Supplementary Materials for

Direct observation of ultrafast collective motions in CO myoglobin upon ligand dissociation

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Table S2
Materials and Methods

Crystallization

Horse heart myoglobin (Sigma) was crystallized in batch in glass vials. Solid ammonium sulfate was added to a solution of 60 mg/ml protein in 50 mM Tris HCl pH 7.4-8.0 until the protein started to precipitate (~3.1 M ammonium sulfate). Plate-shaped crystals appeared overnight. At the LCLS, the crystalline slurry (4 ml aliquots) was filled into a Schlenk flask, and solid sodium dithionite (32 mg) was filled into a solids addition flask attached to the Schlenk flask. The apparatus was evacuated using a handheld vacuum pump (Arbor Scientific) and flushed with CO gas. This was repeated several times. Dithionite and the crystalline slurry were then mixed under a CO atmosphere and the dithionite dissolved by gentle shaking. The color of the crystalline slurry changed from brown to dark red to raspberry-red (Fig. S16). The flask was transferred to a nitrogen-flushed glove bag (AtmosBag, Aldrich) and CO was bubbled through the crystalline slurry using a Pasteur pipette. In the glove bag the bright pink solution was transferred to a 15 ml Greiner tube, topped up with CO, and centrifuged for 5 min at 5000 rpm. The supernatant was removed in the nitrogen-filled glove bag and the crystalline pellet was resuspended in 10 ml CO-saturated storage solution (3.2 M ammonium sulfate, 100 mM Tris/HCl pH 8.0) and topped off with CO. After centrifugation, the washing step was repeated twice and the crystals were resuspended in CO-saturated storage solution.

The crystalline slurry was filtered using a tandem array of 20, 10, and 5 μm stainless steel ¼ inch diameter filters. Care was taken to minimize air contact and all storage vials were flushed with CO. When using this precaution the crystals retained their pink color, indicative for the CO complex, for several weeks. The average size of the crushed crystals was 15×5×3μm³.

Data collection

The diffraction experiments were performed in the microfocus chamber of the CXI instrument of the LCLS at SLAC National Accelerator Laboratory in July 2014 (proposal LD60). Due to a fire at LCLS a few days before our beam time, the accelerator performance was severely impaired and limited to a maximal photon energy of 6.7 – 6.9 keV and an electron pulse length of 100-120 fs (70-80 fs photon pulse length). This, in combination with the detector size and minimum sample detector distance, limited the resolution of the diffraction data geometrically to 1.8 Å. The X-ray focus was ~1.3 μm diameter, the average pulse energy was 1.2 mJ, and the beamline transmission was 40 % resulting in a pulse energy of 0.5 mJ at the sample position.

The MbCO microcrystals were injected into the FEL beam in a liquid microjet (diameter 5± 1μm) as described previously and shown in Fig. S1 using a flowrate of ~30 μl/min. SFX data were collected at 120 Hz with time delays between the optical pump pulse and the probing FEL pulse of 0.5 ps, 3 ps, 10 ps, 50 ps, and 150 ps, as described below. These “light on” exposures were interleaved with non-uniformly spaced “light off” FEL-only exposures (22% of the total number of exposures), serving as a control dark data set. We decided against the conventional dark-light sequence to maximize the output given the ongoing machine problems and to avoid the possibility of systematic FEL-related effects since the FEL can behave like two distinct (though very similar) 60 Hz sources that are interleaved.
Pump Probe Setup

The myoglobin Fe-CO bond was photolyzed using 532 nm laser light produced at the output of an optical parametric amplifier (OPA) (Topas Prime, Light Conversion). The OPA input was from a Ti-Sapphire laser pumped with a frequency-doubled Nd:YLF laser. The laser pulses were stretched to 150 fs. The laser focus diameter was 150 µm (1/e^2 of the peak intensity, 90 µm FWHM). The optical laser intersected the FEL at an angle of ~70°. The small crystal and jet sizes allowed for uniform illumination of the entire sample thickness, thus permitting this non-collinear geometry. The sample jet was kept at the same position along the beam direction using an optical camera to allow for accurate timing. The optical beam was circularly polarized to minimize photo-selection. The laser energy was set to 5 µJ/pulse. This corresponds to ~ one photon/heme given the experimental parameters, the protein concentration in the crystals (51 mM), and an average crystal thickness of 6 µm. (This value has a large variation given the plate-like shape of the crystals and the fact that we had no control over their orientation.) Thus, assuming a 90 µm spot, the power density was 380 GW/cm^2.

We tested for heating of the sample by analyzing the SAXS patterns of MbCO buffer and buffer-only solutions, respectively, that were collected 10 ps after exposure with the optical laser. A laser power titration (5, 11, 56 µJ) did not show an increase of the water peak signal (data not shown), as for example observed by Arnlund et al. (10).

To test for an influence of the high intensity excitation on structural features we also performed a laser power titration for SFX data in April 2015 and collected 3 ps time delay data using laser powers of 0.6, 6 and 20 µJ, corresponding to 45, 450, and 1500 GW/cm^2. We then calculated F(light)-F(dark) difference maps for these power settings. At a laser power of 0.6 µJ, no electron density peak for a CO molecule at the B-state position was observed, whereas at 6 and 20 µJ, the peak heights for the B-state CO were 10.8 and 11.2 σ, respectively. Thus, for our experimental conditions, the occupancy of the B-state appears to reach unity somewhere below 6 µJ.

We then performed one round of refinement of our 3 ps structure against these datasets (not shown) and calculated distance difference matrices (Fig. S13). These show, as expected, that the magnitude of displacement at 6 µJ is comparable to that observed in our 3 ps time point. However, at 20 µJ photolysis laser energy the displacement of the F-helix is larger and there are now also noticeable displacements in the BC corner, which is not observed in any of our time points. Moreover, the 20 µJ F(light)-F(dark) difference map shows negative difference density for one of the heme propionates as well as for several water molecules close to the heme binding pocket. As we observe these effects at 20 µJ but not at 6 µJ, we believe that the analysis of our 5 µJ laser power time-resolved data is largely unaffected by these effects. However, the fact that the F-helix displacement can apparently be exaggerated by using a high laser power illustrates that laser excitation on a fs timescale must be performed with care (5).

