

Watching a protein as it functions: real-time observations using picosecond crystallography

Friedrich Schotte¹, Michael Wulff², and Philip Anfinrud¹

¹ Laboratory of Chemical Physics, NIDDK, National Institutes of Health, Bethesda, MD 20892, USA

² European Synchrotron Radiation Facility, Grenoble Cedex 38043, FRANCE

Abstract. The structural changes associated with ligand translocation in myoglobin have been investigated with picosecond time-resolved x-ray crystallography. These studies, conducted on the ID09 beamline at the ESRF in Grenoble, France, unveil the protein's structural evolution with $< 2 \text{ \AA}$ resolution.

1. Introduction

Proteins are engaged in a myriad of tasks that are essential to life. To understand in mechanistic detail of how proteins function, it is crucial to know the time ordering of events that give rise to their designed function. Myoglobin (Mb), a ligand-binding heme protein, has long served as a model system for investigating ligand transport and binding in proteins. Using femtosecond time-resolved polarized IR spectroscopy, the dynamics of CO motion after photodetachment from MbCO have been probed [1, 2]. Those studies revealed the time-dependent orientation of CO, the presence of a docking site that mediates the transport of ligands to and from the active binding site, as well as the dynamics of ligand binding and escape. In addition, site-specific mutations among the highly conserved residues circumscribing the ligand docking site were found to have a marked influence on the dynamics of ligand binding and escape. While much has been learned about ligand dynamics in Mb, much less is known about the structural evolution that accompanies ligand translocation. To probe the structural evolution of a protein as it executes its designed function, a multinational collaboration has been established to acquire time-resolved x-ray crystal structures on the ID09B beam line at the European Synchrotron and Radiation Facility [3]. Recent improvements in the experimental methodology have extended the time resolution down to 150 ps and improved the spatial resolution of the diffraction images to below 2 \AA .

2. Experimental Methods

Time-resolved x-ray diffraction images are acquired using the pump-probe method: a femtosecond or nanosecond laser pulse triggers ligand dissociation in a ~ 250 micron P6 MbCO crystal and a variably delayed 150 ps x-ray pulse probes its structure. The x-ray pulses, which are generated when a relativistic electron bunch (6 GeV at the ESRF) passes through the gap of an undulator, can be

efficiently focused to the dimensions of the protein crystal. The diffracted x-ray photons are detected with high quantum efficiency using a MAR CCD. Reconstruction of the protein structure with atomic resolution requires a series of diffraction images with crystal orientations spanning 60 degrees (owing to the symmetry of the crystal). With undulator radiation, the x-ray bandwidth was sufficient to obtain redundant data with images collected every 2 degrees. To obtain high dynamic range diffraction images with the available x-ray flux, approximately 64 x-ray shots were integrated on the MAR CCD between readouts. Because the protein crystal requires sufficient time to recover between photolysis pulses, which are intense enough to excite a significant fraction of the protein molecules, the maximum repetition rate used was 3 Hz. Diffraction images were accumulated with and without photolysis to generate accurate difference-diffraction data.

To pursue these studies, the synchrotron is operated in a rare “single bunch” mode, where a single electron bunch is injected into the storage ring. Given the 844 m circumference of the ESRF storage ring, x-ray pulses are generated at 355 kHz. Single pulse selection from this pulse train is accomplished with a high speed synchronous x-ray chopper running at 900 Hz in combination with a single-shot operated millisecond shutter. Synchronization between the fs laser and x-ray pulses is accomplished with ps precision using a Synchro-Lock-equipped Coherent Mira Ti:sapphire laser; synchronization with a ns laser is accomplished with a digital delay generator.

3. Results and Discussion

A high-resolution electron difference density map of MbCO is shown in Fig. 1. This image is the first of a time series that reveal, with atomic resolution, the order of events that accompany ligand translocation. Numerous features are observed including the displacement of the heme iron toward the proximal histidine, tilting of the heme, the docking of CO in a site near the heme iron, and the correlated motion of protein side chains in the vicinity of the active binding site. For example, the distal histidine (residue 64) shifts toward the site once occupied by CO, possibly raising the barrier to geminate recombination. The photodetached CO, found in a heme pocket docking site only about 2 angstroms away from the binding site, causes a displacement of the nearby isoleucine (residue 107). The CO translocation and heme displacement are also evident in a time-resolved structure determined at 150 ps, suggesting that the protein response to the ligand translocation event is ultrafast, perhaps as fast as the 1.6 ps time constant assigned to protein relaxation in an earlier ultrafast time-resolved mid-IR study of photolyzed MbCO in solution [2]. On the time scale of a few hundred ns (not shown), the “docked” CO slips around to the proximal side of the heme and is found in the so-called Xe1 docking site, as observed in low temperature trapping studies [4,5] as well as in earlier time-resolved studies [6]. Within a few microseconds, the CO escapes from this site into the surrounding solvent. The structural changes that accompany ligand translocation help explain how the protein is able to excrete toxic CO with high efficiency, even though the CO is temporarily located so close to the active binding site.

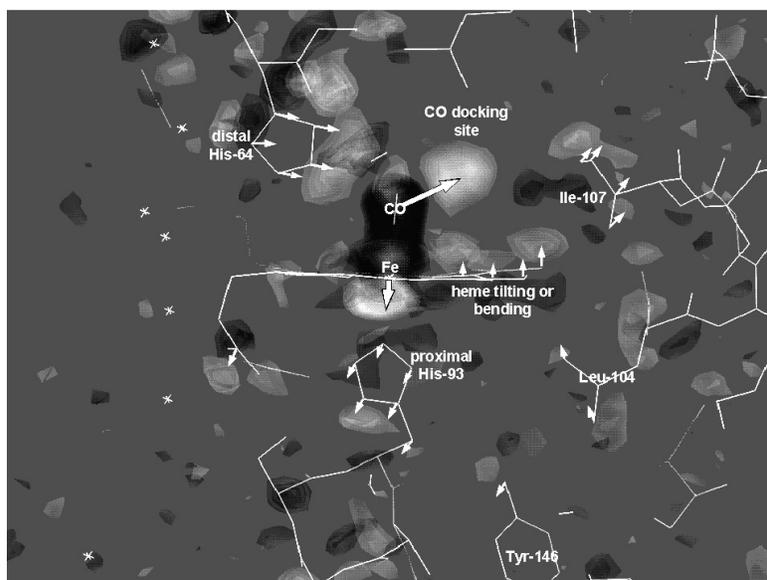


Fig. 1. Difference electron density map of MbCO determined 3 ns after photolysis of a P6 MbCO crystal at 10 °C. The dark (negative) and light (positive) regions correspond to photolysis-induced change in the electron density. The arrows indicate the direction of motion of the CO and the surrounding side chains.

4. Conclusions

Complementary time-resolved spectroscopic and crystallographic studies promise to unveil at a high level of structural detail the conformational changes that accompany protein function in this and other protein systems.

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