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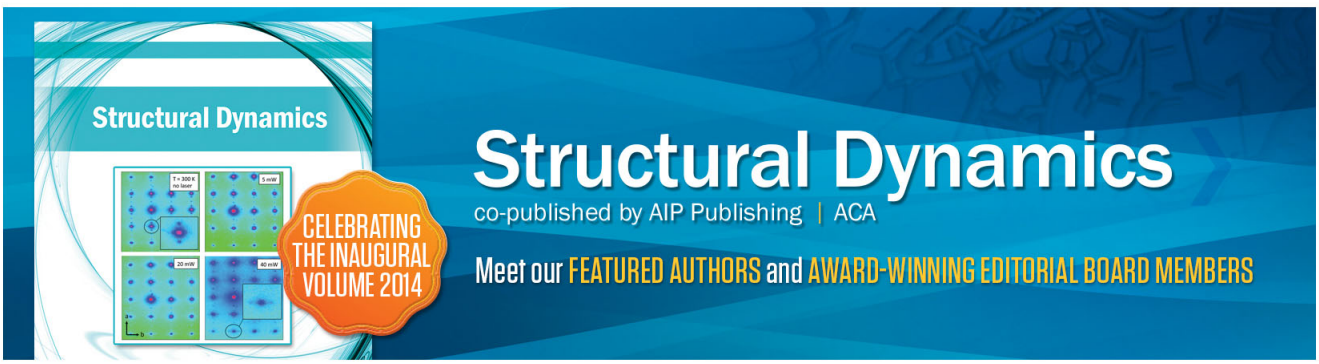
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Observing heme doming in myoglobin with femtosecond X-ray absorption spectroscopy[a)]

M. Levantino,[1] H. T. Lemke,[2] G. Schirò,[3] M. Glownia,[2] A. Cupane,[1] and M. Cammarata[4,b)]
[1]*Department of Physics and Chemistry, University of Palermo, Viale delle Scienze, 90128 Palermo, Italy*
[2]*LCLS, SLAC National Accelerator Laboratory, Menlo Park, California 94025, USA*
[3]*CNRS - Institut de Biologie Structurale, Grenoble 38044, France*
[4]*Department of Physics, UMR UR1-CNRS 6251, University of Rennes 1, Rennes, France*

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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*.
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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 photoexcited 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 (O_2, CO

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[b)]Author to whom correspondence should be addressed. Electronic mail: marco.cammarata@univ-rennes1.fr

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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 photoproduct 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_ε 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 μs 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 μs) 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 μs 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

