Tryptophan-to-heme electron transfer in ferrous myoglobins

Roberto Monni, André Al Haddad, Frank van Mourik, Gerald Auböck, and Majed Chergui

Laboratoire de Spectroscopie Ultrarapide, Institut de Sciences et Ingénierie Chimiques, École Polytechnique Fédérale de Lausanne, CH-1015 Lausanne, Switzerland

Edited by Harry B. Gray, California Institute of Technology, Pasadena, CA, and approved March 31, 2015 (received for review December 5, 2014)

It was recently demonstrated that in ferrous myoglobins (Mb) the fluorescence quenching of the photoexcited tryptophan 14 (\(\text{Trp}^{14}\)) residue is in part due to an electron transfer to the heme porphyrin (porph), turning it to the ferrous state. However, the invariance of \(\text{Trp}\) decay times in ferric and ferrous Mbs raises the question as to whether electron transfer may also be operative in the latter. Using UV pump/visible probe transient absorption, we show that this is indeed the case for deoxy-Mb. We observe that the reduction generates (with a yield of about 30\%) a low-valence Fe-porphyrin \(\pi\) anion radical, which we observe for the first time to our knowledge under physiological conditions. We suggest that the pathway for the electron transfer proceeds via the leucine 69 (Leu\(^{69}\)) and valine 68 (Val\(^{68}\)) residues. The results on ferric Mbs and the present ones highlight the generality of Trp-porphyrin electron transfer in heme proteins.

Electron transfer plays a fundamental role in many biological systems (1–3) ranging from photosynthetic proteins (4) to iron–sulfur (5), copper (6), and heme (7, 8) proteins. It was demonstrated that electron transfer can be used to produce from heme proteins in situ drugs with antimalarial activity (9) and it might have a role in protein folding (2). In general, electron transfer in proteins can occur over long distances (>10 Å) by hopping through different residues, thus reducing the time that would be needed for a single step tunneling from the donor to the acceptor (10–12). Aromatic amino acids and Tryptophan (Trp) in particular can act as a relay in such processes (13–19). Trp also acts as a phototriggered electron donor, e.g., in DNA repair by photolyase (16–18) and in cryptochromes (20, 21). When no obvious electron acceptors are present, excited Trp or (\(\text{Trp}^*\)) still displays shorter lifetimes than its nanosecond decay times in solution (22, 23). This is due to its strong tendency to act as an electron donor, undergoing electron transfer toward the protein’s backbone as in the case of apo-myoglobin mutants (24), small cyclic peptides (25), and human \(γ\)-t-crystallin (26). It is interesting to note that in wild-type horse heart (WT-HH) apo-myoglobin the fluorescence lifetime of the two \(\text{Trp}\) residues was reported to be comparable to that in water (27), demonstrating the absence of deactivation mechanisms, either by energy or by electron transfer.

The protein visible absorption spectrum is dominated by their cofactors, e.g., heme or flavins, whereas the UV absorption in the region between 250 nm and 300 nm is mainly due to the three aromatic amino acids, Trp, tyrosine (Tyr), and phenylalanine (Phe) (28), with Trp having the highest molar extinction coefficient. The high sensitivity of Trp to the local environment and the possibility to correlate it with its fluorescence response (28) have led to its widespread use as a local natural probe of protein structure and dynamics in time-resolved fluorescence resonance energy transfer (FRET) studies, and it has emerged as the “spectroscopic ruler” in such studies (28–30). FRET is mediated by dipole–dipole coupling between a donor \(\text{Trp}\) and an acceptor molecule, and its rate is inversely proportional to the sixth power of the distance between them and to the relative orientation of their dipoles.

