Observing heme doming in myoglobin with femtosecond X-ray absorption spectroscopy

M. Levantino, H. T. Lemke, Giorgio Schirò, M. Glownia, A. Cupane, Marco Cammarata

To cite this version:
M. Levantino, H. T. Lemke, Giorgio Schirò, M. Glownia, A. Cupane, et al.. Observing heme doming in myoglobin with femtosecond X-ray absorption spectroscopy). Structural Dynamics, 2015, 2 (4), pp.041713. 10.1063/1.4921907. hal-01168333
Observing heme doming in myoglobin with femtosecond X-ray absorption spectroscopy

M. Levantino, H. T. Lemke, G. Schirò, M. Glownia, A. Cupane, and M. Cammarata

Citation: Structural Dynamics 2, 041713 (2015); doi: 10.1063/1.4921907
View online: http://dx.doi.org/10.1063/1.4921907
View Table of Contents: http://scitation.aip.org/content/aca/journal/sdy/2/4?ver=pdfcov
Published by the American Crystallographic Association, Inc.

Articles you may be interested in

Ultraviolet photochemical reaction of [Fe(III)(C2O4)3]3− in aqueous solutions studied by femtosecond time-resolved X-ray absorption spectroscopy using an X-ray free electron laser
Struct. Dyn. 2, 034901 (2015); 10.1063/1.4918803

Ultrafast strong-field dissociative ionization dynamics of C H 2 Br 2 probed by femtosecond soft x-ray transient absorption spectroscopy
J. Chem. Phys. 128, 204302 (2008); 10.1063/1.2925268

Soft x-ray imaging system for picosecond time-resolved absorption spectroscopy using a femtosecond-laser-plasma source
Rev. Sci. Instrum. 77, 046105 (2006); 10.1063/1.2194476

Wavelength selective modulation in femtosecond pump–probe spectroscopy and its application to heme proteins
J. Chem. Phys. 114, 10884 (2001); 10.1063/1.1363673

A split beam method for measuring time-resolved circular dichroism
Rev. Sci. Instrum. 68, 1886 (1997); 10.1063/1.1147962
Observing heme doming in myoglobin with femtosecond X-ray absorption spectroscopy

M. Levantino, H. T. Lemke, G. Schirò, M. Glownia, A. Cupane, and M. Cammarata

Department of Physics and Chemistry, University of Palermo, Viale delle Scienze, 90128 Palermo, Italy
LCLS, SLAC National Accelerator Laboratory, Menlo Park, California 94025, USA
CNRS - Institut de Biologie Structurale, Grenoble 38044, France
Department of Physics, UMR UR1-CNRS 6251, University of Rennes 1, Rennes, France

(Received 5 March 2015; accepted 20 May 2015; published online 29 May 2015)

We report time-resolved X-ray absorption measurements after photolysis of carbonmonoxy myoglobin performed at the LCLS X-ray free electron laser with nearly 100 fs (FWHM) time resolution. Data at the Fe K-edge reveal that the photoinduced structural changes at the heme occur in two steps, with a faster (~70 fs) relaxation preceding a slower (~400 fs) one. We tentatively attribute the first relaxation to a structural rearrangement induced by photolysis involving essentially only the heme chromophore and the second relaxation to a residual Fe motion out of the heme plane that is coupled to the displacement of myoglobin F-helix. © 2015 Author(s). All article content, except where otherwise noted, is licensed under a Creative Commons Attribution 3.0 Unported License.

I. INTRODUCTION

Investigating chemical reactions in the femtosecond timescale with direct structural sensitive techniques is one of the scientific challenges that can now be approached thanks to the advent of X-ray free electron lasers (XFELs). Ultrafast nuclear and electronic rearrangements of transition metal complexes occurring after photoinduced spin crossover transitions have recently been reported both for molecular complexes dissolved in water (Bressler et al., 2009; Lemke et al., 2013; and Zhang et al., 2014) or in the crystalline state (Cammarata et al., 2014). In the case of proteins, ultrafast nuclear motions localized at the active site level similar to those observed in transition metal complexes are expected to drive biologically relevant conformational changes of the entire protein (Ansari et al., 1985). Indeed, the propagation of the perturbation from photexcited chromophores to the global polypeptide chain conformation has been recently observed through XFEL time-resolved X-ray scattering both in a bacterial reaction center (Arnlund et al., 2014) and in the hemeprotein myoglobin (Mb) (Levantino et al., 2015). However, in view of the limited spatial resolution of X-ray solution scattering (Cammarata et al., 2008), these studies were not able to provide information on the time evolution of the chromophore structural rearrangement that occurs after light absorption and precedes the polypeptide response (Martin and Vos, 1992). In the case of Mb, one of the most extensively studied systems in biology (Frauenfelder et al., 2003), information relevant to ultrafast structural changes of the heme chromophore has been obtained through time-resolved spectroscopy (Martin et al., 1983; Findsen et al., 1985; Petrich et al., 1988; Zhu et al., 1994; Franzen et al., 1995a; Mizutani and Kitagawa, 2001; and Sato et al., 2007). In particular, it is well-known that the bond between the protein and physiologically relevant diatomic ligands (O₂, CO
or NO) can be photolyzed within tens of fs from heme light absorption (Petrich et al., 1988). In carbonmonoxy Mb (MbCO), the heme is 6-coordinate with the low spin Fe(II) at its center coordinating four nitrogen atoms of the porphyrin, one nitrogen atom of the proximal histidine (His 93) side chain, and the carbon atom of the CO ligand (Figure 1(a)). After photolysis, the Fe(II) spin changes from $S = 0$ to $S = 2$, and the heme adopts a domed structure with the Fe moving out of the mean heme plane towards the proximal histidine (Figure 1(b)) (Teng et al., 1994; Schlichting et al., 1994).