We also compared the structural displacements taking place after a 150 ps pump delay in our SFX data of monoclinic horse heart myoglobin to the ones reported by Anfinrud and coworkers (13) (Fig. S5A) for a 100 ps delay structure of a functional sperm whale mutant (L29F) crystallized in a hexagonal space group using synchrotron-based Laue crystallography and a 10 ps laser pulse of likely significantly lower power density than we used. Given these experimental differences, the agreement between the two ~150
ps delay structures is remarkably high which also argues against a dominant effect of multiphoton absorption or ionization in our data.

**Time tool setup and calibration**

The spatial overlap between optical- and X-ray lasers was determined visually. The temporal overlap was measured using the concepts described by Bionta et al.\((17)\) which is based on X-ray-induced ultrafast changes in optical properties of transparent materials. A glass slide, which becomes instantaneously and reversibly more opaque for a short time (picoseconds) after being hit by sufficiently intense FEL pulses, was placed at the FEL/optical laser interaction point and a diode was used to measure the transmission losses through this glass to find time zero at the sample, where time zero denotes the time point where the fs optical laser and the FEL arrive simultaneously at the sample. This was repeated on a YAG screen in the time tool diagnostic to create a reference of time zero at the time tool, at a separate plane along the X-ray beam axis in the beamline (~2.5 m upstream of the sample location).

Then, for every pulse in the experiment, a Si$_3$N$_4$ foil was inserted at the same location as the YAG screen in the time tool, and the reduction in transmission caused by the X-ray beam arriving before the laser was used to encode the laser arrival time, which was recorded for every pulse. In this particular setup of the time tool, the wavelength of chirped white light encodes time. By using a visible light spectrometer to measure the transmission of this chirped white light as a function of wavelength, timing information is obtained by observing a drop in intensity for the components of the spectrum that correspond to times after the X-rays arrive at the time tool.

**Hit identification and sorting of 0.5 ps data**

CASS\((16)\) was used for online feedback on the hit rate and offline data evaluation and reduction. Frames classified as hits were written to individual HDF5 files together with additional information about the specific shot, such as the pulse and photon energies. CASS was also employed to sort the nominal 500 fs delay data by extracting the exact time-delay value from the recorded raw time tool data. The temporal jitter between the optical fs pump laser and the FEL probe pulse is on the order of 300 fs r.m.s and the exact time delay between the pulses was measured using the LCLS time tool that encodes the time delay as a drop in a spectrum as described above. To identify this drop, the following protocol was used:

1). Horizontally project the signal containing part of the image and subtract the local background, which is a projection close to the signal containing part.

2). Subtract a reference signal that contains the signal from the fs laser generated white light without the FEL from the current signal and divide by the reference.

3). Convolute the resulting signal with a digital filter signal that was provided by the LCLS DAQ Team.

4). Find the highest point within the convoluted signal, which identifies the pixel that corresponds to the position in the spectrum where the drop occurred.
To find the conversion from the measured pixel from the camera, to a value in femtoseconds, one averages the measured pixels at a number of set delay time points and approximates the resulting curve using a linear fit. Along with the information about time zero as described in the pump-probe section, using the fit parameters allows converting the retrieved pixel into the delay in fs. This correction resulted in ~50 fs effective time jitter between optical and X-ray pulses. The main contributions to the overall time resolution of our experiment, which we estimate to be ~250 fs, are the duration of the optical pump pulse and the way we binned the diffraction data (see below).

**Detector geometry refinement and indexing ambiguity resolution**

Data processed with CASS(16) were indexed and integrated using CrystFEL(18) (see Table S1 for data collection statistics). Importantly, we used a custom-written program to refine the positions and orientations of each of the 64 modules of the CSPAD(46) detector. Briefly, the program matches strong diffraction spots to the closest prediction and calculates the difference in position between them for as many spot/prediction pairs as possible on each module. It then adjusts the position and orientation of each module so as to minimize the discrepancy. This resulted in slightly higher indexing rates (28 % before refinement to 31 % after refinement for the dark dataset), but also resolved an indexing ambiguity. Due to their unit cell parameters ($P2_1$, $a=64$ Å, $b=29$ Å, $c=36$ Å, $\beta=107^\circ$), the crystals used here have two indexing possibilities, related by the reindexing operator $h+l$, -k, -l. The unit cell lengths of the two indexing choices are similar, to within an Angstrom. Before detector geometry refinement, CrystFEL could not distinguish between the two choices for all images, causing one part of the images to be indexed in the one setting and another part in the other setting. This resulted in an apparent twinning of the data, as indicated by a strong peak in the $\kappa=180^\circ$ section of the self-rotation function. Structure refinement using the $h+l$, -k, -l twin law as well as image-by-image intensity comparisons with a reference dataset calculated from pdb entry 1DWR(19) suggested that about 70 % of the images were indexed in one setting and about 30 % in the other. After detector geometry refinement, however, the apparent twinning had completely disappeared as judged by the self-rotation function and refinement of the twinning fraction. Final indexing rates for all time points ranged from 24% to 37 %. All datasets were processed using the same unit cell. This ensures identical Fourier sampling which was found to be essential for the subsequent analysis steps. Final overall data statistics are reported in Supplemental Table S1 and resolution-dependent $R_{split}$, CC and CC* values are listed at the end of the supplement.