Electron transfer | heme proteins | tryptophan | picosecond | low valence heme

Myoglobin (Mb) is a small heme protein composed of ~150 residues (31) arranged in eight \(α\)-helices (from A to H) (SI Appendix, Fig. S7), whose biological function is to store molecular oxygen in muscles of vertebrates (32). This is accomplished by its prosthetic group: a Fe–Protoporphyrin IX complex bound to the protein structure via the proximal histidine (His\(^\alpha\)) (SI Appendix, Fig. S7). Both ferric and ferrous hemes tend to bind small diatomic molecules (e.g., O\(_2\), CO, NO, and CN) at the Fe site. Mb has two Trp residues that are situated in the \(α\)-helix A: \(\text{Trp}^1\) (Heme) and \(\text{Trp}^{14}\) (Heme) center-to-center distances are 21.2 Å and 15.1 Å, respectively (33, 39) (SI Appendix, Fig. S7). We recently showed, using ultrafast 2D-UV and visible transient absorption (TA) spectroscopy, that in the ferric myoglobins (MbCN and MbH\(_2\)O) the relaxation pathway of \(\text{Trp}^{1}\)-Heme and \(\text{Trp}^{14}\)-Heme center-to-center distances are 21.2 Å and 15.1 Å, respectively (33, 39) (SI Appendix, Fig. S7). We recently showed, using ultrafast 2D-UV and visible transient absorption (TA) spectroscopy, that in the ferric myoglobins (MbCN and MbH\(_2\)O) the relaxation pathway of \(\text{Trp}^{1}\)-Heme and \(\text{Trp}^{14}\)-Heme center-to-center distances are 21.2 Å and 15.1 Å, respectively (33, 39) (SI Appendix, Fig. S7). We recently showed, using ultrafast 2D-UV and visible transient absorption (TA) spectroscopy, that in the ferric myoglobins (MbCN and MbH\(_2\)O) the relaxation pathway of \(\text{Trp}^{1}\)-Heme and \(\text{Trp}^{14}\)-Heme center-to-center distances are 21.2 Å and 15.1 Å, respectively (33, 39) (SI Appendix, Fig. S7). We recently showed, using ultrafast 2D-UV and visible transient absorption (TA) spectroscopy, that in the ferric myoglobins (MbCN and MbH\(_2\)O) the relaxation pathway of \(\text{Trp}^{1}\)-Heme and \(\text{Trp}^{14}\)-Heme center-to-center distances are 21.2 Å and 15.1 Å, respectively (33, 39) (SI Appendix, Fig. S7). We recently showed, using ultrafast 2D-UV and visible transient absorption (TA) spectroscopy, that in the ferric myoglobins (MbCN and MbH\(_2\)O) the relaxation pathway of \(\text{Trp}^{1}\)-Heme and \(\text{Trp}^{14}\)-Heme center-to-center distances are 21.2 Å and 15.1 Å, respectively (33, 39) (SI Appendix, Fig. S7). We recently showed, using ultrafast 2D-UV and visible transient absorption (TA) spectroscopy, that in the ferric myoglobins (MbCN and MbH\(_2\)O) the relaxation pathway of \(\text{Trp}^{1}\)-Heme and \(\text{Trp}^{14}\)-Heme center-to-center distances are 21.2 Å and 15.1 Å, respectively (33, 39) (SI Appendix, Fig. S7). We recently showed, using ultrafast 2D-UV and visible transient absorption (TA) spectroscopy, that in the ferric myoglobins (MbCN and MbH\(_2\)O) the relaxation pathway of \(\text{Trp}^{1}\)-Heme and \(\text{Trp}^{14}\)-Heme center-to-center distances are 21.2 Å and 15.1 Å, respectively (33, 39) (SI Appendix, Fig. S7).

Electron donor, undergoing electron transfer toward the protein’s backbone as in the case of apo-myooglobin mutants (24), small cyclic peptides (25), and human \(γ\)-t-crystallin (26). It is interesting to note that in wild-type horse heart (WT-HH) apo-myoglobin the fluorescence lifetime of the two \(\text{Trp}\) residues was reported to be comparable to that in water (27), demonstrating the absence of deactivation mechanisms, either by energy or by electron transfer.

The protein visible absorption spectrum is dominated by their cofactors, e.g., heme or flavins, whereas the UV absorption in the region between 250 nm and 300 nm is mainly due to the three aromatic amino acids, Trp, tyrosine (Tyr), and phenylalanine (Phe) (28), with Trp having the highest molar extinction coefficient. The high sensitivity of Trp to the local environment and the possibility to correlate it with its fluorescence response (28) have led to its widespread use as a local natural probe of protein structure and dynamics in time-resolved fluorescence resonance energy transfer (FRET) studies, and it has emerged as the “spectroscopic ruler” in such studies (28–30). FRET is mediated by dipole–dipole coupling between a donor \(\text{Trp}\) and an acceptor molecule, and its rate is inversely proportional to the sixth power of the distance between them and to the relative orientation of their dipoles.

**Significance**

We demonstrate the occurrence of tryptophan (Trp) to heme electron transfer (ET) in ferrous myoglobins by ultrafast UV spectroscopy. The ET gives rise to the theoretically predicted, low-valence Fe(II)(porph\(^\bullet\)) anion radical, which we observe for the first time to our knowledge under physiological conditions. These results highlight the generality of Trp–porphyrin electron transfer events in heme proteins and question the systematic use of Trp fluorescence in FRET studies of protein dynamics.

Author contributions: M.C. designed research; R.M., A.A.H., F.V.M., and G.A. performed research; R.M., G.A., and M.C. analyzed data; and R.M., G.A., and M.C. wrote the paper.

The authors declare no conflict of interest.

This article is a PNAS Direct Submission.

Freely available online through the PNAS open access option.

To whom correspondence should be addressed. Email: majed.chergui@epfl.ch.

