocket and less than 1% NO. Most remarkably, CO rebinding occurs for 95%, predominantly in 1.2 ns. The general CO rebinding properties are unexpectedly robust against changes in the interactions with close by aromatic residues Trp88 (G8) and Tyr36 (CD1). Molecular dynamics simulations of the CO complex suggest that interactions of the ligand with structural water molecules as well as its rotational freedom play a role in the high reactivity of the ligand and the heme. The slow exchange of ligands between heme and environment may result from a combination of hindered ligand access to the heme pocket by the network of distal aromatic residues, and low escape probability from the pocket.

1. Introduction

Many heme proteins have the ability to bind diatomic ligands (O_2, NO, CO, CN) to the heme iron. This property is used in a wide variety of physiological functions, including storage, transport, sensing, and catalysis. The overall binding and release properties for different ligands are essentially controlled by the protein matrix. The dynamics of the heme and of the ligand in the heme vicinity can be studied by time-resolved photolysis experiments, as has been extensively done in the classic model oxygen-storage protein myoglobin (Mb) [1,2]. In particular, dynamics of 'geminate' NO recombination occurring on the picosecond timescale has been proven exquisitely sensitive to the close heme environment [3–5]. In contrast to NO, ultrafast rebinding of dissociated CO and heme prior to ligand escape from the heme pocket is generally inefficient in Mb-like proteins [2], and therefore does not provide information on the heme environment. In recent years, a number of proteins have been discovered that belong to the hemoglobin (Hb) family (as Mb does), but displaying strikingly varying ligand-binding properties [6–8]. An interesting example is the truncated hemoglobin HbO from *Mycobacterium tuberculosis* [9] (further referred to as trHbO), one of two different 'truncated' hemoglobins found in this bacterial pathogen. The precise function(s) of this protein remain(s) unelucidated, but may include oxygen, NO scavenging [10] and peroxidatic activity [11].

TrHbO displays very slow exchange of O_2 and CO with the aqueous surroundings (low values for both k_{on} and k_{off}), combined with a two orders of magnitude higher overall affinity for O_2 than Mb [12]. The yield of CO escape from the protein upon photodissociation of the heme–CO bond was found to be extremely low [12,13]. These properties have been suggested to stem from a unique hydrogen bonding network and strong interactions between aromatic residues (in particular Trp88 (G8) and Tyr36 (CD1)) at the external ligand binding side of the heme, which could effectively cage ligands [12–16]. Heme–CO geminate recombination has been studied with a time resolution of a few nanoseconds in combination with molecular modeling of CO migration pathways on this timescale [13], and a role of Trp88 in accessing different ligand cavities from the heme pocket and escape via a 'tunnel' has been inferred. Partial picosecond geminate recombination of CO and heme has been reported for the related *Bacillus subtilis* truncated hemoglobin (*Bs*trHb) [17], which also has Trp at the G8 position,

* Corresponding author at: Laboratory for Optical Biosciences, CNRS, Ecole Polytechnique, 91128 Palaiseau, France. Tel.: +33 169335066; fax: +33 169335084.
E-mail address: marten.vos@polytechnique.edu(M.H. Vos).
[1] Present address: Institut Curie (CNRS UMR144), 26 rue d'Ulm, 75248 Paris cedex 05, France.
[2] Present address: Department of Pharmaceutical Chemistry, Genentech Hall, University of California, San Francisco, Box 2280, 600 16th Street, N554, San Francisco, CA 94158-2517, USA.

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doi:10.1016/j.chemphys.2011.04.003

but otherwise displays some differences in the distal heme pocket, including Phe instead of Tyr at position CD1. Here, we focus on the reactivity of ligands with the heme in the *M. tuberculosis* trHbO by studying picosecond and early nanosecond ligand rebinding from the distal heme pocket upon femtosecond flash photolysis, in combination with molecular dynamics simulations on the femtosecond/picosecond time scale. The results indicate that O_2 and NO virtually do not escape from the heme pocket and that the reactivity of CO with heme is even higher than deduced from previous studies on longer timescales. The general CO binding properties are surprisingly robust against point mutations of crucial residues in the distal pocket.

2. Materials and methods

WT [12] and W88F, Y36F and W88F/Y36F mutant [16] trHbO proteins from *M. tuberculosis* were prepared as described and used at a concentration of ~50 μM, in 100 mM Tris, pH 8.0, in 1-mm path length gastight optical cells. After degazing in the cell, sodium ascorbate (for the O_2 and NO bound forms) or sodium dithionite (for the CO bound form) were anaerobically added to reduce the heme. Subsequently the samples were equilibrated with 1 atm. O_2, 0.01 atm. NO, or 1 atm. CO to obtain the respective liganded forms.

Multicolor femtosecond absorption experiments were performed as described [18] with a 30 fs pump pulse centered at 565 nm and a <30 fs white light continuum probe pulse, at a repetition rate of 30 Hz. Full spectra of the test and reference beams were recorded using a combination of a polychromator and a CCD camera. The sample was continuously moved perpendicular to the beams to ensure sample renewal between shots.