The heme doming structural change can be directly monitored through resonance Raman spectroscopy since the Raman active Fe-His stretching vibration is resonantly coupled to heme absorption (Bangcharoenpaurpong et al., 1984) and its intensity is proportional to the distance of the Fe(II) with respect to the heme macrocycle plane (Stavrov, 1993). Time-resolved resonance Raman investigations have demonstrated that heme doming occurs within 700 fs (Franzen et al., 1995a; 1995b; and Kruglik et al., 2010). However, the temporal evolution of the iron out-of-plane motion could not be resolved in those experiments in view of the time resolution of those experiments. An experimental technique potentially able to monitor the structural changes at the heme with fs time resolution is X-ray absorption spectroscopy (XAS). Indeed, X-ray Absorption Near Edge Structure (XANES) spectroscopy is very sensitive to both the electronic distribution and the position of the nuclei around the absorbing metal (Lima et al., 2014; Shelby et al., 2014). In particular, it has been shown that the Fe(II) K-edge XANES spectrum of MbCO photoprodut is sensitive to the distances and bond angles around the heme iron (Della Longa et al., 2001; Arcovito et al., 2005) and that one of the most relevant parameters affecting the spectrum shape is the distance between the Fe and the N_e of the proximal histidine (Arcovito et al., 2005). Time-resolved XAS has been used in the past to investigate the structural dynamics of Mb. Pioneering work by Mills et al. (1984) showed that within 300 $\mu$s from photolysis of MbCO, the Fe K-edge energy position ($\sim$7.1 keV) is red-shifted by $\sim$3 eV with respect to the MbCO equilibrium spectrum. Many years later, Wang et al. repeated the same experiment with a slightly shorter time resolution (100 $\mu$s) but obtaining much higher signal-to-noise ratio XANES spectra thanks to the $\sim$100 times higher repetition rate of photolysis employed (Wang et al., 2005). Their data show that the Fe K-edge XANES spectrum at 100 $\mu$s from photolysis of MbCO is characterized by a relative broad pre-edge peak associated to weak Fe 1s to 3d transitions typical of high spin Fe(II) systems. Full advantage of the time resolution available at third generation X-ray synchrotron sources was
recently exploited by Chergui group (Lima et al., 2011; Lima et al., 2012) and the Chen group (Stickrath et al., 2013). However, no significant difference between the 100 ps spectrum and those measured at longer time delays could be observed (Stickrath et al., 2013) (Figure 2), indicating that nuclear and electronic rearrangements at the heme level are already completed within 100 ps, in agreement with time-resolved resonance Raman results (Franzen et al., 1995a; 1995b; Mizutani and Kitagawa, 2001; Sato et al., 2007; and Kruglik et al., 2010).

In order to track in real-time, the structural transition taking place at the heme after photolysis of MbCO, we have performed a time-resolved X-ray absorption experiment at the Linear Coherent Light Source (LCLS) XFEL. By exploiting the femtosecond X-ray pulses available at the LCLS, we have been able to monitor the time-evolution of the Fe K-edge absorption signal at selected energies. Our data show that the heme structural transition consists of two steps, with an initial response (~70 fs) associated to a partial heme doming and a slower component (~400 fs) that we tentatively attribute to a residual Fe out-of-plane motion that is slaved to the displacement of Mb F-helix.

II. EXPERIMENTAL METHODS

A. Sample preparation and handling

The sample was a solution of horse MbCO in CO saturated 0.1 M phosphate buffer at pH 7.4. The protein concentration was 5.6 mM (~100 mg/ml). A few molar excess of sodium dithionite was anaerobically added to the solution while the sample was saturated with CO to ensure full reduction of the heme iron and in order to minimize residuals traces of dissolved molecular oxygen. An aliquot (~10 ml) of the MbCO solution was anaerobically transferred to a vial connected to a closed loop liquid circulation system that was previously flushed thoroughly with N2 and then with CO. A slight positive pressure of CO gas was maintained in the sample reservoir during the experiments. The liquid circulation system was connected to a capillary nozzle which generated a stable 100 µm round liquid jet inside a He purged sample chamber. The flow rate was set to 2 ml/min. The optical absorption spectrum of microliter samples was measured before, after, and during the X-ray absorption data collection to ensure that close to 100% of the myoglobin molecules were in the MbCO ligation state.

