Ultrafast Tryptophan-to-Heme Electron Transfer in Myoglobins Revealed by UV 2D Spectroscopy
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of Bi. At sufficiently high Cr doping, the SOC is not strong enough to invert the bands, leading to a trivial bulk topology (19). In contrast, for Cr-doped Bi$_2$Te$_3$ our DFT calculations show that the bulk band remains inverted for Cr content up to $y = 0.25$ (figs. S8 and S9). The more robust band inversion is a result of the larger SOC strength of Te as compared with Se. The calculated band structures of Bi$_{1.75}$Cr$_{0.25}$(Se$_{0.6}$Te$_{0.4}$)$_3$ with varied $x$ (Fig. 4, A to E) show a transition from inverted to normal bands caused by the reduced SOC strength with increasing Se/Te ratio. The calculated bulk gap at the $\Gamma$ point is summarized in Fig. 4F, which clearly shows a topological QPT near $x = 0.66$, which is in agreement with the experiments.

With the correlation between the magnetic and topological QPTs firmly established, we turned to a more fundamental question: Which phase transition is the driving force, and which one is the consequence? Two pieces of evidence support the scenario that topology determines the magnetic ordering. First, in our Bi$_{1.75}$Cr$_{0.25}$(Se$_{0.6}$Te$_{0.4}$)$_3$ samples the Cr content is fixed, and only the Se/Te ratio is varied. This provides a knob for fine-tuning the SOC strength—hence, the bulk band topology—but the magnetic property is not directly affected. Therefore, the magnetic QPT should be a secondary effect of the topological QPT. Second, the ARPES results show that even at high $T$ when all the samples are in the FM state, the two regimes separated by the QCP already develop different topologies. At low $T$, the magnetic ground states form following the preformed topological character, with the FM phase resulting from the nontrivial topology and transitioning to the PM phase when the bulk turns topologically trivial.

The topological origin of the magnetic QPT is further supported by the effective model calculations (17). We calculated the $\Sigma$-direction spin susceptibility ($\chi_{zz}$) of eight QL magnetically doped Ti films using an effective four-band model (Fig. 4G) as a function of the chemical potential ($\mu$) and the mass term ($M_0$). In the inverted regime with $M_0 < 0$, $\chi_{zz}$ always remains a large value when $\mu$ is around the gap, as a consequence of the van Vleck mechanism (5); the second-order matrix element is strongly enhanced when the bulk bands become inverted. The topologically nontrivial phase thus strongly favors an FM ordering, which naturally explains the topology-driven magnetic QPT discovered in the experiments. The van Vleck mechanism is further supported by the magnetization measurements (Fig. S10), which show that the ferromagnetism occurs in the bulk rather than on the surfaces (25, 26). The out-of-plane magnetic anisotropy (Fig. S11) is also consistent with the van Vleck–type FM order in TiS (5).

To reveal the physical origin of the AHE sign change at the QCP, we calculated $\sigma_{xy}$ based on the four-band model, with two additional Zeeman splitting terms, $G_{z^2}$ and $G_{x^2}$, from the exchange coupling between the electrons and magnetic impurities (17). The $\sigma_{xy}$ value is summarized in Fig. 4H as a function of $M_0$ with fixed chemical potential, which clearly uncovers a sign change when the band gap is reversed, which is in good agreement with the experimental observation. The close correlation between the sign of AHE and topological QPT suggests that it can be used as a transport fingerprint for the bulk topology. This is not unexpected given the growing recognition of the topological nature of the intrinsic AHE in recent years (27, 28). The extrinsic AHE, which may be present in realistic materials, is ignored here because it typically dominates in highly metallic materials, whereas the disordered Ti films studied here are poorly conductive (20).

The topologically nontrivial FM states with tunable magnetic properties revealed here provide an ideal platform for realizing the exotic magneto-electric effects proposed by theory. The topology-driven magnetic QPT may also inspire new ideas for topological-magnetic phenomena and spintronic applications in TIs with broken TRS. We cannot completely rule out all other possibilities for the disappearance of FM ordering across the topological QPT. For example, the ARPES results (Fig. 3) show that the properties of itinerant carriers also change with Se content, which may affect an itinerant-driven FM mechanism.