**Data set construction**

For the dark reference data set, all available indexed pump-laser off images were used (96,503 images in total). For the 3, 10, 50 and 150 ps datasets, 20,000 indexed images were used to construct each final dataset to ensure the results were comparable. To obtain the sub-ps time delay datasets, the 64,982 indexed images collected with the time delay set to a nominal value of 0.5 ps were sorted by their time delay as reported by the timing tool. After sorting, starting at the negative time delay end (i.e. where the pump pulse came before the probe), a 20,000 image-wide window of images was combined into a dataset. Then, the window was progressively shifted towards increasingly positive time delays in 5000 image steps, combining all images in the window into datasets. In this
way, windows were created with average time delays of -89 ± 124 fs (which we called “-0.1 ps”), 10 ± 95 fs (“0 ps”), 93 ± 91 fs (“0.1 ps”), 175 ± 98 fs (“0.2 ps”), 264 ± 106 fs (“0.3 ps”), 360 ± 115 fs (“0.4 ps”), 462 ± 122 fs (“0.5 ps”), 574 ± 134 fs (“0.6 ps”), see Fig. S2. It must be stressed that the standard deviations given here underestimate the true width of the windows, as the distribution of time delays within each window is not Gaussian but almost flat, and the total width of these windows is approximately 200 fs on either side (see Fig. S2). An additional window with an average time delay of 734 fs ± 216 fs was discarded because the way in which the timing tool data was evaluated is not reliable at these relatively long time delays.

Structure solution and refinement
To obtain the dark state structure, molecular replacement was performed with PHASER(47) using 1DWR(19) as the search model without the CO ligand, water molecules and sulfate ions. A clear peak for a heme-bound CO was observed in the density maps. Therefore, the CO was built into this position for the dark-state structure. After several cycles of rebuilding and refinement with REFMAC5(48) and PHENIX(49), a final model was obtained with excellent R-factors and geometry (see Table S1). All other structures were obtained by iterative cycles of refinement and rebuilding, starting from the dark structure. Care was taken to use the same parameters and the same number of cycles for each structure. In all pump-laser-on structures, a clear difference density peak for a photodissociated CO was observed atop the heme porphyrin plane, at the position expected from other crystallographic studies. Consequently, for these structures the CO was built into this position, even though the earliest time points show some evidence of partial photolysis (see Fig. S3). Importantly, the CO was built as an “alternate conformation” with respect to the rest of the structure (using an occupancy of 1.0 for all atoms), thus removing all distance restraints between the CO and the rest of the model, as modelling it otherwise resulted in clashes that clearly moved some residues out of their electron density. This also ensured that any displacements observed in the refined structures are truly in the data, and are not caused or exaggerated by geometrical constraints imposed by the refinement programs. Moreover, no additional restraints were imposed on the distance between the heme iron and the proximal histidine 93. Given our resolution of 1.8 Å we used rather strong geometrical constraints which may have reduced some of the structural changes, in particular heme doming. For all time points, structures with excellent R-factors and geometry were obtained, with over 98.7% of residues in the favored regions of the Ramachandran plot, and no outliers. “Light-Dark” electron density maps were calculated using PHENIX(49), using the dark-state structure as the source of phases, using data between 15 and 1.8 Å resolution. The average coordinate error for the crystal structures as estimated from the maximum-likelihood refinement is 0.26 Å on average, and ranges from 0.21 Å for the dark state structure to 0.33 Å for the 150 ps time point structure. However, these estimates are based in part on the sigma values for the intensities, which are not normally distributed since they are derived from Monte-Carlo integration of the SFX data. Moreover, they are averages over the whole structure, i.e. they include the errors in the positions of e.g. mobile side chains on the surface of the protein, which are probably much larger than the positional errors for atoms in the core of the protein.
To obtain rough estimates of the precision of our structures, we first added random shifts with a standard deviation of 0.3 Å to the x, y and z coordinates of the dark state and 0.5 ps structures. This process was carried out three times, resulting in three strongly distorted structures for both states, with very poor geometries and R-factors of ~40%. We then refined these three structures against the respective datasets and calculated the r.m.s. distance of each atom to the average position (including the original refined coordinate sets in the calculation). This resulted in average r.m.s. displacements of 0.012 and 0.015 Å for the dark and 0.5 ps states, respectively. Coloring the structures according to these r.m.s. positional differences revealed that as expected, atoms in the center of the protein had very small r.m.s. positional differences, with the largest differences at the surface of the protein. Interestingly, the CD corner showed considerable r.m.s. positional differences, also for main-chain atoms.

We then took the original 0.5 ps dataset and randomly split the diffraction images into two half-datasets, which were processed independently. We refined one of the distorted structures described above against both half-datasets independently, and compared the resulting structures. Upon least-squares superposition using all atoms, an r.m.s. positional deviation of 0.12 Å was found. To obtain an estimate for the errors in our sequence displacement plots (Fig. 3), we calculated a sequence displacement plot between the structures refined against the two half-datasets. This plot (Fig. S15B) showed no systematic variations, but a random distribution of displacements around 0 Å with a standard deviation of 0.04 Å. This shows that the effects observed in the sequence displacement plots are highly significant. We also calculated a difference distance matrix between the two structures. Plotting this matrix (Fig. S15C) again revealed a largely random pattern, uncorrelated to the secondary structure and with only small displacements.

Thus, it appears that the uncertainty in the individual coordinates is considerably smaller than what would be expected from the maximum-likelihood refinement. Moreover, as described in the main text, the sequence displacement plots as well as the difference distance matrices show that the various structural elements move as more or less rigid bodies upon photolysis. This increases the confidence in the observed effects further, as the errors in the displacements of the various helices etc. which consist of multiple atoms are smaller than those for individual atoms. In addition to these motions, these are seen for several time points, several of them increasing smoothly with time.

To obtain estimates for the significance of the oscillations shown in Fig. 4 we calculated the difference of the quantities plotted in Fig. 4 between the structures refined against the two half-datasets. For the Phe43 and Val68 χ1 angles, the difference was 0.6°, whereas it was 0.2° for the His93 χ1 angle. However, for the Ile107 χ2 angle the difference was 3.4°. The difference in distance between the heme O1 and the His97 Nε2 was 0.07 Å and the difference in distance between the Lys98 O and the Lys42 Nζ atoms was 0.24 Å. Importantly, several of the observed oscillations share the same frequencies, and these frequencies are comparable to what is expected from earlier experiments.