This article contains supporting information online at www.pnas.org/lookup/suppl/doi:10.1073/pnas.1423186112/-/DCSupplemental.
To address these questions, here we present a UV-pump/visible-probe TA study of ferrous Mbs. In the latter case with apical diatomic ligands, e.g., MbNO and MbCO, heme photoexcitation leads to dissociation of the ligand, followed by its recombination to the heme, which can be both geminate (the ligand stays inside the protein scaffold) and nongeminate (the ligand migrates out of the protein scaffold) (46–48). For the NO ligand, recombination timescales are typically ∼10 ps, ∼30 ps, and ∼200 ps (46, 47), whereas for CO they span up to the millisecond range (46, 49–51). The presence of recombination timescales in the order of *Trp decay times leads to additional signal contributions, which complicate the analysis of the data. These problems are avoided using deoxy-Mb, which has a penta-coordinated heme bound only to the His53. Upon heme photoexcitation, the system recovers to the ground state within a few picoseconds (46, 52). This allows investigating the *Trp–heme interaction without any overlapping contributions.

We show here that just as in the ferric Mbs (40), also in deoxy-Mb does *Trp partly decay to the heme by electron transfer, competing with the FRET pathway. We find that the transferred electron is localized on the porphyrin ring, contrary to the ferric case where it resides on the metal center. This is due to the highly negative reduction potential of the FeII/FeI couple (53, 54), which is close to the porphyrin reduction potential (55). To our knowledge, this is the first report of a low-valent myoglobin, under physiological conditions.

The experimental setup, the sample preparation, and the data analysis are described in SI Appendix.

Results and Discussion

The static absorption spectra of deoxy-Mb and Trp (SI Appendix, Fig. S1) show that at wavelengths <310 nm, the contributions of the heme and Trp overlap, excluding selective excitation of the Trp residues. To disentangle the Trp and heme contributions we performed TA measurements exciting deoxy-Mb at 315 nm, where only the heme absorbs, and compared them to those exciting at 290 nm where both Trp and heme absorb. Assuming that the heme response is similar for both excitation wavelengths, this allows separation of the heme and the Trp contributions in the transient absorption signal. It must be noted that in the probe range used in our experiments (390–730 nm), Trp excited-state absorption (ESA) and stimulated emission (SE) as well as an eventual Trp–photoproduction absorption may contribute to the transient signal (22).

Photoexcitation at 315 nm. Fig. 1 shows TA spectra, at selected pump–probe time delays, obtained upon 315-nm excitation of deoxy-Mb (more TA spectra are shown in SI Appendix, Fig. S5). Two negative features appear at ∼430 nm and ∼550 nm that are due to ground state bleach (GSB) of the Soret and Q bands, respectively (SI Appendix, Fig. S1). Positive features due to ESA are observed at 450 nm and 600 nm, respectively (46, 52), which shift to the blue within the first 10–15 ps, while becoming weaker. The apparent shift of the GSB features results from the dynamics of the overlapping ESA contributions. Two mechanisms were proposed to explain the heme photocycle, namely the system undergoes vibrational relaxation (46, 52) or relaxes by cascading through spin states (56). However, our purpose here is not to discuss these mechanisms as they occur in the first few picoseconds or so and do not influence the *Trp kinetics we are investigating.

The timescales related to the relaxation of deoxy-Mb were retrieved by both a singular value decomposition (SVD) analysis and a global fit (GF). The fit function, used to recover the involved timescales, is a sum of exponential decays convoluted with the instrumental response function (IRF) (∼300 fs), assumed to be Gaussian. The timescales, obtained by a GF of the kinetic traces (SI Appendix, Fig. S3), are 280 ± 60 fs, 1.6 ± 0.2 ps, and 4.0 ± 0.4 ps, in agreement with the literature (49, 52, 56). The large error for the 280-fs contribution results from its proximity to the duration of the IRF. In Fig. 1B we show the decay-associated spectra (DAS) obtained from the SVD analysis. The DASs are due to the amplitudes of the exponential decay functions used to best fit the data points, allowing us to define whether a certain feature is decaying or rising. A DAS is related to a particular timescale and it can be read by comparing it with the transient spectrum at the corresponding time delay. If the amplitude of the DAS has the same sign as the spectrum, the feature is decaying (e.g., a positive DAS in the GSB region), whereas if the DAS has opposite sign with respect to the spectrum, the spectral feature is rising (e.g., a negative DAS in the spectral region corresponding to an ESA feature).

As mentioned above, the interpretation of the mechanism related to the heme relaxation is still a subject of debate (46, 52, 56–58). Our aim here is not to discuss these mechanisms. Important is that the longest timescale in the heme photocycle is ∼4 ps, which is much shorter than the Trp decay times (∼20 ps and ∼120 ps).