References and Notes
2. M. Z. Hasan, C. L. Kane, Rev. Mod. Phys. 82, 3045 (2010).
17. Materials and methods are available as supplementary materials on Science Online.

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Ultrafast Tryptophan-to-Heme Electron Transfer in Myoglobins Revealed by UV 2D Spectroscopy
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Tryptophan is commonly used to study protein structure and dynamics, such as protein folding, as a donor in fluorescence resonant energy transfer (FRET) studies. By using ultra-broadband ultrafast two-dimensional (2D) spectroscopy in the ultraviolet (UV) and transient absorption in the visible range, we have disentangled the excited state decay pathways of the tryptophan amino acid residues in ferric myoglobin (MbCn and metMb). Whereas the more distant tryptophan (Trp)$^2$ relaxes by energy transfer to the heme, Trp$^1$ excitation predominantly decays by electron transfer to the heme. The excited Trp$^1$$\rightarrow$ heme electron transfer occurs in <40 picoseconds with a quantum yield of more than 60%, over an edge-to-edge distance below ~10 angstroms, outcompeting the FRET process. Our results raise the question of whether such electron transfer pathways occur in a larger class of proteins.

The advent of optical-domain multidimensional spectroscopies has opened entirely new perspectives for the study of biochemical dynamics, thanks to their abilities to visualize correlations and interactions between chromophores of the protein (1). This is particularly interesting when it comes to finding the pathways of electron and/or energy transfer in proteins, which are key processes in bioenergetics. Whereas vibrational multidimensional spectroscopy in the infrared (IR), which detects the couplings between vibrational dipoles, was established in the early 1990s, it was only in mid-2000 that the first study

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of multidimensional electronic spectroscopy appeared using continua in the 800-nm region (2). The authors monitored the transport of energy and the excitonic couplings between seven chromophores of the Fenna-Matthews-Olson photosynthetic light-harvesting protein. The extension of multidimensional spectroscopies to shorter wavelengths is an obvious goal considering the rich variety of biologically relevant chromophores that absorb from the green to the ultraviolet (UV). In particular, amino acids and nucleic acids absorb below 300 nm. Among the amino acid residues, Trp is found in most proteins and has become a routine probe of protein structure and dynamics using fluorescence resonance energy transfer (FRET), (3–9) Myoglobins contain two Trp residues (Trp14 and Trp17), located in the α helix A (Fig. 1), fluorescence of which is quenched on time scales of 20 to 30 ps and 110 to 140 ps, respectively (10–12). These times are consistent with calculated quenching rates for a FRET process to the heme. (7, 12) The lack of Trp fluorescence quenching in apomyoglobins was taken as further evidence of FRET to the heme (12).

Trp is also involved in electron transfer processes as the primary donor in DNA repair by photo-tolyase (13), with carbonyl- and sulfur-containing residues (14), and was suggested to reduce Cu(II) in azurin (15). It is also an intermediate in functional multistep hopping processes, for example, in class I ribonucleotide reductase (16), in cytochrome c peroxidase (17), and in azurin tagged with a rhenium carbonyl diimine complex (18).

In general, it is difficult to distinguish electron transfer from FRET if the acceptor does not fluorescence, which is the case with heme proteins. Here, two-dimensional (2D) spectroscopy with broad UV continua [detailed in (19, 20)] is implemented to study the photocycle of ferric CN- and water-ligated horse myoglobins (MbCN and metMb, respectively). This technique allows us to disentangle the overlapping spectral contributions of the various chromophores (Trp17, Trp14, and heme) in the protein. Ferric Mbs are ideal to address the Trp-heme interaction because heme ground-state recovery is complete <10 ps after phototexcitation (21–24). We unravel a Trp14 → heme electron transfer process proceeding in ≤40 ps through ~8 Å, which efficiently competes with FRET. The Trp-mediated heme reduction is further confirmed by transient absorption spectroscopy in the visible range.