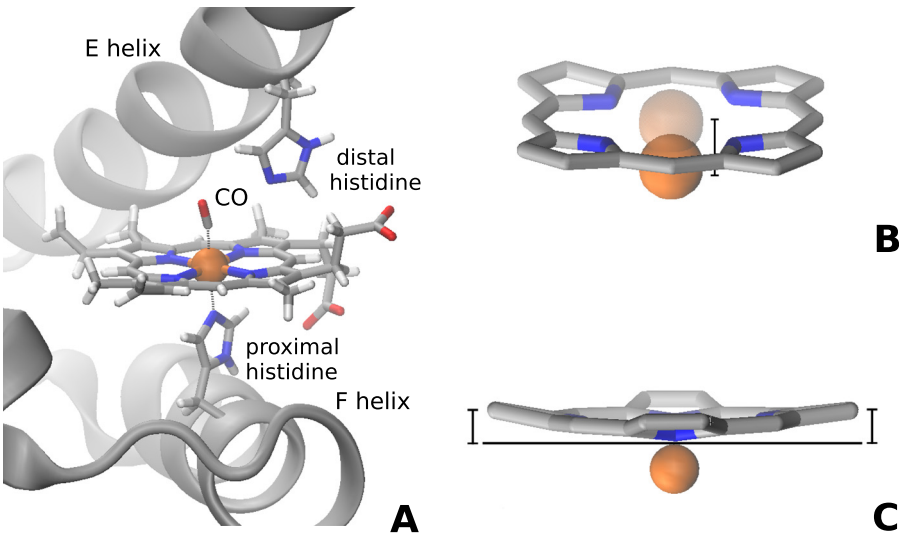


FIG. 1. Schematics of myoglobin active site. (a) In MbCO the Fe(II) at the heme center coordinates the four pyrrole nitrogens, the N_ε atom of the proximal histidine, and the carbon atom of the CO ligand. After photolysis, the Fe(II) spin changes from $S=0$ to $S=2$ and the heme adopts a domed structure: (b) the Fe(II) moves out of the mean heme plane and (c) the porphyrin pyrrole rings tilt slightly with respect to the mean heme plane.

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 N_2 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.

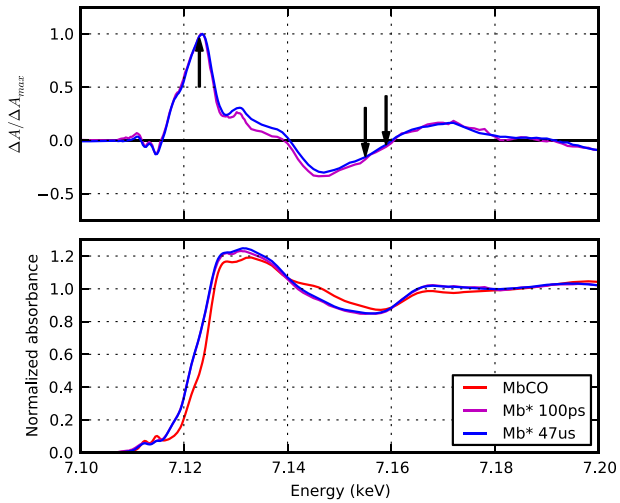


FIG. 2. XANES spectra of myoglobin at the Fe K-edge. XANES spectra obtained by Stickrath *et al.* (2013) at the advanced photon source synchrotron are reported. Bottom panel: the steady-state MbCO spectrum (red) is compared with the photoproduct (Mb*) spectra at 100 ps (magenta) and at 47 μs (blue) from 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

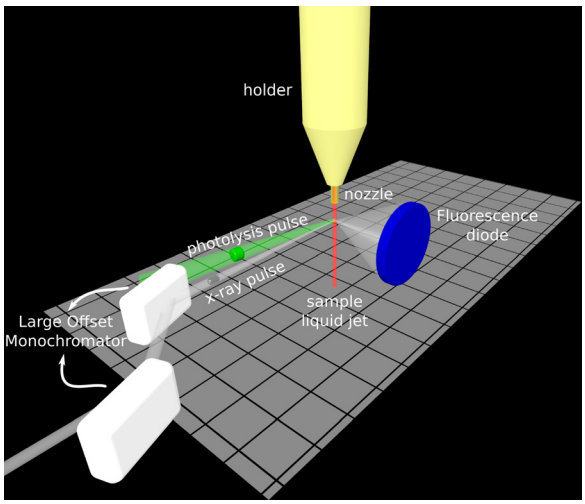


FIG. 3. Schematic representation of the experimental setup. A ∼100 μm thick liquid jet of the sample (a solution of ∼5.6 mM MbCO in 0.1 M phosphate buffer at pH 7.4, in red) was produced with a homemade nozzle. The sample was photoexcited with ∼70 fs optical laser pulses at 538 nm (green beam) and probed 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 ∼125 fs, is the velocity mismatch of the optical and X-ray beams through the 100 μm thick liquid jet (∼100 fs).