Photoexcitation at 290 nm. Fig. 2A shows TA spectra at selected time delays, obtained upon 290-nm excitation (more TA spectra are shown in SI Appendix, Fig. S6). They display GSB features at ∼430 nm and 550 nm due to the Soret and Q bands, respectively. The latter is overlapped with a very broad unstructured positive contribution that we assign to ESA of the photoexcited Trp residues (22). Additionally the two ESA features of the heme (∼450 nm and ∼600 nm, corresponding to the Soret band and the Q band, respectively) are observed for small pump–probe delays. For time delays <15 ps, the TA spectra exhibit the same behavior as upon 315-nm excitation, namely the ESA features shift to the blue and decrease in intensity while the GSB decreases in intensity. However, at longer pump–probe delays the TA signals show a persistent GSB feature at ∼430 nm and two ESA features, at ∼460 nm and at ∼610 nm, respectively (Fig. 3). The TA spectra maintain the same shape from delay times of ∼40 ps up to 1 ns, except for a small reduction in intensity (over the entire probe range) in the first 100 ps due to the disappearance of the *Trp and *Trp ESA. Fig. 3 compares the TA spectra at 900 ps upon 315-nm (Fig. 3A) and 290-nm (Fig. 3B) excitation. The shape and the amplitude of these transients differ significantly, suggesting the formation of a long-lived (LL) photoproduction for 290-nm excitation. As this photoproduction spectrum displays clear features in the Soret- and Q-band region, it indicates a modification...
of the heme group. Further, it cannot result from a *Trp–heme
FRET process, because the heme photocycle is very short. The
*Trp FRET rate would be the rate-limiting step and no LL pho-
toprocess would be observed.

An SVD analysis and a GF (SI Appendix, Fig. S3) were per-
formed to determine the kinetics of the spectral evolution. The
fits, using six exponential components, yielded time constants of
230 ± 60 fs, 1.5 ± 0.2 ps, 4.4 ± 0.4 ps, 18 ± 2 ps, and 106 ± 12 ps
and a long component (set to 5 ns) that accounts for the LL
signal. All time constants (except for the 5 ns) were free pa-
rameters of the fit and the results are in excellent agreement with
the decay times for the heme obtained upon 315-nm excitation
(see above) and with the literature values for the *Trp decay
times (35, 37).

Fig. 2B shows the DASs obtained for the *Trp7 and *Trp14
decay times (106 ps and 18 ps, respectively). Additionally the
DAS corresponding to the LL photoproduct is shown (SI Ap-
pendix, Fig. S4 presents the full set of DASs). The DASs assigned
or *Trp and *Trp14 differs significantly, indicating dif-
ferent relaxation pathways. The former contains a decay of
the *Trp ESA as well as a response of the heme observed on the
same timescale, because FRET is the rate-limiting step. The
18-ps DAS (Trp14) is almost a mirror image of the LL DAS.
More precisely, around 430 nm the positive feature in the Trp14
DAS mirrors the negative feature present in the LL DAS, al-
though it is somewhat narrower. Furthermore, the two DASs
mirror each other in the entire range from 460 nm to 730 nm,
bearing in mind an overall small positive offset in the Trp14
DAS. This strongly suggests that *Trp14 decay feeds the LL pho-
toprocess population. The spectral response to excitation of
the two Trp residues is likely similar, except for the rise of the
photoproduct spectrum that occurs only upon excitation of the
Trp14 residue. Thus, it should be possible to reproduce the Trp14
DAS with a linear combination (LC) of the Trp14 DAS, which
represents the response from Trp excitation, and the inverted LL
spectrum representing the rise of the photoproduct. This is shown
in Fig. 2C, where we compare the linear combination −LL DAS +
Trp7 DAS with the Trp14 DAS and find excellent agreement
demonstrating that indeed, the LL state grows out of relaxation of
the *Trp14 residue.

As mentioned above, the LL photoprocess must be related to
a change of the heme group and is not due to a *Trp-to-heme
FRET. Because a phototriggered Trp14-to-heme electron trans-
ferral was already reported for ferric Mbs (40) and because the
*Trp decay times are almost invariant for all Mbs (SI Appendix,
Table S1), this suggests that a photoinduced Trp†-to-heme
electron transfer also occurs in the ferrous deoxy-Mb. The
resulting low-valent heme could be either an FeI heme or an FeII-
porphyrin π-anion radical [FeI(porph unharn)-] complex, if the
additional electron resides on the porphyrin ring (59–63).