Figure 2 (A to C) shows a set of 2D UV transient absorption spectra of MbCN at different delay times, in which the transient absorption signal is plotted as a function of excitation and detection wavelength (λ). In Fig. 2A, a bleach feature centered at ~335 nm appears for λpump > 305 nm, which completely recovers within 10 ps (red dashed trace in Fig. 2E). For λpump < 305 nm, the signal is dominated by excited state absorption (ESA) below λprobe ≈ 330 nm, with a dip along the diagonal near 290 nm because of the ground state bleach (GSB) of Trp. The ESA signal persists for over 100 ps. The transition between these two spectral regions reflects the transition from exclusive heme absorption (λpump > 305 nm) to overlapping heme and Trp absorptions (λpump < 305 nm). Thus, the short-lived transient observed for λpump > 305 nm can be ascribed to the heme relaxation, in line with the previously reported <10-ps photocycle (22).

The longer-lived features produced by λpump < 305 nm show a significant spectral evolution from tens to hundreds of ps (Fig. 2, B and C). In particular, an ESA signal (Fig. 2C) appears in the 290- to 340-nm region, which differs from the ESA at earlier times (Fig. 2, A and B) because it lacks the absorption below 280 nm, and its maximum is in the region between 305 and 318 nm. It arises exclusively through Trp excitation (Fig. 2E).

To rationalize this complex temporal and spectral evolution, we performed a singular value decomposition and a global analysis (20) of the time-resolved 2D spectra. By modelling the overall kinetics with a multieponential framework,
the characteristic time constants and respective amplitudes were obtained for each pair of (λ_pump, λprobe) wavelengths [Fig. 3 and figs. S1 (MbCN) and S3 (metMb)]. The plots in these figures constitute the decay-associated dispersed action spectra (DADAS), which are the 2D assemblies of decay-associated spectra (DAS) [see (20) for more details]. A cut of these spectra along the probe axis provides the conventional (1D) DAS, that is, the spectra associated with the respective time constants for a fixed excitation wavelength. A cut along the excitation axis provides the action spectrum of the observed kinetics.

Six time constants are necessary to describe the observed transients. For MbCN, the three extracted fast components, <300 fs, 1.1 ± 0.1 ps, and 4.4 ± 0.2 ps (error bars represent standard deviations, table S1 and fig. S1), are in good agreement with previous ultrafast visible and IR studies (22, 24, 25). These components are detected at all excitation wavelengths, and their action spectra (Fig. 3D and figs. S1 and S2) agree with the MbCN heme absorption; that is, they reflect the heme relaxation.

Three additional time constants (19 ± 1 ps, 140 ± 10 ps, and ≥2.5 ns; table S1 and Fig. 3, A to C) are necessary to describe the system kinetics for excitation below ~305 nm. The 19- and 140-ps components agree with the known Trp14 fluorescence decays, respectively (7, 11, 12). The transient spectra along the λ_probe axis are dominated by the Trp ESA, with two strong absorption features at ~303 nm and below 275 nm, separated by a minimum because of the Trp GSB. The same pattern of results and time scales is found for metMb (table S1 and fig. S3).

The ≥2.5-ns (≥0.7-ns in metMb) component, in contrast, was not reported before. Although this state is only produced by excitation below ~305 nm, its probe spectrum (Figs. 2C and 3, C and D) is significantly different from the 19- and 140-ps DAS. None of the Trp ESA bands are present, whereas an absorption band appears at 325 nm with a shoulder at 307 nm (Fig. 3E). This strongly suggests that the state responsible for this transient cannot be associated with an excited Trp. The 19-ps DAS (Fig. 3E) contains, in addition to the typical features of the Trp decay (see trace of the 140-ps DAS), a component that matches the mirror image of the >2.5-ns DAS, indicating a population growth of the long-lived state with a time scale of 19 ps, suggesting that it is generated by the decay of Trp14. The data analysis for metMb yields similar temporal and spectral behaviors (fig. S3 and table S1).

Figure 3F shows typical cuts of the >10-ns DADAS. For the decay of the two Trp molecules and the 2.5-ns component, the traces are in agreement with the spectral shape of the Trp steady-state absorption in a pH = 7 buffer, but they all show a small red shift with respect to it. The Trp14 absorption and the action spectrum of the ≥2.5-ns component coincide and show a small red shift (~1 nm) with respect to the Trp14 absorption. A similar effect is observed also in metMb (Fig. 3F, inset), but the shift of the Trp14 absorption is larger (2.5 to 3 nm). This is briefly discussed in the supplementary materials.