During the time-resolved data collection, we continuously monitored the ground state fluorescence signal (I_{off}) by dropping the photolysis pulse every 17 shots. This allows to track with high fidelity relative changes (I_{on}/I_{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 (∼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 ± 5) fs and (400 ± 25) fs, respectively. Both exponentials have been convoluted with a Gaussian instrument response function having a (53 ± 2) fs width (corresponding to 125 fs FWHM). The faster exponential process accounts for ∼80% (±3%) of the observed signal increase, indicating that significant electronic and nuclear rearrangements around the iron occur at ∼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

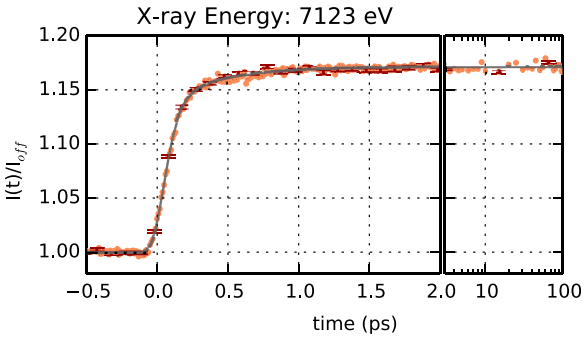


FIG. 4. Time dependence of the absorption at the Fe K-edge (7123 eV) after photolysis of MbCO. The ratio between the total fluorescence signal, I(t), and the average signal in the absence of photolysis, I_{off}, is plotted as a function of time (orange symbols). Left panel: data between −0.5 and 2 ps. 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 $\tau_1 = 73 \pm 5$ fs $(80 \pm 3\%)$ and $\tau_2 = 400 \pm 20$ fs $(20 \pm 3\%)$; the Gaussian width is 53 ± 2 fs (RMS).

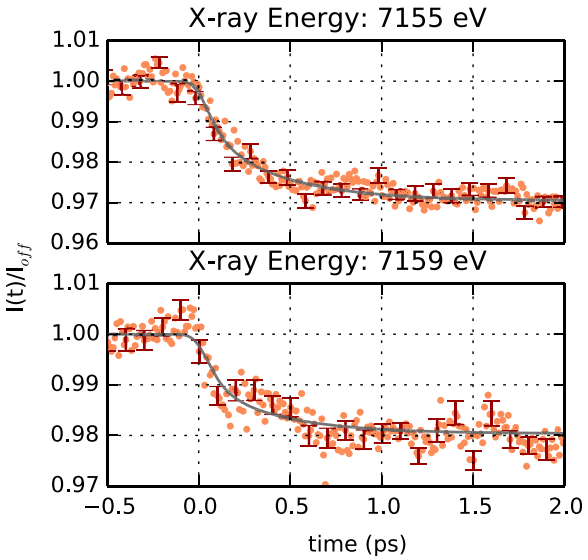


FIG. 5. Time dependence of the absorption at 7155 and 7159 eV after photolysis of MbCO. The ratio between the total fluorescence signal, I(t), and the average signal in the absence of photolysis, I_{off}, is plotted as a function of time (orange symbols) between −0.5 and 2 ps. Errorbars (corresponding to one standard deviation) are shown every 10 experimental points in red. The time evolutions observed at 7155 eV (upper panel) and at 7159 eV (bottom panel) can be satisfactorily described using the same exponential characteristic times obtained from the fit of the data obtained a 7123 eV. The relative weight of the 73 fs component with respect to the 400 fs one, are $40 \pm 3\%$ and $48 \pm 4\%$ for the data at 7155 and 7159 eV, respectively.

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 (±0.03) and 0.48 (±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 ∼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 ∼70 fs relaxation time obtained in the present experiment is shorter than the ∼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}, corresponding to an oscillation period of ∼150 fs) observed in both equilibrium deoxygenated Mb and photolyzed MbCO (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 ∼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 ∼70 fs relaxation phase in the present experiment, we propose that the ∼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 ∼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 proteinquake (Ansari *et al.*, 1985). The F helix motion occurs at ∼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 ∼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 ∼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 (∼70 fs) and the second (∼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 (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 photoexcitation of a macromolecule chromophore and demonstrates that the significantly improved time resolution (∼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).

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