Several studies were performed, with a wide variety of tech-
niques, on low-valent iron complexes, both as FeII–porphyrin and
FeIII(porph unharn)- (42, 53, 54, 60–64). However, a large part of
these studies focuses on tetraphenyl-porphyrins (TPP) and octaethyl-
porphyrins (OEP) in organic solvents (61, 64). It was concluded
that formation of FeII-porph or FeIII(porph unharn)- depends sen-
sitively on the relative energy of the iron dπ eg orbitals and the
porphyrin eπ orbitals (SI Appendix, Scheme S1) (62). One way to
experimentally affect the relative energies of these orbitals is
substitution of the hydrogens in the porphyrin meso positions
(e.g., in TPP and OEP) (62). If electron-withdrawing substituents
are introduced in the ring, the energy of the eπ orbitals will decrease,
making the π-anion radicals more likely (62). On the other hand,
if electron-donor groups are present in the ring, the energy of
the eπ orbitals will become higher, leading to FeII complexes (62, 63,
65). In the case of the FeIII(porph unharn)- species, absorption spectra
display a broad band centered at ~700 nm and ~450 nm, and the
Q and Soret bands disappear (62).

The LL photoproduct absorption spectrum (Fig. 3C) is obtained
by subtracting the GSB contribution to the transient signal at 900 ps (Fig. 3B). It is comparable to the absorption spectrum of the reduced FeII-(NO2-OEP), which generates a porphyrin π-anion radical (62). It is comparable to the LL photoproduct absorption spectrum (Fig. 3C) is obtained by subtracting the GSB contribution to the transient signal at 900 ps (Fig. 3B). It is comparable to the absorption spectrum of the reduced FeII-(NO2-OEP), which generates a porphyrin π-anion radical (62). However, in mind that this com-
parison is qualitative as the porphyrin, the solvent, and the en-
vironment differ. In Fig. 3C, the Soret band and the Q band are
nearly vanished and new bands arise around 450 nm and 600 nm.
This comparison leads us to conclude that the anion radical
FeII(porph unharn)- is formed.

This is further supported by cryo-radioisotopes experiments (41,
42). EPR/ENDOR studies of Mb at ~70 K show that upon γ-ray
irradiation, a mixture of FeI-Mb and FeII(porph unharn)-Mb is gen-
erated, in a 9:1 ratio (42). The authors suggested that different
conformations in the frozen protein complexes might explain the
simultaneous observation of both species. Annealing experi-
ments hint at the possibility that the decay of FeII species could
involve intramolecular electron transfer, leading to the formation of

![Fig. 2. (A) Transient absorption spectra, at selected pump-probe delays, of deoxy-Mb upon 290-nm photoexcitation. (B) DASs obtained by SVD analysis. (C) Comparison of the Trp14 DAS with the linear combination DAS LC = −LL DAS + DAS Trp7. The regions above 500 nm are multiplied by 3.](image-url)
FeI\(^+(\text{porph}^\bullet)\) (42). The latter results suggest that the \(c_\beta\) and \(d_{\alpha-\gamma}\) orbitals are close in energy, leading to the Fe\(^+(\text{porph}^\bullet)\) when the system has the possibility to relax. Low-valent heme species, their nature, and relevance under physiological conditions were also investigated in theoretical studies (43–45), which also suggest formation of an FeI\(^+(\text{porph}^\bullet)\).

The photoproducts of *Trp\(^-\)to-heme electron transfer can be Trp\(^+\) and/or FeI\(^+(\text{porph}^\bullet)\) or *FeI\(^+(\text{porph}^\bullet)\). Because the transient spectra at delay times >40 ps do not display any changes (except for a small vertical offset due to *Trp\(^-\) and *Trp\(^+\)ESA), it is safe to assume that the FeI\(^+(\text{porph}^\bullet)\) product is generated. In the opposite case [generation of *FeI\(^+(\text{porph}^\bullet)\)] different spectral features should have been present in the transient spectra, together with their evolution. Further, if Trp\(^+\) is generated, an ESA feature at 560 nm [absorption band of Trp\(^+\) (66)] should arise with the *Trp\(^+\) decay time; if instead *FeI\(^+(\text{porph}^\bullet)\) is generated, some transient features should appear somewhere in the probing region (note that no information is available on *Trp\(^+\) absorption bands, but an ESA feature should at least show up in the probed region). However, despite this fact, the transient spectra do not display any ESA feature around 560 nm, suggesting that the molar extinction coefficient of the generated Trp\(^+\) (or *FeI\(^+(\text{porph}^\bullet)\)) is too small to detect the produced species. This is in line with the results on MbCN and met-Mb, in which the Trp radical cation was not detected either in its ground or in its excited state (40). Of course, if the signals of the products in their excited state fall outside the region of our probe and/or they decay to their ground state on a timescale that is too fast to be measurable with our setup, these considerations are no longer valid.

To estimate the quantum yield (QY) for electron transfer, the deoxy-Mb static spectrum (SI Appendix, Fig. S1) has been rescaled to the GSB amplitude of the LL transient spectrum at 900 ps, allowing a rough estimate of the proportion of Mb\(\text{s}\) with a *Trp14-to-heme electron transfer reaction in ferrous Mb\(\text{s}\). Hemoglobin (Hb) has six Trp residues: one in each α-subunit (α14) and two in each β-subunit (β15 and β37). Quite remarkably, their fluorescence lifetimes (68, 69) are comparable to the Mb values in SI Appendix, Table S1. As a matter of fact, in deoxy-, oxy-, and carboxy-Hb, the Trp residues are at typical distances of 13–18 Å from a heme porphyrin, which is comparable to the Trp\(^+\)-heme distance of 15.1 Å in Mb. We predict that a Trp-to-heme electron transfer also occurs in hemoglobins.