The good agreement of the action spectrum of the long-lived state with the Trp14 absorption confirms that this state is populated by the decay of excited Trp14. To identify the nature of this long-lived photoproduct, we carried out (1D) visible transient white-light probe measurements upon 290-nm excitation. Figure 4A shows the time-wavelength plot for MbCN (fig. S5 for metMb). In the Q-band region (500 to 600 nm), a double-peaked structure grows on a 20-ps time scale and lasts for over 700 ps (the limit of our measurement). In the Soret-band region (380 to 450 nm), a similar rise is observed for negative and positive transient bands, centered at 419 and 438 nm, respectively. The spectral shape of this long-lived state (Fig. 4B) agrees well with the difference between ferrous and ferric MbCN spectra, indicating the formation of a ferrous-MbCN (metMb) photoproduct (26). The reduction quantum yield
(QYr) upon 290-nm excitation is estimated from the fraction of excited molecules and the number of photo-reduced molecules, which is in turn obtained from the amplitude of the DAS associated with the long-lived state compared with the difference between the reduced and non-reduced MbCN (metMb) absorption spectra (see supplementary materials). We find a quantum yield of 11 to 12% (corresponding to a reduction cross section $\sigma_{\text{red}} \approx 0.56$ cm$^2$) in both MbCN and metMb. Under static conditions $\sigma_{\text{red}} \approx 5 \times 10^{-19}$ cm$^2$ was measured for metMb under 290-nm irradiation (27), which implies that most of the hemes photo-reduced in our experiment re-oxidize on time scales beyond our measurement window. In fact, for both samples a decay of the reduced state occurs on a 100- to 200-ps time scale, which accounts for only 30 to 40% of the photo-reduced hemes (Fig. 4 and figs. S4 and S5). This may indicate a partial, fast back electron transfer. The Trp cation radical is likely to be produced in an excited state from the Trp-to-heme electron transfer (similar to (26)). A branching between deprotonation and intersystem crossing to the ground state in its relaxation may explain the partial electron hole recombination: TrpH$^+$ in the ground state would allow for a fast back electron transfer, whereas deprotonated Trp would prohibit it. Such an explanation is reasonable but needs further confirmation.

The above data unambiguously support reduction of the heme by Trp$^+$, overwhelming the FRET process. No detectable electron transfer from Trp$^-$ is found. At 290 nm, Trp$^-$ excitation accounts for ~18% of the total number of excited molecules (29). Thus, the quantum yield (QY) of the Trp$^-$→heme electron transfer is ~60% in MbCN and >50% in metMb. The time constants for electron transfer obtained from the QY and the observed decay times of excited Trp$^{14+}$ in MbCN (metMb) are ~34 ps (~40 ps).

Long-range (~5 Å) electron transfer in proteins occurs either by tunnelling through covalent bonds forming the secondary structure (21, 30) or by hopping across specific intermediates (13, 16, 17, 31). The distance dependence of the tunnelling probability through the protein medium follows an exponential behavior $\exp(-b\beta)$ (32), where $\beta$ can be empirically determined, and differs for different protein secondary structures (33). Pathway (34–36) and density-packing (35, 36) models account in more detail for the molecular protein structure. The edge-to-edge distance between Trp$^{14+}$ and the porphyrin ring is ~8 Å; the distance to the Fe atom is ~12 Å. Our observed rate is consistent with Marcus theory (21, 37) assuming a well-optimized process (Gibbs free energy of the redox reaction equals reorganization energy) and $\beta \sim 1.1$ to 1.3 Å$^{-1}$, which is in good agreement with density-packing predictions (35). The pathway analysis (34, 38) allowed determination of a large number of electron transfer paths that are of relevance here. The electron transfer is mediated by the residues of the α-helix E located between the heme and the indole ring of Trp$^{14+}$ and in (or close to) van der Waals contact with the latter (Fig. 1 and fig. S7). The estimated time of ~40 ns for electron transfer through the ~13.5 Å Trp$^{14+}$-heme edge-to-edge distance, compared with the 140-ps FRET time, explains the dominance of the latter. Although it is not clear whether electron transfer also occurs in ferrous myoglobins, the invariance of the Trp$^{14+}$ lifetimes with redox state and nature of ligand (11) strongly suggests this to be the case.