**Deoxymyoglobin structure determination**

Macroscopic hhMb crystals were obtained essentially as described(19). The 3.2 M ammonium sulfate storage solution was made by dissolving solid ammonium sulfate in thoroughly degassed and then nitrogen-saturated Tris HCl (0.1 M, pH8.0). The hhMb
crystals (~100 × 20 × 500 µm³) were transferred to this storage solution and then moved into a glove box (Belle Technology, UK). In the glove box, the crystals were transferred to a reducing solution (10 mg dithionite/ml ammonium sulfate storage solution) and left to equilibrate for 3 minutes. After three dithionite-solution exchanges, the bright red crystals were transferred into 0.3 mm diameter quartz capillaries completely filled with reducing solution. The crystal containing capillaries were sealed and then moved out of the glove box. Prior to data collection at the PXIII beamline at the Swiss Light Source, the fully immersed crystals had become immobilized by wedging themselves into the tapered capillaries. During data collection the part of the capillary containing the crystal was cooled to 7°C using an Oxford cryostream. Three crystals were used to collect a composite dataset, with each crystal contributing two 50° wedges that were separated by a crystal translation of at least 100 µm. With an X-ray focus of ~90 × ~90 µm², an exposure time of 0.1 s/degree, a flux of 3.9 × 10¹¹ photons/s, and a photon energy of 12.39 keV (1 Å wavelength) the dose was ~0.1 MGy as calculated by Raddose 3D. Data were processed using XDS. Structure solution and refinement were performed as described for the SFX data, fixing the unit cell parameters to those of the SFX data to enable accurate comparisons.

Structure interpretation and analysis of motions

The plots in Fig. 2A,B and S4,5,14,15B were prepared by calculating the change in distance between each Ca atom and the center of mass of the four heme nitrogen atoms upon illumination as described in (27) for each refined structure. These values where then used to color-code the structures shown in Fig. 2C and S6. Here, too, blue indicates a decrease in distance and red indicates an increase in distance. Figures S8 and S9 were produced in the same way, but using different reference points. All structural figures were prepared using PyMOL. The plots in Fig. 3 and Fig. S10,13,15C were constructed using custom-written software that reads pdb files and calculates a matrix of all pairwise Ca distances |Δr|. For each refined structure, the matrix of the “dark” structure was then subtracted from the matrix of the respective “light” structure. In this way, a matrix with changes in Ca-Ca distances Δ|Δr| was obtained. These were plotted using a blue-to-red color scale, where red indicates a positive “light”-“dark” distance change, i.e. the distance has increased, and blue indicates a negative distance change, i.e. a decrease in distance. Torsion angles and distances were extracted from the refined structures and plotted as a function of delay time. The resulting time traces were analysed with the data fitting program Grafit 7 (Erithacus Software, UK) that searches for least-squares minima with a non-linear optimization routine. Data points for changes in torsion angles χ₁ and χ₂ or distance changes relative to the dark state were analyzed with equations that either describe a damped oscillation or a single exponential response.

The equation for a damped oscillation is:

\[ S(t) = A \sin(2\pi ft + \phi_0) \cdot e^{-at} + S_0 \]

Where S is the signal, A is the signal amplitude, f the oscillation frequency in THz, t is time, \(\phi_0\) is the phase shift, a the damping constant and \(S_0\) the signal offset. It should be
noted that in a few cases the damping constant converged to a negative value, resulting in negative damping. We consider this phenomenon to be caused by a lack of sufficient time points after the initial, fast phase and/or additional events occurring after the initial 0.6 ps time window.

**QM/MM simulations**

A molecular simulation model was built from the structure of CO-bound myoglobin\(^{(19)}\) (PDB code 1DWR). Histidines 36, 64, 82, 97, and 119 were protonated at the epsilon nitrogen, and the others at the delta position. Asp, Glu, Arg, Lys, and heme propionates were charged. Retaining crystal water, the protein was solvated with TIP3P water\(^{(53)}\) and 50 mM NaCl, resulting in an overall neutral, periodic simulation box of \(63.2 \times 66.7 \times 68.6\) Å\(^{3}\) containing 33791 atoms. The simulation setup was prepared with xLeap\(^{(54)}\). After energy minimization, classical equilibration simulations were performed for 5 ns at constant temperature (288 K) and pressure\(^{(55)}\) (1 atm) with GROMACS 4.6.7\(^{(56-58)}\) using the AMBER99SB all-atom forcefield\(^{(59)}\) with sidechain torsional angle corrections\(^{(60)}\) and the Giammona-Case CO-bound heme model\(^{(61)}\). A 1 fs timestep was used. Backbone atoms were restrained. Particle-Mesh Ewald electrostatics\(^{(62, 63)}\) was used with a non-bonded cutoff of 0.8 nm.

The QM/MM simulations were performed using the implementation by Laio et al.\(^{(64)}\), which combines CPMD\(^{(65)}\) and GROMOS96\(^{(66, 67)}\) codes using the Car-Parrinello MD\(^{(68)}\) approach. Density Functional Theory (DFT) with the BP86 functional\(^{(69, 70)}\) was used for the QM region, as in earlier studies of heme proteins\(^{(71-76)}\) and other metal-dependent proteins\(^{(77)}\). The QM/MM electrostatic interactions were treated via a fully Hamiltonian coupling scheme. Short-range electrostatic interactions were explicitly taken into account with an appropriately modified Coulomb potential that ensures that no unphysical escape of the electronic density occurred. Long-range interactions were treated via a multipole expansion. The covalent bonds across the QM/MM interface were handled using a link-atom pseudopotential that saturates the QM region\(^{(78)}\), and cross-interface van-der-Waals interactions were treated classically according to the force fields listed above. Classical long-range electrostatic interactions were calculated within the P3M implementation\(^{(79)}\) with a mesh of \(64 \times 64 \times 64\).