An extreme case of Trp fluorescence quenching in heme proteins was found for ferrous and ferric cytochrome c (Cyt\(c\)) with, respectively, decay times of 350 fs and 770 fs (57, 58). Trp is at van der Waals distances of the porphyrin (3–5 Å; SI Appendix, Fig. S8) in Cyt\(c\), but the Trp quenching was clearly identified as being due to FRET, at least to 85%. In this case the FRET may well be mediated by an exchange (Dexter) mechanism, in which the donor loses an electron from its excited state that is donated back to its ground state by the acceptor. In the light of these and the present results, it seems that electron transfer can compete with FRET only when the latter is less efficient (due to distance and orientation of the donor/acceptor dipoles) and/or when residues between the Trp and the porphyrin are present that can mediate it. In any case, more studies are needed to fully understand the competition between FRET and electron transfer in hemoproteins.

Conclusions
Femtosecond UV-visible transient absorption experiments were performed on deoxy-Mb for excitation wavelengths near 300 nm. They reveal the formation of a long-lived photoprocess, which results from a *Trp\(^+\)-to-heme electron transfer with a quantum yield of ~30%. This species is an FeI\(^+(\text{porph}^\bullet)\)-anion radical that has a lifetime exceeding our measurement window of 1 ns. To our knowledge this is the first observation of such a low-valent heme complex under physiological conditions, although their existence and biological importance as intermediates in the production pathway of active species in cytochrome P450 (43) as well as for CO\(_2\) reduction (44) were discussed earlier.

The similarity to our previous results on ferric Mb\(\text{s}\) (40) and the invariance of the *Trp lifetimes for all myoglobin species suggests that the *Trp\(^+\)-to-heme electron transfer is likely operative in the ligated ferrous Mbs.

We propose a single-step tunneling pathway for the electron transfer that involves the Leu\(^\bullet\) and Val\(^\bullet\) residues that lower the tunneling energy. Finally, as previously stressed (40, 70), care is advised when using *Trp fluorescence as a spectroscopic ruler, assuming that its fluorescence decay is due to FRET. This is surely an important tool in studies of protein dynamics but more often than previously thought, parallel electron transfer pathways may also contribute to its quenching.

Associated Content
Sample preparation, optical setup details, power dependence, data analysis details, and extra figures are available in SI Appendix.

ACKNOWLEDGMENTS. We thank Dr. Cristina Consani for useful discussions. This work was supported by the Swiss National Science Foundation via the National Centre for Competence in Research: Molecular Ultrafast Science and Technology.


Monni et al.


**Supplementary information**

Tryptophan-to-haem electron transfer in ferrous myoglobins

Roberto Monni, Andre Al Haddad, Frank van Mourik, Gerald Auböck and Majed Chergui*

Laboratoire de Spectroscopie Ultrarapide, École Polytechnique Fédérale de Lausanne, FSB- ISIC, CH-1015 Lausanne, Switzerland

**SI. Material and Methods**

**SI.1 Sample preparation**

The following doses were used to prepare 100 mL of 0.1 M phosphate buffer: 30 mL of a sodium hydroxide (NaOH) 0.1 M aqueous solution were added to 50 mL of a potassium orthophosphate (KH₂PO₄) 0.1 M aqueous solution, and the mixture was then diluted to 100 mL with 18 mQ distilled water. The KH₂PO₄ solution was prepared by dissolving 0.68 g of the latter in 50 mL of 18 mQ distilled water, while the NaOH solution was obtained by dissolving 0.20 g of NaOH in 50 mL of the same type of distilled water. The pH was controlled by a pH-meter and adjusted, if necessary, with the rest of NaOH solution or by addition of a dilute Hydrochloric acid (HCl) solution (0.05 M). The buffer solution used for the experiments was further diluted to a concentration of 10 mM.

Lyophilized horse heart met-Mb and sodium dithionite (Na₂S₂O₄), were purchased from Sigma Aldrich and used as delivered without any further purification. The deoxy-Mb was prepared under inert gas environment, by flowing Argon (Ar) in a portable glove box, to avoid both oxidation of the reagents and consequent formation of the oxy-Mb complex. The met-Mb was flowed with Ar in the glove box for ~30 min and then dissolved in a degased phosphate buffer solution (10 mM) at pH 7. A 2-fold excess of sodium dithionite, with respect to the equimolar ratio, was added to the met-Mb solution to obtain the deoxy-Mb. Figure S1 shows the static absorption spectrum of the deoxy-Mb of a solution of ~0.3 mM in a 0.2 mm-thick cell. This was enough to obtain an optical density of ~0.15 at the excitation wavelength (315 nm or 290 nm). The deoxy-Mb solution was handled and held in an air-tight container.
with an inlet to introduce a small overpressure of Ar gas during both transportation and the entire measuring time, so as to avoid possible oxygen leaks. The deoxy-Mb’s static spectrum (Fig. S1) was checked before and after each experiment to control integrity of the sample.