The present studies, along with the examples of Trp-mediated electron transfer in other proteins (13–18), raise questions about its widespread use in FRET studies of protein dynamics, especially when the acceptor is not fluorescent.

References and Notes

20. The experimental procedures and analysis of data are described in the supplementary materials.
29. This value can be estimated from the extinction coefficient of Trp as compared with those of myoglobin.
33. Typical Trp lifetimes are 1.0 for tunnelling through β sheets, 1.3 through α helices.

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Supplementary Materials for

Ultrafast Tryptophan-to-Haem Electron Transfer in Myoglobin Revealed by 2D UV Spectroscopy

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SI. Material and Methods:

SI.1 The setup:

The two-dimensional (2D) setup was already described in ref. (19). Within the 2D transient absorption (TA) scheme, the wavelength of the narrow band pump beam is scanned over the desired range, while the broad-band probe beam is spectrally dispersed for detection.

Briefly, ~ 0.4 mJ of the output of a 20 kHz amplified laser system (KMLabs, Halcyon + Wyvern500, providing 800 nm, typically ~ 0.6 mJ pulses of ~ 50 fs) are injected into a TOPAS-White (Light Conversion) which is operated to obtain sub-90 fs broad-bandwidth pulses in the 540 – 700 nm range. The typical output energy is 13 µJ. About 60% of it is used to generate the narrowband pump pulses. The pulses are focused onto a 2 mm thick BBO crystal for frequency doubling whose wavelength is controlled by rotation of the crystal around the ordinary axis. The typical pump band-width is 1.5 nm Full Width Half Maximum (FWHM). Maximum excitation energy was ~ 120 nJ; the signal was checked for linearity with the pump power.

The remaining TOPAS intensity is used to generate the broad-band UV probe pulses through the achromatic doubling scheme (39). The beam is finally focused onto the sample with a typical focal spot size of 40 µm. The typical dimension of the pump focus is 90-100 µm FWHM, which results in a homogeneous illumination of the probed region. After the sample, the broadband probe beam is focused in a multi-mode optical UV fiber (100 µm, Avantes), which is coupled to the entrance slit of a 0.25 m imaging spectrograph (Chromex 250is). The beam is dispersed by a 150 gr/mm holographic grating and imaged onto a multichannel detector consisting of a 512 pixel CMOS linear sensor (Hamamatsu S11105, 12.5 x 250 um pixel size) with up to 50 MHz pixel readout, so the maximum read-out rate per spectrum (almost 100 kHz) allows us to perform shot-to-shot detection easily.(40)

All 2D measurements were performed in parallel polarization. The investigated spectral range spans ~ 50 nm along the excitation axis, around 300 nm, and exceeds 75 nm along the probe axis. To better address the entire transient dynamics, a full set of 2D spectra was recorded as a function of time. The high sampling density along the time axis, comparable with those of a normal transient absorption experiment, guarantees detailed characterization of the sub-ns time scales. The temporal resolution was ~150 fs, but the first 250 fs were removed from the analysis due to cross-phase modulation. The pump power is recorded on a shot-to-shot basis by a calibrated photodiode for each pump wavelength, allowing for the reconstruction of the 2D spectra corrected for the pump power.
The transient absorption (1D) UV-pump/visible continuum probe setup uses the same pump path as in the 2D experiments. The visible continuum is obtained by focusing a small fraction (~ 1 µJ) of the fundamental at 800 nm in a continuously moving CaF2 window. The generated continuum is focused onto the sample with a parabolic reflector on a spot of ~ 30 µm diameter, and detected with the same detector system as described above. Two consecutive scans with different grating angles were necessary to cover the desired spectral range. The polarization of the 800 nm pulse was controlled by a half waveplate and set to the magic angle (MA) with respect to the pump.

We also used another UV pump/UV-visible continuum probe setup, which is described in references (41, 42). Briefly, the output of a 1 kHz regenerative amplifier is used to pump two home-built Non-collinear Optical Parametric Amplifiers (NOPAs) which deliver typical pulses of 1-2 µJ and 80 nm bandwidth at 600 nm, with a pulse duration of ~ 30 fs. The output of one NOPA is used to generate the UV excitation pulse by narrow band frequency doubling in a 0.25 mm thick BBO crystal. The output of the other NOPA is used to generate a broadband UV pulse via achromatic doubling in a 0.15 mm thick BBO crystal. The experimental scheme here is quite different from the one described above, and the bandwidth of the generated broad band pulses is narrower. Three scans are necessary to cover the 270 – 380 nm spectral region. The visible probe is obtained by focusing ~ 1 µJ of the amplifier output in a moving CaF2 window.