The 99-atom QM region included the heme group, the CO, and the proximal and distal histidines truncated at the C\(_\beta\) atom. Norm-conserving Troullier-Martins pseudopotentials were used for all elements\(^{(80)}\) except iron, for which an eight valence electrons supplemented with non-linear core corrections (NLCC)\(^{(81)}\) description was employed. Kohn-Sham orbitals were expanded in a plane-wave basis set with an energy cutoff of 70 Ry, contained within a \(21.7 \times 20.6 \times 19.6\) Å\(^{3}\) subcell of the simulation box. All QM/MM CPMD simulations were performed within the spin-unrestricted density functional theory (DFT) framework, using a time step of 5 a.u., a fictitious electron mass of 800 a.u., and all QM hydrogen atoms replaced by deuterium.

The classically equilibrated structure of the CO-bound myoglobin was optimized by a series of QM/MM simulations in the singlet spin state. The ionic temperature was annealed, starting with position restraints on the MM region and the Fe-CO bond distance that were removed in steps until the maximum gradient of the ionic degrees of freedom was less than \(5 \times 10^{-3}\) a.u.
The resulting structure of the CO-bound low-spin state was submitted to 9 ps of NPT QM/MM molecular dynamics simulations using a Nosé-Hoover chain thermostat(82-85) to keep the temperature constant at 288 K. After the first 1.8 ps, snapshots were taken every 1000 MD steps (120 fs) and used as initial coordinates and ionic velocities for the photolysis simulations. Photolysis was simulated by continuing the QM/MM runs on the dissociative high-spin (quintet) state, after reoptimization of the wave function. A total of 44 photolysis simulations of at least 2 ps duration were performed.

Excited-state quantum-chemical calculations and validation of QM/MM photolysis protocol

The QM/MM photolysis protocol was validated by excited-state electronic structure calculations for a small myoglobin-CO model complex, involving a Fe²⁺-porphyrin moiety coupled to CO and an imidazole ligand to mimic the influence of the proximal histidine at the axial position of the complex (see previous related studies(86)). Time-Dependent Density Functional Theory (TD-DFT(87)) was used throughout. Geometries of stationary points were fully optimized without any symmetry constraints at the (u)B3LYP(69, 88-90)/LANL2DZ(91, 92) level, followed by frequency calculations to verify the minima by analysis of the Hessian. Natural transition orbital analysis(93) was employed to identify the character of the excitations in terms of orbital interaction patterns. Further, potential energy scans as a function of CO-Fe distance and tilt coordinates were carried out using the unrestricted Kohn-Sham formalism, highlighting a complex scenario of multiple potential crossings. The most relevant photochemical pathways were characterized by identifying a series of potential crossings, from the initial porphyrin Soret band (S2) to the lowest porphyrin singlet state (S1), which in turn couples to the triplet and quintet manifolds that exhibit ligand to metal charge transfer character(94, 95). The main results of these calculations are summarized in Fig. S17. The quintet high-spin state is dissociative, and represents the ground state at heme-CO distances beyond ca. 2.5 Å, validating the QM/MM procedure which proceeds from the quintet surface. Removal of the CO ligand modifies the octahedral Fe²⁺ ligand field and generates a pentacoordinated metal complex where the iron moves out of plane, towards the proximal histidine. As shown for similar iron complexes(96), the spin cross-over dynamics is ultrafast and proceeds through a cascade of internal conversion processes that sequentially involve singlet, triplet, and quintet manifolds.

Excited-state electronic structure calculations

Fig. S17 summarizes the results of quantum-chemical calculations of heme-CO ground and excited-state energy surfaces in different spin states. As can be inferred from the figure, initial excitation to the Soret band (S2) at the Franck-Condon (FC) geometry is followed by rapid internal conversion to the lowest porphyrin singlet state (S1), which in turn couples to the triplet and quintet manifolds that exhibit ligand to metal charge transfer character(94, 95). The quintet ground state is dissociative and is taken as the starting surface for the QM/MM calculations reported in this work. As can be seen from the relaxed scan (Qrelaxed), the quintet high-spin state is significantly stabilized and is found to be the energetically lowest state for Fe-C distances beyond ca. 2.5 Å in our calculations. Removal of the CO ligand modifies the octahedral Fe²⁺ ligand field and
generates a pentacoordinated metal complex where the iron moves out of plane, towards the proximal histidine. As shown for similar iron complexes(96), the spin cross-over dynamics is ultrafast and proceeds through a cascade of internal conversion processes that sequentially involve singlet, triplet, and quintuplet manifold.

**Fig. S1. Setup of the pump-probe experiment.**
MbCO microcrystals were injected into the X-ray FEL beam in a thin liquid jet. They were photolyzed with a 150 fs optical laser pulse and then, after a variable time delay, exposed to the X-ray FEL probe beam. The diffraction data were collected with a CSPAD detector at 120 Hz.
Fig. S2. Time window size.
Graph showing the distributions of the pump-probe delay times in the -0.1 to 0.6 ps windows. The circles indicate the average time delay, the black error bars the standard deviation, and the red error bars the total distribution of delay times.
Fig. S3. CO photodissociation.
Structures of the dark state and the time points up to 0.6 ps. The 2mFo-DFc (blue, 1.0 σ) and the mFo-DFc (green: +3.0 σ, red: -3.0 σ) electron density maps after refinement of the structures are shown, as well as the distance between the iron atom and the carbon atom of the CO molecule (upper right hand corner of each panel). The earliest time points show a mixture of bound and photodissociated CO(97). This is apparent from the Fe-CO bond distance and the elongated electron density. The electron densities of bound and photolyzed CO (caused by partial photolysis) can only be distinguished at a resolution of better than 1.5 Å. At 2 Å resolution, an electron density peak appears at the weighted average position and a variation in photolysis yield seems to indicate ligand migration(97).
Fig. S4. Sequence displacement plots\(^{(27)}\) from experiment and simulations. 
Displacement of N, Cα, and C atoms towards the heme or away from it plotted as a function of the residue number. The blue and green curves show the experimental results at 0.5 and 3 ps, respectively, the orange and red curves the QM/MM results averaged over 146 trajectories at 462 fs and 2.1 ps, respectively. The experimental and theoretical results agree qualitatively.
Fig. S5. Comparison of the displacements to those observed in other crystallographic experiments.