SI.2 Optical setup

The experiments were performed on a 2D-UV transient absorption setup, which has been described elsewhere.(2, 3) Briefly, ~0.4 mJ of the output of a 20 kHz, cryogenically-cooled, amplifier (Wyvern, KMLabs, 780 nm, 50 fs, ~0.6 mJ) are injected into a Non-collinear Optical Parametric Amplifier (NOPA) (Topas White, Light Conversion) to provide pulses in the visible range between 560 nm and 650 nm. The typical output energy is ~15 µJ and ~60% of it is used to generate narrow-band pump pulses. The visible pulse is focused on a 0.5 mm-thick BBO crystal for frequency doubling, in order to generate UV pump pulses (290 nm and 315 nm) with typical band-width of ~2 nm and energies of ~150 nJ. The latter is chopped at half the laser repetition rate and focused onto the sample with typical spot-size of 110 µm x 110 µm. In our experiments the pump pulse-length was ~200 fs Full Width at Half Maximum (FWHM), leading to an instrument response function (IRF) of ~300 fs FWHM.

The visible probe pulses were generated by focusing ~0.5% (~1 µJ) of the remaining output of the amplifier (~0.2 mJ) onto a 5 mm-thick CaF₂ window, in order to generate a white light super-continuum. The probe region used in our experiments spans from 390 nm to 730 nm, covering the main absorption features of the haem (Soret- and Q-band).(4, 5) After focusing

![Figure S1: Static spectra of deoxy-Mb 0.27 mM (solid yellow line), Trp in water 0.54 mM (solid blue line) and difference between deoxy-Mb and Trp spectra (dotted red).](image)
onto the sample (30 µm x 30 µm spot-size) the probe is coupled into a 100 µm multi-mode fiber and sent to a spectrometer to be spectrally resolved and detected on a single shot basis by means of a fast CMOS array detector.

SI.3 Power dependence

Figure S2 shows the power dependence of the signal upon 315 nm (Panels A and B) and 290 nm excitation (Panel C). Each point in Figure S2 was obtained by integration of the absolute transient signal in the region between 387 and 580 nm, in order to have a better signal to noise ratio. However, the integration of the absolute value of the signal leads to a positive offset due to integration of the noise of the measurement. The latter does not influence the results of the power dependence since it only adds a rigid shift of each point.

The points were fit using a power law given by

\[ y = y_0 + A x^z \]  

(S1)

Where \( y_0 \) indicates the vertical offset, \( A \) is the amplitude of the power law and \( z \) is the power of \( x \), which is indicated as “pow” in the fit results shown in the insets of Fig. S2.

The power dependence of deoxy-Mb upon 315 nm photo-excitation was measured at two different time-delays, namely 1.5 ps and 600 ps, and the results are reported in Figure S2A and B, respectively. As shown in Figure S2A, at 1.5 ps delay-time the dependence is well described by a fit with \( z = 1.0 \pm 0.1 \). At 600 ps (Fig. S2B), the fit yields \( z = 2.5 \pm 0.4 \), implying that this signal is due to a two-photon absorption processes. It is important to note that this quadratic behavior is visible only at long time-delays where only a small signal from

![Figure S2](image)

Figure S2: (A) Power dependence of deoxy-Mb upon 315 nm excitation at a pump-probe delay of 1.5 ps. (B) Power dependence upon 315 nm excitation at a pump-probe delay of 600 ps. (C) Power dependence upon 290 nm excitation at 600 ps delay. In each panel the fit coefficients are given.
a long lived photoproduct remains. At short time delays the signal appears linear because the small two-photon signal is covered by a much larger signal resulting from the normal haem photo-cycle.

Figure S2C shows the power dependence at 600 ps pump-probe delay upon 290 nm excitation, in order to concentrate on the long-lived (LL) transient signal resulting from the Trp\textsuperscript{14} to haem electron transfer (see main article). The fit of the power dependence gave $z = 0.9 \pm 0.5$, meaning that the process is linear and not related to multi-photon absorption processes.

As a consequence, we performed the measurements at a fluence of ~0.8 mJ/cm\textsuperscript{2} at both excitation wavelengths, which is the best compromise between minimal multi-photon absorption and good signal-to-noise ratio.