FIG. 2. XANES spectra of myoglobin at the Fe K-edge. XANES spectra obtained by Stickrath et al. (2013) at the advanced photon source synchrotron are reported. Bottom panel: the steady-state MbCO spectrum (red) is compared with the photo-product (Mb*) spectra at 100 ps (magenta) and at 47 µs (blue) from photolysis of MbCO (all data were provided by Prof. Lin Chen, Northwestern University). Upper panel: normalized difference spectra calculated from the data reported in the lower panel show that no significant change is observed between 100 ps (magenta) and 47 µs (blue) from photolysis; black arrows indicate the X-ray energies of 7.123, 7.155, and 7.159 keV at which XFEL time-scans have been recorded during the LCLS experiment.
B. Time-resolved X-ray absorption setup

Time-resolved X-ray absorption measurements at the Fe K-edge have been performed at the XPP endstation of the LCLS (Figure 3). The 100 μm liquid jet was vertically oriented with respect to both the incoming optical and X-ray beams. Photolysis of the bond between Mb and CO was achieved with linearly polarized optical pulses (~70 fs FWHM; 538 nm; 20 μJ per pulse, vertically polarized) focused to a 250 μm (FWHM) circular spot at the sample. This corresponds to an estimated average of 2.2 photons absorbed per heme at each photolysis pulse. A nearly collinear (~1°) geometry between optical and X-rays beams was adopted. X-rays of ~1 eV bandwidth were selected from the FEL spectrum using a Si(111) double-crystal monochromator (so called Large Offset Double Crystal Monochromator). This monochromator relies on two independent crystals with an horizontal offset of 600 mm thus allowing the XPP instrument in a location to remain in place (in the 600 mm offset position) while three instruments located in the far experimental hall of LCLS take the beam. This setup, while allowing to rapidly switch between beamlines and even to perform different experiments at the same time (multiplexed mode), has the disadvantage of not being suitable for rapidly changing the X-ray energy (Zhu et al., 2012), and thus no time-resolved X-ray spectra could be measured during our available beamtime. The X-ray beam was focused downstream the sample by means of Be lenses, the beamsize at the sample position was ~30 μm (FWHM). The X-ray pulses duration was ~30 fs (FWHM) and the estimated number of photons is of the order of 4 × 10^10 for the intense shots at the sample position. The X-ray absorption of the sample was monitored in fluorescence mode (Jaklevic et al., 1993) by measuring the total fluorescence signal in the vicinity of the Fe K-edge. The X-ray fluorescence signal was collected with a Si diode (Canberra FD450–18-300RM, diameter 2.54 cm) positioned at a 90° angle in the horizontal plane with respect to the X-ray beam propagation direction (detector-sample distance of ~5 cm). Diode nonlinearity was corrected using the same procedure described by Lemke et al. (2013). Moreover, by using the recently developed XPP timing tool, the collected data have been time sorted on the basis of independent shot-by-shot measurements of the arrival times of pump and probe pulses (Harmand et al., 2013). This correction resulted in ~10 fs effective time jitter between optical and X-ray pulses, which is significantly shorter than the intrinsic uncorrected shot-to-shot time jitter (~300 fs). The main contribution to the overall time resolution of our

FIG. 3. Schematic representation of the experimental setup. A ~100 μm thick liquid jet of the sample (a solution of ~5.6 mM MbCO in 0.1 M phosphate buffer at pH 7.4, in red) was produced with a homemade nozzle. The sample was phototexcited with ~70 fs optical laser pulses at 538 nm (green beam) and probed with ~30 fs monochromatic X-ray pulses (white beam) in nearly collinear (~1°) geometry (the angle between the pump and the probe beams is exaggerated in the figure for the sake of clarity). The large offset monochromator of XPP is sketched in white (Zhu et al., 2012). An X-ray diode (in blue) was used to measure the total fluorescence yield signal as a function of the delay between pump and probe pulses.
experiment, which we estimate to be $\sim$125 fs, is the velocity mismatch of the optical and X-ray beams through the 100 $\mu$m thick liquid jet ($\sim$100 fs).

During the time-resolved data collection, we continuously monitored the ground state fluorescence signal ($I_{\text{off}}$) by dropping the photolysis pulse every 17 shots. This allows to track with high fidelity relative changes ($I_{\text{on}}/I_{\text{off}}$) in that it minimizes the effects of slow drifts (e.g., X-ray beam pointing). Data have been analyzed using a script based on the freely available ixppy package (https://github.com/htlemke/ixppy).