After the sample, the probe beam is dispersed by a 830 gr/mm transmission grating and focused on a 512 pixel photodiode array.

Typical pump and probe focus dimensions are 120 µm and 60-80 (UV-visible) µm. Pump energies were set to 120 nJ, within the region of signal linearity as function of pump power. Polarization of the pump pulses was controlled by a half-wave plate and set at the MA.

SI.2 Sample preparation:

Dehydrated myoglobin powder from horse skeletal muscle was purchased from Sigma Aldrich. The aqueous sample of metMb was prepared by dissolving the powder in 20 mM phosphate buffer at pH 7.0 without further purification.

MbCN was prepared by dissolving the powder in 20 mM phosphate buffer at pH 6.9. Complex formation upon addition of a ~ 3-fold excess of NaCN to the solution was monitored by measurement of the UV-vis steady state absorption spectrum. The final pH of the solution was 7.2.
The concentration of the sample was adjusted such as to have a $\sim 0.35$ optical density (OD) at 280 nm for the 2D measurements, corresponding to $\sim 0.57$ mM for MbCN (0.2 mm flow cell) and $\sim 0.22$ mM for metMb (0.5 mm flow cell). The concentration was further increased to $\sim 0.4$ OD at 280 nm for the TA UV pump/visible continuum probe measurements. All measurements were performed on freshly prepared samples, continuously flowing to avoid photodamage.

**SI.3 Data treatment and analysis:**

Although the prism compressor efficiently removes the linear chirp from the broadband UV light, higher order terms result in a relatively long ($\sim 300$ fs FWHM) pulses. In the broadband detection scheme, this does not result in a significant deterioration of the single-frequency temporal resolution, and the group-velocity-dispersion is corrected in the analysis, as for the TA (1D) UV-pump/ broadband UV-visible probe measurements.

Global analysis of the GVD-corrected time-resolved 1D and 2D transients was performed by singular value decomposition (SVD) technique, which is known to efficiently act both as an instrument capable to reduce the fit dimensionality, and as a noise filter (43, 44). A typical SVD analysis of the 1D transient absorption measurements ($M(\lambda,t)_{\text{data}}$ data matrices of signal as function of wavelength and time), decomposes them into a product of three matrices

$$M(\lambda,t) = S(\lambda)_{\text{diag}} \times W_{\text{diag}} \times T(t)_{\text{diag}}^T$$

In the hypothesis of purely stochastic noise with zero average and if the data matrix can be described by a discrete linear combination of terms, each of them factorized into a temporal and a spectral dependence, then the columns of $S$ and $T$ can be interpreted as the singular spectral and kinetic vectors (often called "eigenspectra" and "eigentraces"). $W_{\text{diag}}$ is a matrix which is zero except for its diagonal entries $w_{ii}$, called singular values and representing the weight with which each singular eigen spectrum and its corresponding eigentrace contribute to the total signal. For noise-free data, there are exactly N non-vanishing singular values, which are conventionally arranged in a non-increasing order: $w_{11} \geq w_{22} \geq ... \geq w_{NN} > 0$. The presence of noise acts as a perturbation of the singular values and the respective singular vectors. It was demonstrated that the amount of perturbation can be directly related to the "isolation" of the singular values (44). For any practical application, the only relevant singular values satisfy the condition $w_{ii} - w_{i+1,j+1} \gg w_{NN}$, the latter being entirely associated to experimental noise. Multi-exponential global least-squares fit of the relevant eigentraces allows retrieving the characteristic times $\tau_i$ with
their uncertainties and the respective amplitudes $a_{j\tau}$. The latter are used to construct the Decay Associated Spectra, i.e. the spectral contribution associated to each specific time constant $\tau_j$, as

$$DAS_j(\lambda) = \sum_j a_{j\tau} w_{j\lambda} U_j(\lambda)$$

where $U_j(\lambda)$ is the eigenspectrum associated to the j-th eigentrace. Note that an implicit condition for application of the SVD is that the kinetic components describing the signal have an overall amplitude evolution but their spectral shapes do not evolve in time.