(A) Sequence displacement plots of our time delay data of wildtype horse heart (hh) myoglobin (colored lines) as also shown in Fig. 3A compared with that of the 100 ps delay data of the L29F mutant of sperm whale (sw) myoglobin by Aranda et al. (13) (black line). The displacements in the Aranda et al. (13) structure agree quantitatively with our observations at 150 ps. Differences in displacement for the B-helix, the G- and H-helices were also observed (27) when comparing different CO-bound and deoxy structures of sw Mb determined previously (98, 99) and related to differences in
experimental parameters. Sw and hh Mb crystallize in different space groups, and they have different crystal contacts, in particular at the G and H helices. These may affect the mobility of Tyr96 which forms a hydrogen bond with the backbone oxygen of Ile99. Ile99 is located right beneath the pyrrole C ring and needs to move downwards to accommodate heme doming. We observe a lesser extent of the heme plane movement. We cannot distinguish whether this motion is damped because of the crystal contact or whether this effect is due to multiphoton excitation, but it may also reflect the rather strong geometrical restraints used in our refinement due to the lower resolution compared to the Aranda et al. data(13). (B) Comparison of sequence displacement plots of our time delay data (0.5, 3, 10 and 150 ps, green-red) with a sequence displacement plot obtained using our room temperature hh deoxymyoglobin structure. The time-resolved SFX structures largely reach the same displacements as those observed in deoxymyoglobin, apart from some minor residual motion in the E-helix and the C-terminus of the F-helix.
Fig. S6. Motion of structural elements with respect to the heme center.
Cartoon representations of myoglobin colored according to the difference in distance of N, Ca, and C atoms to the center of the heme nitrogen atoms at various time points with respect to the dark state structure. The color scale runs from -0.3 Å towards the heme (blue) to +0.3 Å away from the heme (red).
Fig. S7. Principal Component Analysis (PCA) of the motion of structural elements with respect to the heme center.

(A). First eigenvector extracted using PCA from the time-dependent differences in distance of N, Cα, and C atoms to the center of the heme nitrogen atoms with respect to the dark state structure. Motions towards the heme are shown in blue, motions away from the heme in red. (B) Time dependence of the amplitude of the first eigenvector. A fast initial motion along the eigenvector is observed, followed by a slower phase.
Fig. S8. Motion of structural elements with respect to the center-of-mass of the protein.
Cartoon representations myoglobin, colored according to the difference in distance of N, Ca, and C atoms to the center-of-mass of the protein at each time point with respect to the dark state structure. The color scale runs from -0.3 Å towards the center-of-mass (blue) to +0.3 Å away from the center of mass (red).
Fig. S9. Motion of structural elements with respect to the dark-state positions.
Cartoon representations of myoglobin, colored according to the distance of N, Ca, and C atoms at each time point to the position of these atoms in the dark state structure. The color scale runs from 0.0 Å (white) to +0.3 Å away from the dark-state position (red).
Fig. S10. Motion of structural elements with respect to each other.
Matrices showing the difference in pair-wise Cα-Cα distances with respect to the dark state structure for each time point. Red denotes an increase in distance between Cα atoms, blue denotes a decrease in distance. The helices are indicated on the diagonal.
From the appearance of the plots it is clear that there is a single, dominant structural change that all time points have in common to different extents. Indeed, the correlation coefficients between the difference distance matrix at 150 ps and the matrices at the other time points vary between 41% and 61%. 
Fig. S11. Time dependence of side chain torsion angles.

The side-chain torsion angles of Phe43 ($\chi_2$), Leu29 ($\chi_1, \chi_2$), Ile107($\chi_2$), and His93 ($\chi_2$) seem to oscillate with time at sub-ps time delays, as do the Lys98 O-Lys42 Nζ and heme O1-His97 Ne. Several of these oscillations share approximately the same frequencies. Moreover, as expected, the $\chi_2$ angles oscillate at higher frequencies than the $\chi_1$ angles (see Figure 4 for the His93 $\chi_1$ angle). The iron out-of-plane oscillation has a period of ~300-320 fs which is ~three times the period of the $\gamma_7$ iron-out-of-plane mode (~350 cm$^{-1}$, 95 fs). Interestingly, overtones of this frequency have been predicted to be strongly coupled to the $\nu_7$ mode and to contribute to its relaxation(100).
Fig. S12. Heme breathing motion.

(A) The ν₇ heme breathing mode. (B) Amplitude of the ν₇ heme breathing mode from QM/MM simulations (blue), averaged over 146 CO photodissociation trajectories. The fit (red) is a sinusoidal wave whose amplitude is modulated with a period of 353 fs (green) and exponentially damped with a time constant of 1.3 ps.
Fig. S13. Distance difference matrices at various pump laser pulse energies.
(A) Displacements at 20 µJ. (B) Displacements at 6 µJ. There are noticeable differences in the displacement of the F-helix, which appears more pronounced at the higher laser power. (C) Difference between the 20 and 6 µJ distance difference matrices. (D) For comparison, the distance difference matrix for the 3 ps time delay is reproduced here. The differences observed are comparable in magnitude (or even smaller) than at 6 µJ.
Fig. S14. Sequence displacement plots for the “cold” heme system.
Displacement of N, Cα and C atoms towards the heme or away from it plotted as a function of the residue number for the 0.5 ps SFX structure (black line) as well as for the average over 146 QM/MM simulations starting from a cold heme (0 K) at 0.462 ps (blue line). QM/MM data include time points within 100 fs of the target time. To study possible heating effects computationally without the need to run at shorter time steps, “cold” runs were performed in which the initial velocities of atoms in the QM region were artificially set to zero. As dark state reference, we used the average structure of the trajectory segment from which the QM/MM photodissociation simulations were initiated. The resulting displacements (blue line) were found to be in qualitative agreement with those found experimentally (black line). Moreover, they are comparable to those in the simulations with a “hot” heme (Fig. S3), or if anything slightly larger, consistent with only small effects of variations in the initial heme temperature.
Fig. S15. Significance of the observed motions.