III. RESULTS

The time evolution of the sample X-ray absorption near the Fe K-edge has been monitored at selected energies (7123, 7155, and 7159 eV). No full XANES spectra could be measured during the experiment in view of the large offset monochromator available during our beamtime (see Methods). Figure 4 reports the signal obtained at an X-ray energy of 7123 eV, which corresponds to the average Fe K-edge position and to the maximum of the deoxyMb-MbCO XANES difference spectrum (Lima et al., 2011). Structural changes at the level of the heme chromophore, such as the elongation of the bonds between the Fe ion and the pyrrole N atoms are expected to induce a shift in the Fe K-edge position that can be probed by monitoring the time evolution of the absorption at 7123 eV. Figure 4 shows that, indeed, a large signal change is observed in the subpicosecond timescale, as hinted by previous optical spectroscopy studies (Martin et al., 1983; Petrich et al., 1988; Franzen et al., 1995a; 1995b; Mizutani and Kitagawa, 2001; Sato et al., 2007; and Kruglik et al., 2010). Such ultrafast signal change could not be observed in previous time-resolved X-ray absorption studies performed at synchrotrons, in view of the significantly lower ($\sim$3 orders of magnitude) time resolution of those experiments. The sudden increase of the signal around time $t = 0$ indicates that we are observing the contribution of relaxation processes having a characteristic time longer than the effective time resolution of the measurement. Indeed, the data in Figure 4 can be accurately described in terms of the sum of two exponential processes having characteristic times of $(73 \pm 5)$ fs and $(400 \pm 25)$ fs, respectively. Both exponentials have been convoluted with a Gaussian instrument response function having a $(53 \pm 2)$ fs width (corresponding to 125 fs FWHM). The faster exponential process accounts for $\sim$80% ($\pm$3%) of the observed signal increase, indicating that significant electronic and nuclear rearrangements around the iron occur at $\sim$70 fs from photolysis of MbCO. These results are compatible also with the signal evolution obtained with X-ray energies of 7155 and 7159 eV (Figure 5). These two X-ray energies correspond to emitted photoelectrons having an energy in the 30–40 eV region for which the mean free path is close to a

![FIG. 4. Time dependence of the absorption at the Fe K-edge (7123 eV) after photolysis of MbCO. The ratio between the total fluorescence signal, $I(t)$, and the average signal in the absence of photolysis, $I_{\text{off}}$, is plotted as a function of time (orange symbols). Left panel: data between $-0.5$ and 2 ps. Right panel: data between 2 and 100 ps plotted in a logarithmic time scale. Negative times correspond to X-ray pulses arriving at the sample before the optical pulses. Errorbars (corresponding to one standard deviation) are shown every 10 experimental points (red symbols). The experimental time evolution was fitted in terms of the convolution between a normalized Gaussian function and the sum of two exponential functions (black line) (Lemke et al., 2013). The fitting parameters for the two exponential functions are $t_1 = 73 \pm 5$ fs (80 $\pm$ 3%) and $t_2 = 400 \pm 20$ fs (20 $\pm$ 3%); the Gaussian width is 53 $\pm$ 2 fs (RMS).](https://example.com/figure4.png)
minimum (Muller et al., 1982; Tanuma et al., 1994), thus having the highest sensitivity to the position of iron nearest neighbour atoms. Since the signal-to-noise ratio at these energies is quite low, the fitting parameters are less reliable than those obtained from the signal at 7123 eV. However, as shown by the continuous line in Figure 5, the same relaxation times are able to describe satisfactorily the observed time evolutions obtained at 7155 and 7159 eV. Indeed, by using only the relative weight of the exponentials as a free fitting parameter, one can satisfactorily reproduce the experimental data. The relative weight of the fast component is found to be 0.40 \(\pm 0.03\) and 0.48 \(\pm 0.04\) for 7155 and 7159 eV, respectively. Within the signal-to-noise ratio of our data, there is no evidence of any relevant further signal evolution at times longer than \(\sim 1\) ps, indicating that the most significant structural changes around the Fe(II) take place in the subpicosecond timescale. Moreover, no periodic modulation of the X-ray absorption signal is evident in our data. Although the time-resolution of our experiment is sufficiently high to observe the iron-histidine (\(\sim 220\) cm\(^{-1}\)) and/or the heme doming (\(\sim 50\) cm\(^{-1}\)) vibrational modes (Zhu et al., 1994), we cannot exclude that a small amplitude modulation is within the noise level of our data. More experiments with significantly improved statistics would be needed to test whether this is the case or not.