Since typical SVD algorithms act on matrices, their application to the 3D data set (signal as function of pump wavelength, probe wavelength, time) of the 2D experiment requires an initial dimensional transformation.

The 3D data $S(\lambda_{probe}, \lambda_{pump}, t)$ are arranged in a matrix $S(\lambda', t)$, where the index $\lambda'$ runs over all $(\lambda_{probe}, \lambda_{pump})$ pairs. SVD analysis is performed on $S(\lambda', t)$ as previously described, and each obtained DAS relative to a time constant $\tau_i$ are re-arranged into matrices $DADAS_i(\lambda_{probe}, \lambda_{pump})$.

Since the excitation axis of these matrices contains the action spectra as a function of the probe wavelength for a specific characteristic time, we call the 2D DAS Decay Associated Dispersed Action Spectra (DADAS).

### SII. Supplementary Text

#### SII.1 Estimation of the photoreduction quantum yield:

The reduction quantum yield ($QY_r$) can be determined as:

$$QY_r = \frac{\text{number molecules undergoing reduction/number of photoexcited molecules}}{N_r \over N_{exc}} \quad \text{where} \quad N_{exc} = \rho_{ph} \sigma N_m$$

where the number of excited molecules $N_{exc}$ can be calculated from the experimental conditions, knowing the number of molecules $N_m$ in the illuminated volume, the photon density $\rho_{ph}$ and the absorption cross section $\sigma$ :

In our experiments, the fraction of excited molecules is estimated to be $8 \pm 1\%$. 

5
Let us call $S_{PR}$ the difference between the static spectra of the reduced and oxidized states, scaled to the number of excited molecules. Then the fraction of photoreduced molecules ($Q_Y^R$) is obtained by comparing $S_{PR}$ with the DAS accounting for the decay of the photoreduced state.

By comparing the extinction coefficients of the Trp with those of myoglobin at the desired excitation energy (in our case 290 nm) we can estimate that haem photoexcitation occurs in $\sim 64\%$ of the excited molecules, while each Trp’s contributes for $\sim 18\%$. Finally, the Trp14 $\rightarrow$ haem electron transfer QY ($Q_Y^R$) is obtained as:

$$Q_Y^R = \frac{Q_Y^R}{0.19}$$

**SI.2 Batochromic shift of Trp absorption**

Several properties of the Trp amino acid residue make it a unique probe of the electrostatic properties of its surrounding. Among them, strong solvatochromic effects in fluorescence, affecting its energy (11) and shape (12, 13), or the QY of energy or electron transfer to other amino acids or protein functional groups, affecting emission intensities and characteristic times (11). Trp absorption is also environment-sensitive as manifested in energy shifts as a function of local polarity and/or presence of H-bonds (14, 15).

Our data allow to access the spectrum of Trp in the protein through the action spectra of a given DAS. They show that the spectra of Trp7 and Trp 14 can be spectrally distinguished, as they exhibit differential energy shifts. In both myoglobins, the spectrum of Trp7 presents a small red-shift (~ 1 nm) with respect to aqueous Trp, while Trp14 shows an additional red-shift of ~ 1 nm in MbCN and ~ 2.5-3 nm in metMb.

It is not surprising that Trp7, which is in contact with the solvent, has an absorption band closer to that of aqueous Trp than Trp14, which is embedded in a relatively hydrophobic environment. More surprising is the different energy shift observed for the latter in the two proteins. This speaks for a different polarity, and thus local structure, around Trp14, primarily caused by the substitution of CN by H$_2$O as an Fe axial ligand. Indeed, X-ray crystallography of met- and CN-myoglobin shows differences in the proximity of Trp14 (PDB files 1YMC and 1YMB), among them a different relative orientation and distance of the positively charged NH$_3$ side chain of Lysine77 and the negatively charged CO$_2$ side chain of Glu18 with respect to the Trp plane (see figure S7).
SII.3 Summary of measured time constants

**Table S1:** Time constants observed upon UV excitation of MbCN and metMb. Errors correspond to one standard deviation and are obtained from the least-squares fit of the SVD eigentraces. Interpretation is according to the main article. * In met Mb, additional 1D visible pump/visible probe experiments reveal an additional sub-100 fs time constant.