Molecular dynamics simulations were performed using CHARMM [19] (version 30b1). Two models based on structural variants found in the dodecameric crystal structure of the WT cyanide-poised protein, based on subunits B and H (with and without a Tyr36–Tyr23 covalent bond, respectively) [14] were investigated and gave qualitatively similar results. The models were constructed by replacing CN by CO, taking the FeII parameters for the heme, and embedding the protein in a shell of 1270 H_2O molecules. Unless indicated otherwise, the structural water molecules present in the CN-structure were maintained. The simulation protocol was similar to that in Ref. [20]. After energy minimization and equilibration of the system, the H-bonding network, including Trp88, Tyr36, the structural water molecules and the heme propionates, remains similar as in the crystal structure of the CN-complex [14], and the oxygen atom of CO interacts with both, Tyr36 and Trp88.

CO dissociation was simulated by deletion of the bond between the heme iron and the carbonmonoxy ligand from the bond list and simultaneous switching of heme parameters from six-coordinate to five-coordinate. Different starting conditions of the model before ligand dissociation were obtained by prolongation of the free dynamic phase of the CO-bound form by subsequent periods of 50 ps. Atom coordinates of the simulated structures were saved each picosecond for further analysis. For comparison, similar simulations were performed on a model based on the structure of the CO complex of sperm whale myoglobin.

3. Results

3.1. O_2 dissociation and rebinding

Upon excitation in the lowest-energy α band of the heme of the trHbO–O_2 complex, the Soret band of the heme shifts to longer wavelengths (Fig. 1) A. After decay of the subpicosecond phases due to heme photophysics (that are generally observed in heme-proteins [21]), the transient spectra decay for ~98% on the picosecond time scale. With a global exponential analysis a very good fit was obtained with on the timescale >1 ps a dominant component with a time constant of 4.8 ps and a minor component of ~20 ps (Fig. 1B and C). The induced absorption (product state) lobe of the transient spectrum associated with the 4.8 ps decay phase is severely perturbed with respect to the steady state (deoxy-*minus*-oxy) spectrum. Based on analysis of very similar features in the O_2 complex of the heme domain of the bacterial oxygen sensor FixL [22,23], we identify the corresponding state as a thermally excited 6-coordinate O_2-bound state rather than an actual O_2-dissociated state.[3] The ~20 ps phase may reflect rebinding of a small fraction of dissociated O_2, and the spectral shape of the remaining phase is similar to the steady state deoxy-*minus*-oxy spectrum. Comparing the relative amplitudes of the asymptotic and initial phases in HbO with those in FixL and taking into account the photodissociation quantum yield of the latter, we estimate that only ~1% of excited heme leads to dissociated O_2 on the time scale of >50 ps. This result is in agreement with the reported lack of O_2 dissociation observed using nanosecond excitation pulses [16].

3.2. NO dissociation and rebinding

Excitation of the NO complex leads to NO dissociation as indicated by the transient spectra on the picosecond timescale. These are similar (but not identical) to the deoxy-*minus*-nitroxy spectrum (Fig. 2) and, unlike in the oxycomplex, the shape of the spectrum remains virtually unchanged at $t > 1$ ps The dissociated NO subsequently rebinds virtually completely (>99%) on the picosecond time scale; this rebinding can be described by two exponential phases of 5.1 ps (75%) and 18 ps (25%). Rebinding of dissociated NO to heme generally occurs on this time scale and is often multiphasic. The near-unity amplitude of the rebinding in trHbO implies that NO has a very low probability to leave the heme pocket, as is for instance also the case in the NO receptor guanylate cyclase [24].

3.3. CO rebinding experiments

Upon excitation of the trHbO–CO complex, after decay of the subpicosecond phases due to heme photophysics [21], the transient spectra are assigned to CO-dissociated heme. For many heme proteins, including myoglobin [23], dissociated CO virtually does not rebind to the heme on the time scale up to a few nanoseconds. However, the CO rebinding characteristics of trHbO are highly unusual (Fig. 3): at $t = 4$ ns, most dissociated CO has rebound with the heme. Predominantly this occurs with a 1.2 ns time constant; kinetic analysis indicates that after this phase only ~5% of heme remains unliganded (Table 1). The spectrum associated with the major 1.2-ns CO rebinding phase is fairly similar to the steady-state difference spectrum. This observation, consistent with CO photoproduct resonance Raman spectra using 8-ns pulses [25], indicates that the photodissociated heme does relax to the steady-state deoxy configuration within ~100 ps. Using a simple model of competition between CO rebinding (~1.2 ns) and escape from the heme pocket (~5% amplitude), we can estimate the intrinsic time constant of escape at ~20 ns.

A small (~15%) additional 37-ps recombination phase is also observed (Fig. 3B and C). The spectral properties associated with this phase (less extensive shift than the main 1.2-ns phase) indicate that it corresponds to a fraction of unrelaxed heme. This phase

[3] Spectral diffusion may play a role in the cooling process. This process is likely not to be fully captured in an analysis in a global exponential analysis. However the quality of the fit indicated that, on the timescale >1 ps, the main features could be described in this framework and no efforts were made for a more detailed analysis.