IV. DISCUSSION

The \(\sim 70\) fs relaxation time obtained in the present experiment is shorter than the \(\sim 170\) fs characteristic timescale of the signal change in iron coordination complexes (Bressler et al., 2009; Lemke et al., 2013) at the same X-ray photon energy and attributed to the structural change accompanying the iron low-spin to high-spin transition, but is longer than a quarter-period of the Fe-His stretching vibration (\(\nu_{\text{Fe-His}} \sim 220\) cm\(^{-1}\)) and/or the heme doming (\(\sim 50\) cm\(^{-1}\)) vibrational modes (Findsen et al., 1985; Franzen et al., 1995a). Moreover, this relaxation time is close to predictions based on theoretical calculations and molecular dynamics (MD) simulations (Henry et al., 1985; Petrich et al., 1991; and Li et al., 1993). Indeed, by combining quantum mechanical calculations to model the 5-coordinate heme metastable state generated right after photolysis with
MD simulations on both heme model compounds or entire protein models, an iron out-of-plane motion with an average relaxation half-time of $\sim 50$ fs was invariably obtained. In light of our recent time-resolved X-ray scattering data (Levantino et al., 2015) and of the observation of a $\sim 70$ fs relaxation phase in the present experiment, we propose that the $\sim 400$ fs relaxation is a residual iron out-of-plane motion that is coupled to the motion of the F helix of Mb polypeptide chain. Indeed, the $\sim 70$ fs relaxation is likely to result in the elongation of the bonds between the iron and the pyrrole N atoms and in a corresponding compression of the iron-histidine bond; this latter compression is expected to trigger the F helix motion, thus initiating Mb protein quake (Ansari et al., 1985). The F helix motion occurs at $\sim 400$ fs from photolysis and propagates to the global protein structure within 1 ps (Levantino et al., 2015), in agreement with the observation of a fully relaxed Fe-His stretching mode within $\sim 700$ fs from photolysis of MbCO (Franzen et al., 1995a; 1995b). It must be noted that previous time-resolved optical absorption spectroscopy experiments have observed a single $\sim 300$ fs relaxation process, which has been attributed to the (de)population of different possible intermediates with partially domed heme (Martin et al., 1983; Petrich et al., 1988; and Franzen et al., 2001). Ye et al. (2002) interpreted alternatively this relaxation as an evidence of the 5-coordinate heme vibrational cooling based on a comparison with results obtained on photoexcited deoxyMb. Our data cannot rule out this alternative interpretation, although simulative (Henry et al., 1986; Sagnella and Straub, 2001; and Zhang et al., 2007), theoretical (Li and Champion, 1994), and time-resolved Raman (Lingle et al., 1991; Mizutani and Kitagawa, 1997) investigations point to a heme cooling process occurring after several picoseconds from photolysis of MbCO.

In order to fully characterize the structural dynamics of the heme, the extent of motion and changes in electronic configuration involved during the first ($\sim 70$ fs) and the second ($\sim 400$ fs) relaxation steps, full femtosecond XANES spectra (over a 70–100 eV range) would be needed. Indeed, different hypotheses on the attribution of the observed X-ray absorption signal time evolutions to specific structural entities could be verified only through an in depth analysis of XANES spectra as it has been shown for Mb equilibrium states or cryogenic photoproducts evolutions to specific structural entities could be verified only through an in depth analysis of XANES spectra (Della Longa et al., 2001; Arcovito et al., 2005; and Lima et al., 2014). The work presented here is a first step in the direction of elucidating the ultrafast structural changes occurring after photexcitation of a macromolecule chromophore and demonstrates that the significantly improved time resolution ($\sim 100$ fs) now available at XFELs may allow to monitor electronic and nuclear rearrangements at chemical relevant timescales, thus extending and complementing time-resolved solution (Levantino et al., 2015) and crystallography studies (Schotte et al., 2003; Srajer and Royer, 2012).

ACKNOWLEDGMENTS

Portions of this research were carried out at the Linac Coherent Light Source (LCLS) at the SLAC National Accelerator Laboratory. LCLS is an Office of Science User Facility operated for the U.S. Department of Energy Office of Science by Stanford University. M.C. acknowledges the PEPS project “SASLELX” of the CNRS and the University of Rennes 1 for financial support. M.L. acknowledges the financial support of the University of Palermo (FFR program 2012/2013 and CoRI program 2013). The authors would like to thank Lin Chen (Northwestern University) for kindly providing the data shown in Figure 2.

Ansari, A., Berendzen, J., Bowne, S. F., Frauenfelder, H., Iben, I. E., Sauke, T. B., Shyamsunder, E., and Young, R. D., “Protein states and protein quakes,” Proc. Natl. Acad. Sci. U.S.A. 82, 5000–5004 (1985).

Arcovito, A., Lamb, D., Nienhaus, G., Hazemann, J., Benfatto, M., and Della Longa, S., “Light-induced relaxation of photooxidized carbonmonoxide myoglobin: A temperature-dependent x-ray absorption near-edge structure (XANES) study,” Biophys. J. 88(4), 2954–2964 (2005).