(A) Scatter plot showing the distance differences $\Delta|\Delta r|$ at 0.5 ps shown in Figure 3A (purple dots) from the SFX structure (vertical axis) vs. those from the QM/MM simulations (horizontal axis). The blue line shows perfect correspondence ($y=x$), the yellow line a linear fit ($y=0.91x-0.004$). The green error bars show the average and the standard deviation of the experimental distance differences, calculated in bins of 0.01 Å width. (B) Difference distance matrices between two structures refined independently against two half-datasets constructed from the 0.5 ps dataset (left) and between the full
0.5 ps dataset and the dark data. The matrix on the left shows a random pattern, whereas the matrix on the right shows correlated motions of structural elements. (C) Sequence displacement plots for the light-dark differences for all time points (black lines) as well as for the difference between the structures refined independently against the two half-datasets for the 0.5 ps time point. Here, too, the light-dark curves show correlated motions of structural elements (black lines), whereas the comparison of half-datasets only shows random noise (red line). It should be noted that the reduction in multiplicity caused by splitting the 0.5 ps data into two halves has likely increased the noise significantly.
Fig. S16. hhMb microcrystals.
Crystalline slurries of met myoglobin (right, brown) and carbonmonoxy myoglobin (left, pink) differ in color.
Fig. S17. Excited-state electronic structure calculations.  
(A) Heme model system, involving a CO-bound Fe$^{2+}$-porphyrin moiety coupled to an imidazole ligand to mimic the influence of the proximal histidine at the axial position of the complex(86).  (B) Potential energy surfaces for singlet ground and excited states $S_0$, $S_1$, and $S_2$ (solid grey), triplet states (dashed), and lowest quintet state $Q$ (solid red), plotted as a function of the heme iron – CO carbon distance, with CO translated normal to the heme plane and the other atoms fixed. In addition, relaxed, fully energy minimized surfaces for the $S_0$ ground state (dotted grey) and the lowest quintet Q state (dotted red) are shown to illustrate the $S_0$/$Q$ crossing at ca. 2.5 Å. Initial excitation to the Soret band ($S_2$) at the Franck-Condon (FC) geometry is followed by an ultrafast cascade of internal conversion processes leading to the Q ground state that is taken as the starting surface in the QM/MM calculations reported in this work.
Table S1. Data collection and refinement statistics.

96,503 crystals were used for the dark dataset; 20,000 crystals for all other SFX data sets. For the deoxy dataset, 3 capillary-mounted crystals were used.

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<th>~0 ps</th>
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### Data collection

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### Resolution (Å)

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| R
erro | 0.241 | 0.240 | 0.249 | 0.251 | 0.252 | 0.244 | 0.244 |
| I/σI | 3.9 (1.6) | 3.9 (1.6) | 3.9 (1.6) | 3.9 (1.6) | 3.9 (1.7) | 3.9 (1.7) | 3.9 (1.7) |
| Completeness (%) | 100 (100) | 100 (100) | 100 (100) | 100 (100) | 100 (100) | 100 (100) | 100 (100) |
| Multiplicity | 148 (75) | 148 (75) | 148 (75) | 148 (75) | 148 (75) | 148 (75) | 148 (75) |

### Refinement

| Resolution (Å) | 15-1.8 | 15-1.8 | 15-1.8 | 15-1.8 | 15-1.8 | 15-1.8 | 15-1.8 | 15-1.8 |
| No. reflections | 11722 | 11720 | 11724 | 11718 | 11719 | 11721 | 11721 | 11722 |
| R
erro / R
test | 0.175 / 0.166 | 0.176 / 0.176 | 0.176 / 0.172 | 0.172 / 0.172 | 0.171 / 0.171 | 0.171 / 0.171 | 0.171 / 0.171 | 0.171 / 0.171 |
| No. atoms | 1194 | 1194 | 1194 | 1194 | 1194 | 1194 | 1194 | 1194 |
| Protein | 53 | 53 | 53 | 53 | 53 | 53 | 53 | 53 |
| Heme/sulfate | 2 | 2 | 2 | 2 | 2 | 2 | 2 | 2 |
| CO | 28.2 | 29.0 | 29.1 | 29.3 | 29.1 | 28.9 | 29.0 | 28.7 |
| O2 | 30.8 | 31.3 | 31.2 | 32.7 | 31.9 | 31.9 | 31.9 | 31.9 |
| Waters | 36.7 | 35.8 | 35.8 | 36.7 | 36.9 | 38.4 | 38.1 | 38.1 |

### B-factors

| R.m.s deviations (Å) | 0.006 | 0.006 | 0.006 | 0.006 | 0.006 | 0.007 | 0.006 | 0.006 |
| R.m.s deviations (°) | 0.997 | 1.002 | 0.980 | 0.998 | 1.007 | 1.039 | 1.000 | 1.030 |
Table S1. (continued)