<table>
<thead>
<tr>
<th>System</th>
<th>MbCN</th>
<th>metMb</th>
</tr>
</thead>
<tbody>
<tr>
<td>$\lambda_{\text{pump}}$</td>
<td>&lt; 305 nm</td>
<td>&gt; 305 nm</td>
</tr>
<tr>
<td>$\tau$ (Haem) [ps]</td>
<td>&lt; 0.3</td>
<td>0.47 ± 0.03 *</td>
</tr>
<tr>
<td></td>
<td>4.4 ± 0.2</td>
<td>4.7 ± 0.1</td>
</tr>
<tr>
<td>$\tau$ (Trp14) [ps]</td>
<td>19 ± 1</td>
<td>-</td>
</tr>
<tr>
<td>$\tau$ (Trp7) [ps]</td>
<td>140 ± 10</td>
<td>-</td>
</tr>
<tr>
<td>$\tau$ (Reduced) [ns]</td>
<td>≥ 2.5</td>
<td>-</td>
</tr>
</tbody>
</table>

**Supplementary Figures:**
**Fig S1**: Haem-related (A-C) and Trp-related (D-F) Decay Associated Dispersed Action Spectra (DADAS) of MbCN obtained from the global analysis of the time-resolved 2D data: (A) Sub-300 fs component; (B) 1.1 ps component; (C) 4.4 ps component; (D) 19 ps component (Trp14 decay + reduced photoproduct rise); (E) 140 ps component (Trp7 decay); (F) \( \geq 2.5 \) ns (reduced MbCN). The color scales in A,B,C and D,E,F are, respectively, the same.
Fig S2: Pump wavelength dependence of the amplitude of the 4 ps MbCN DADAS at a probe wavelength of 300 nm compared to the static absorption of the haem. Data points around 300 nm were removed since they are perturbed by scattered pump light.
**Fig S3:** Haem-related (A-C) and Trp-related (D-F) Decay Associated Dispersed Action Spectra (DADAS) of metMb obtained from the global analysis of the time-resolved 2D data corresponding to the characteristic time constants reported in table S1: (A) 0.47 ps component; (B) 1.14 ps component; (C) 4.7 ps component; (D) 20 ps component (Trp14 decay + rise of the deoxy-Mb signal); (E) 137 ps component (Trp7 decay); (F) >>1 ns (deoxy-Mb). The color scales in A, B, C and D, E, F are, respectively, the same.
Fig S4: Transient absorption of 290 nm-excited metMb. Positive signals are in red, negative ones in blue. The signal intensity is shown in a hyperbolic arcsine scale to enhance the small signals below 380 nm and above 450 nm. Solid line shows the kinetic trace at 438 nm (marked by the dotted line in the plot).
Fig S5: Decay Associated spectra (DAS) of Trp14 (blue), Trp7 (green) and ferrous state (red) resulting from global analysis of the 290 nm excited MbCN (A) and metMb (B). The inset in panel A enlarges the specific double-peaked features between 510-590 nm. In both panels, the Trp14 signal contains a rise of the long-lived component, while its decay is observed on a time scale similar to the Trp7 decay.
**Fig S6:** Most important calculated electron transfer paths from Trp14 to the porphyrin ring in myoglobin (34, 38). The dominant pathways proceed via Leu69, which is in van der Waals contact with Trp14, and Val68. Two views (A and B) are shown for clarity.
Fig S7: Molecular structure of MbCN (A) and metMb (B) in the proximity of Trp14 (45). Residues 69-77 are shown. The positive side chain of Lys77 and the negatively charged CO$_2$ side chain of Glu18 are in the proximity of Trp14. The former exhibits different orientations with respect to Trp14 in the two proteins.
References and Notes


20. The experimental procedures and analysis of data are described in the Supplementary on-line material.


29. This value can be estimated from the extinction coefficient of Trp as compared with those of myoglobin.


33. Typical β values are 1.0 for tunnelling through β-sheets, 1.3 through α-helices)


45. PDB files 1YMC and 1YMB.