Arlund, D., Johansson, L. C., Wickstrand, C., Bart, A., Williams, G. J., Malmerberg, E., Davidson, J., Milathianaki, D., DePonte, D. P., Shoeman, R. L., Wang, D., James, D., Katona, G., Westhoff, S., White, T. A., Aquila, A., Bari, S., Berntsen, P., Bogan, M., Driel, T. B. V., Doak, R. B., Kjær, K. S., Frank, M., Fromme, R., Grotjohann, I., Henning, R., Hunter, M. S., Kirian, R. A., Kosheleva, I., Kupitz, C., Liang, M., Martin, A. V., Nielsen, M. M., Messerschmidt, M., Seibert, M. M., Sjöhann, J., Stellato, F., Weierstall, U., Zatsepin, N. A., Spence, J. C. H., Fromme, P., Schlichting, I., Boutet, S., Groenhof, G., Chapman, H. N., and Neutze, R., “Visualizing a protein quake with time-resolved x-ray scattering at a free-electron laser,” Nat. Methods 11, 923–926 (2014).
Bungcharoenpauppong, O., Schomacker, K. T., and Champion, P. M., “A resonance Raman investigation of myoglobin and hemoglobin,” J. Am. Chem. Soc. 106, 5688–5698 (1984).

Bressler, C., Milne, C., Pham, V.-T., Elahinas, A., van der Veen, R. M., Gaweida, W., Johnson, S., Beaud, P., Grolimund, D., Kaiser, M., Borca, C. N., Ingold, G., Abel, R., and Chergui, M., “Femtosecond XANES study of the light-induced spin crossover dynamics in an iron(II) complex,” Science 323, 489–492 (2009).

Cammarata, M., Levantino, M., Schotte, F., Anfinrud, P. A., Ewald, F., Choi, J., Cupane, A., Wulf, M., and Ihee, H., “Tracking the structural dynamics of proteins in solution using time-resolved wide-angle x-ray scattering,” Nat. Methods 5, 881–886 (2008).

Cammarata, M., Bertoni, R., Lorenz, M. Cuilleau, H., Di Matteo, S., Mauriac, C., Matar, S. F., Lemke, H., Chollet, M., Ravy, S., Lauh, C., Letard, J. F., and Collet, E., “Sequential activation of molecular breathing and bending during spin-crossover photoswitching revealed by femtosecond optical and x-ray absorption spectroscopy,” Phys. Rev. Lett. 113, 227402 (2014).

Della Longa, S., Arcovito, A., Girasole, M., Hazemann, J. L., and Benfatto, M., “Quantitative analysis of x-ray absorption near edge structure data by a full multiple scattering procedure: The Fe-CO geometry in photolyzed carbonmonoxy-myoglobin single crystal,” Phys. Rev. Lett. 87, 155501 (2001).

Findsen, E. W., Scott, T. W., Chance, M. R., Friedman, J. M., and Ondrias, M. R., “Picosecond time-resolved Raman studies of photodissociated carboxymyoglobin,” J. Am. Chem. Soc. 107(11), 3355–3357 (1985).

Franzen, S., Bohn, B., Poyart, S., and Martin, J.-L., “Evidence for sub-picosecond heme doming in hemoglobin and myoglobin: A time-resolved resonance Raman comparison of carbonmonoxy and deoxy species,” Biochemistry 34, 1224–1237 (1995a).

Franzen, S., Bohn, B., Poyart, S., DePillis, G., Boyer, S. G., and Martin, J.-L., “Functional aspects of ultra-rapid heme doming in hemoglobin, myoglobin, and the myoglobin mutant H93G,” J. Biol. Chem. 270, 1718–1720 (1995b).

Franzen, S., Kiger, L., Poyart, C., and Martin, J.-L., “Heme photolysis occurs by ultrafast excited state-metal-to-ring charge transfer,” Biophys. J. 80, 2372–2381 (2001).

Frauenfelder, H., McMahon, B. H., and Fenimore, P. W., “Myoglobin: The hydrogen atom of biology and a paradigm of complexity,” Proc. Natl. Acad. Sci. U.S.A. 100, 8615–8617 (2003).

Harmand, M., Coffee, R., Bionta, M. R., Chollet, M., French, D., Zhu, D., Fritz, D. M., Lemke, H. T., Medvedev, N., Ziaja, R. B., Toleikis, S., and Cammarata, M., “Achieving few-femtosecond time-sorting at hard x-ray free-electron lasers,” Nat. Photonics 7, 215–218 (2013).

Henry, E. R., Levitt, M., and Eaton, W. A., “Molecular dynamics simulation of photodissociation of carbon monoxide from hemoglobin,” Proc. Natl. Acad. Sci. U.S.A. 82, 2034–2038 (1985).

Henry, E. R., Eaton, W. A., and Hochstrasser, R. M., “Molecular dynamics simulations of cooling in laser-excited heme proteins,” Proc. Natl. Acad. Sci. U.S.A. 83, 8982–8986 (1986).