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**Data collection**

| Space group |          |      |      |       |       |      |          |
| P2_1        |          |      |      |       |       |      |          |
| Cell dimensions | a, b, c (Å) |      |      |       |       |      |          |
| 63.6, 28.8, 35.6 | 63.6, 28.8, 35.6 | 63.6, 28.8, 35.6 | 63.6, 28.8, 35.6 | 63.6, 28.8, 35.6 | 63.6, 28.8, 35.6 |
| a, β, γ (°) |          |      |      |       |       |      |          |
| 90, 106.5, 90 | 90, 106.5, 90 | 90, 106.5, 90 | 90, 106.5, 90 | 90, 106.5, 90 | 90, 106.5, 90 |
| Resolution (Å) |          |      |      |       |       |      |          |
| 22-1.8 (1.9) | 22-1.8 (1.9) | 22-1.8 (1.9) | 22-1.8 (1.9) | 22-1.8 (1.9) | 22-1.8 (1.9) |
| Rsplit / Rmerge |          |      |      |       |       |      |          |
| 0.217 (0.481) | 0.214 (0.485) | 0.189 (0.614) | 0.236 (1.36) | 0.108 (0.349) | 0.074 (0.478) |
| I/σ |          |      |      |       |       |      |          |
| 4.6 (2.4) | 4.4 (2.3) | 4.5 (1.6) | 3.4 (0.74) | 7.9 (3.3) | 14.3 (2.9) |
| Completeness (%) |          |      |      |       |       |      |          |
| 100 (100) | 100 (100) | 100 (100) | 100 (100) | 100 (100) | 96.8 (87.9) |
| Multiplicity |          |      |      |       |       |      |          |
| 147 (77) | 148 (76) | 148 (75) | 137 (76) | 606 (188) | 5.0 (3.4) |

**Refinement**

| Resolution (Å) |          |      |      |       |       |      |          |
| 15-1.8        | 15-1.8   | 15-1.8 | 15-1.8 | 15-1.8 | 15-1.8 | 35-1.6 |
| No. reflections | 11723   | 11725 | 11722 | 11706 | 11725 | 16486 |
| Rwork / Rfree | 0.17 / 0.213 | 0.168 / 0.215 | 0.166 / 0.218 | 0.179 / 0.228 | 0.16 / 0.204 | 0.172 / 0.205 |
| No. atoms |          |      |      |       |       |      |          |
| Protein | 1194 | 1194 | 1194 | 1194 | 1194 | 1194 |
| Heme/sulfate | 53 | 53 | 53 | 53 | 53 | 43 |
| CO | 2 | 2 | 2 | 2 | 2 | 0 |
| Waters | 78 | 81 | 68 | 36 | 80 | 120 |
| B-factors |          |      |      |       |       |      |          |
| Protein | 28.8 | 28.9 | 33.1 | 40.9 | 25.5 | 19.6 |
| Heme/sulfate | 29.0 | 29.2 | 33.0 | 40.4 | 24.9 | 14.3 |
| CO | 28.7 | 29.0 | 32.9 | 38.5 | 21.5 | n.a. |
| Waters | 37.3 | 37.1 | 40.7 | 46.2 | 33.6 | 29.6 |
| R.m.s. deviations |          |      |      |       |       |      |          |
| Bond lengths (Å) | 0.007 | 0.007 | 0.006 | 0.007 | 0.007 | 0.007 |
| Bond angles (°) | 1.105 | 1.084 | 1.083 | 1.099 | 0.997 | 1.114 |

*Highest resolution shell is shown in parentheses

1 For the SFX structures, the values of Rsplit are listed, whereas for the synchrotron deoxymyoglobin structure, the conventional Rmerge is reported.

\[
R_{\text{split}} = \frac{1}{\sqrt{2}} \sum \left| \frac{I_{\text{even}} - I_{\text{odd}}}{I_{\text{even}} + I_{\text{odd}}} \right| \quad (18), \quad R_{\text{merge}} = \frac{\sum |I_{h,i} - I_{h}|}{\sum |I_{h,i}|}
\]

2 This is the conventional redundancy, rather than the SFX multiplicity that is reported for the SFX structures.
Additional Data table S2 (separate file)
Detailed SFX data statistics for each time point.
References and Notes


14. Materials and methods are available as supplementary materials on *Science* Online.

15. Previous studies have shown that in the high intensity excitation regime the directed functional protein motions become dominated by either thermal or ionization contributions rather than those of ligand dissociation (5). Our observed structural changes are, however, highly consistent with those expected for ligand dissociation and changes in potential energy surface at the heme. Given our structural results for the 20 μJ (1 TW/cm²) excitation data (fig. S13) it seems that ionization of the heme still appears like a centralized force that displaces the heme and protein in a very similar fashion to ligand dissociation. The process appears to be occurring within a linear response in which case the observed dynamics still capture the protein response function. We cannot exclude that the oscillations of spatial positions of residues coupled to the heme are caused or increased by heating due to high excitation conditions. However, the excellent agreement of our structural observations and their temporal dependence with previous spectroscopic data argues against photolysis artefacts. In addition, our QM/MM simulations starting from cold heme (0 K) agree well with those of the normal system as well as the experimental data (see fig. S14). Moreover, they are in line with the collective modes model (32) and other mode-mode coupling mechanisms (4, 38).


45. At this peak power density, multiphoton absorption and ionization effects become an issue. In fact, multiphoton absorption or ionization effects have been shown to affect signals in fs transient grating experiments (101) and were implicated in changes in ps transient absorption spectra (102). Anti-Stokes resonance Raman spectroscopy (103) showed that after photon absorption, the hot, electronically and vibrationally excited heme relaxes exponentially in a biphasic manner with time constants of $1.9 \pm 0.6$ ps for the vibrational population decay and $3.0 \pm 1.0$ and $25 \pm 14$ ps for temperature relaxation via vibrational cooling through collective motions of the protein (5, 101) and diffusive cooling dissipating energy to the water bath, respectively [see (104) for details]. Goodno et al. (5) suggested that at high power excitation conditions, a larger fraction of the excess thermal energy will be transferred via the protein to the solvent via collective modes rather than through the slower diffusive channel. The reason would be a stronger anharmonic coupling between the collective modes of the protein and an increased spatial dispersion of the excess energy (5).


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