Jaklevic, J., Kirby, J. A., Klein, M. P., Robertson, A. S., Brown, G. S., and Eisenberger, P., “Fluorescence detection of EXAFS: sensitivity enhancement for dilute species and thin films,” Solid State Commun. 88, 1105–1108 (1993).

Kruglik, S. G., Yoo, B.-K., Franzen, S., vows, M. H., Martin, J.-L., and Negerie, M., “Picosecond primary structural transition of the heme is retarded after nitric oxide binding to heme proteins,” Proc. Natl. Acad. Sci. U.S.A. 107, 13678–13683 (2010).

Lemke, H. T., Bressler, C., Chen, L. X., Fritz, D. M., Gaffney, K. J., Galler, A., Gaweida, W., Haldrup, K., Hartsock, R. W., Ihee, H., Kim, J., Kim, K. H., Lee, J. H., Nielsen, M. M., Stickrath, A. B., Zhang, W., Zhu, D., and Cammarata, M., “Femtosecond x-ray absorption Raman at a hard x-ray free electron laser: Application to spin crossover dynamics,” J. Phys. Chem. A 117, 735–740 (2013).

Levantino, M., Schirò, G., Lemke, H. T., Cottone, G., Glownia, J. M., Zhu, D., Chollet, M., Ihee, H., Cupane, A., and Cammarata, M., “Ultrafast myoglobin structural dynamics observed with an x-ray free electron laser,” Nat. Commun. 6, 6772 (2015).

Li, H., Elber, R., and Straub, J. E., “Molecular dynamics simulation of No recombination to myoglobin mutants,” J. Biol. Chem. 268, 17908–17916 (1993), available at http://www.jbc.org/content/268/24/17908.long.

Li, P. and Champion, P. M., “Investigations of the thermal response of laser-excited biomolecules,” Biophys. J. 66, 430–436 (1994).

Lima, F. A., Milne, C. J., Amarasinghe, D. C. V., Rittmann-Frank, M. H., van der Veen, R. M., Reinhard, M., Pham, V.-T., Karlsson, S., Johnson, S. L., Grolimund, D., Borca, C., Huthwelker, T., Janousch, M., Van Mourik, F., Abel, R., and Chergui, M., “A high-repetition rate scheme for synchrotron-based picosecond laser x-ray probe experiments on chemical and biological systems in solution,” Rev. Sci. Instrum. 82, 063111 (2011).

Lima, F. A., Milne, C. J., Rittmann-Frank, M. H., van der Veen, R. M., Reinhard, M., Penfold, T. J., Benfatto, M., and Chergui, M., “X-ray absorption studies of the photo-induced structural changes of myoglobin in physiological,” Research in Optical Sciences, OSA Technical Digest 2012, paper W2D6.2, 2012.

Lima, F. A. Penfold, T. J., van der Veen, R. M., Reinhard, M., Abel, R., Tavernelli, I., Rothlisberger, U., Benfatto, M., Milne, C. J., and Chergui, M., “Probing the electronic and geometric structure of ferric and ferrous myoglobin in physiological solutions by Fe K-edge absorption spectroscopy,” Phys. Chem. Chem. Phys. 16(4), 1617–1631 (2014).

Lingle, R., Xu, X., Zhu, H., Yu, S.-C., and Hopkins, J. B., “Picosecond Raman study of energy flow in a photoexcited heme protein,” J. Phys. Chem. 95, 9320–9331 (1991).

Martin, J.-L., Migus, A., Poyart, C., Lecarpentier, Y., Astier, R., and Antonetti, A., “Femtosecond photolysis of co-ligated prototere and hemoproteins: Appearance of deoxy species with a 550-fsec time constant,” Proc. Natl. Acad. Sci. U.S.A. 80, 173–177 (1983).

Martin, J.-L and Man, M.-H., “Femtosecond biology,” Annu. Rev. Biophys. Biomol. Struct. 21, 199–222 (1992).

Mills, D. M., Lewis, A., Harootunian, A., Huang, J., and Smith, B., “Time-resolved x-ray absorption spectroscopy of carbox monoxy-myooglobin recombination after laser photoysis,” Science 223, 811–813 (1984).

Mizutani, Y. and Kitagawa, T., “Direct observation of cooling of heme upon photodissociation of carbonmonoxy myoglobin,” Science 278, 443–446 (1997).

Mizutani, Y. and Kitagawa, T., “Ultrafast structural relaxation of myoglobin following photodissociation of carbon monoxide probed by time-resolved resonance Raman spectroscopy,” J. Phys. Chem. B 105, 10992–10999 (2001).
Muller, J. E., Jepsen, O., and Wilkins, J. W., “X-ray absorption spectra: K-edges of 3d transition metals, L-edges of 3d and 4d metals, and M-edges of Palladium,” *Solid State Commun.* **42**, 365–368 (1982).

Petrich, J. W., Poyart, S., and Martin, J.-L., “Photophysics and reactivity of hemoglobin, myoglobin, and protoheme,” *Biochemistry* **27**, 4049–4060 (1988).

Petrich, J. W., Lamby, J.-C., Kuczera, K., Karplus, M., Poyart, C., and Martin, J.-L., “Ligand binding and protein relaxation in heme proteins: A room temperature analysis of NO geminate recombination,” *Biochemistry* **30**, 3975–3987 (1991).

Sagnella, D. E. and Straub, J. E., “Directed energy ‘funneling’ mechanism for heme cooling following ligand photolysis or direct excitation in solvated carbonmonoxy myoglobin,” *J. Phys. Chem. B* **105**, 7057–7063 (2001).

Sato, A., Gao, Y., Kitagawa, T., and Mizutani, Y., “Primary protein response after ligand photodissociation in carbonmonoxy myoglobin,” *Proc. Natl. Acad. Sci. U.S.A.* **104**, 9627–9632 (2007).

Schlichting, I., Berendzen, J., Phillips, G. N., and Sweet, R. M., “Crystal structure of photolyzed carbonmonoxy-myoglobin,” *Nature* **371**, 808–812 (1994).

Schotte, F., Lim, M., Jackson, T. A., Smimov, A. V., Soman, J., Olson, J. S., Phillips, Jr., G. N., Wulff, M., and Anfinrud, P. A., “Watching a protein as it functions with 150-ps time-resolved x-ray crystallography,” *Science* **300**, 1944–1947 (2003).

Sato, A., Gao, Y., Kitagawa, T., and Mizutani, Y., “Primary protein response after ligand photodissociation in carbonmonoxy myoglobin,” *Proc. Natl. Acad. Sci. U.S.A.* **104**, 9627–9632 (2007).

Schlichting, I., Berendzen, J., Phillips, G. N., and Sweet, R. M., “Crystal structure of photolyzed carbonmonoxy-myoglobin,” *Nature* **371**, 808–812 (1994).

Stavrov, S. S., “The effect of iron displacement out of the porphyrin plane on the resonance Raman spectra of heme proteins and iron porphyrins,” *Biophys. J.* **65**, 1942–1950 (1993).

Stickrath, A. B., Mara, M. W., Lockard, J. V., Harpham, M. R., Huang, J., Zhang, X., Attenkofer, K., and Chen, L. X., “Detailed transient heme structures of Mb-CO in solution after CO dissociation: An x-ray transient absorption spectroscopic study,” *J. Phys. Chem. B* **117**, 4705–4712 (2013).

Tanuma, S., Powell, C. J., and Penn, D. R., “Calculations of electron inelastic mean free paths,” *Surf. Interface Anal.* **21**, 165–176 (1994).

Teng, T.-Y., Srajer, V., and Moffat, K., “Photolysis-induced structural changes in single crystal of carbonmonoxy myoglobin at 40 K,” *Nat. Struct. Biol.* **1**, 701–705 (1994).

Wang, H., Peng, G., and Cramer, S. P., “Document x-ray absorption spectroscopy of biological photolysis products: Kilohertz photolysis and soft x-ray applications,” *J. Electron. Spectrosc. Relat. Phenom.* **143**, 1–7 (2005).

Ye, X., Demidov, A., and Champion, P. M., “Measurements of the photodissociation quantum yields of MbNO and MbO2 and the vibrational relaxation of the six-coordinate heme species,” *J. Am. Chem. Soc.* **124**, 5914–5924 (2002).

Zhang, Y., Fujisaki, H., and Straub, J. E., “Molecular dynamics study on the solvent dependent heme cooling following ligand photolysis in carbonmonoxy myoglobin,” *J. Phys. Chem. B* **111**, 3243–3250 (2007).

Zhang, W., Alonso-Mori, R., Bergmann, U., Bressler, C., Chollet, M., Galler, A., Gawelda, W., Hadt, R. J., Hartsch, R. W., Kroll, T., Kjær, K. S., Kubicki, K., Lemke, H. T., Liang, H. W., Meyer, D. A., Nielsen, M. M., Purser, C., Robinson, J. S., Solomon, E. I., Sun, Z., Sokaras, D., van Driel, T. B., Yankó, G., Weng, T. C., Zhu, D., and Gaffney, K. J., “Tracking excited-state charge and spin dynamics in iron coordination complexes,” *Nature* **509**, 345–348 (2014).

Zhu, L., Sage, J. T., and Champion, P. M., “Observation of coherent reaction dynamics in heme proteins,” *Science* **266**, 629–632 (1994).

Zhu, D., Cammarata, M., Feldkamp, J. M., Fritz, D. M., Hastings, J. B., Lee, S., Lemke, H. T., Robert, A., Turner, J. L., and Feng, Y., “A single-shot transmissive spectrometer for hard x-ray free electron lasers,” *Appl. Phys. Lett.* **101**, 034103 (2012).