**SII Results**

**SII.1 Trp fluorescence quenching times**

Table S1 shows the time constants of the Trp\textsuperscript{7} and Trp\textsuperscript{14} fluorescence for several Sperm Whale (SW) myoglobin complexes and for horse heart (HH) apo-myoglobin. All Mbs have two Trp residues at positions 7 and 14, at nearly similar distances from the haem.(6, 7) It can be seen that the decay times for the different Mbs do not change dramatically, but that for apo-myoglobin, the decay constant for both Trps are significantly longer and are actually close to those of Trp in water.(8)

<table>
<thead>
<tr>
<th>Myoglobin complex</th>
<th>Trp7</th>
<th>Trp14</th>
</tr>
</thead>
<tbody>
<tr>
<td>DeoxyMb</td>
<td>105(9)</td>
<td>18(9)</td>
</tr>
<tr>
<td>metMb</td>
<td>135(10)</td>
<td>16(10)</td>
</tr>
<tr>
<td></td>
<td>112.5(9)</td>
<td>21.5(9)</td>
</tr>
<tr>
<td>MbCO</td>
<td>132(10)</td>
<td>26(10)</td>
</tr>
<tr>
<td></td>
<td>125.4(9)</td>
<td>23.4(9)</td>
</tr>
<tr>
<td>MbCN</td>
<td>113.2(9)</td>
<td>28.5(9)</td>
</tr>
<tr>
<td>MbN\textsubscript{3}</td>
<td>109.4(9)</td>
<td>27(9)</td>
</tr>
<tr>
<td>MbO\textsubscript{2}</td>
<td>122(9)</td>
<td>24.4(9)</td>
</tr>
<tr>
<td>HH apoMb</td>
<td>2840(11)</td>
<td>2806(11)</td>
</tr>
</tbody>
</table>
SII.2 Data analysis

The white-light super-continuum probe pulses are generated with some chirp and have duration of \( \sim 300 \, \text{fs} \) FWHM. Frequency-resolved detection allows however to correct for a difference of time-zero for different probe frequencies and this does not limit the time-resolution of the experiment.

Global analysis of the time-zero corrected data-matrix \( M(\lambda, t)_{m \times n} \) was performed by a Singular Value Decomposition (SVD) technique. The latter is known to reduce the fit dimensionality and to act as a noise filter.\(^{(12-14)}\) The SVD procedure starts from the data matrix \( M(\lambda, t)_{m \times n} \) and it decomposes the latter in the product of three matrices, as shown in eq. 1:

\[
M(\lambda, t) = S(\lambda)_{m \times m} \times W_{m \times n} \times T(t)_{n \times n}^{T} \tag{S2}
\]

If the data matrix can be described by a discrete linear combination of terms and in the case of pure stochastic noise, the columns present in the \( S(\lambda)_{m \times m} \) and \( T(t)_{n \times n}^{T} \) matrices are known as singular spectra (also called “eigenspectra”) and singular kinetic vectors (also called “eigentraces”) respectively. \( W_{m \times n} \) is a matrix that has \( w_{ij} = w_{ji} = 0 \) while \( w_{ii} \neq 0 \). The latter entries are also called “singular values” and represent the contribution of each eigenspectrum (and the corresponding eigentrace) to the data matrix. In the case of noise-free data there are \( N \) non-vanishing singular values, while the presence of noise acts as a perturbation of the latter and the respective singular vectors.\(^{(12-14)}\)

A GF analysis of the relevant eigentraces allows retrieving the exponential time constants \( \tau \) with their respective uncertainties and amplitudes \( a_{ik} \). The fitting function used to fit the eigentraces is given by

\[
f(t) = H(t) \ast (G(t) \otimes \sum_{i} a_{i} \exp \left( -\frac{t}{\tau_{i}} \right)) \tag{S3}
\]

Where \( H(t) \) is the Heaviside step function, \( G(t) \) is a Gaussian function with a FWHM equal to the IRF of the measurement and the last part is a sum of exponential functions with a given amplitudes \( a_{i} \) and time constants \( \tau_{i} \).

The amplitudes can then be used to construct the Decay Associated Spectra (DAS), which give the spectral contribution associated to a given time-constant as:

\[
DAS_{k}(\lambda) = \sum_{i} a_{ik} w_{ii} U_{k}(\lambda) \tag{S4}
\]
where $U_k (\lambda)$ is the eigenspectrum related to the $k$-th eigentrace.

The data were analyzed both via GF of single kinetic traces and SVD of the entire data matrix. The results obtained with these methods agree with each other, within the experimental error.

Figure S3 shows the result of the GF of several selected kinetic traces, for 315 nm (A) and 290 nm excitation (B). The best-fit of the kinetic traces, obtained by photo-excitation of deoxy-Mb at 315 nm, gave time-scales of: $280 \pm 60$ fs, $1.6 \pm 0.2$ ps and $4.0 \pm 0.4$ ps. After $\sim10$ ps, the system has completely recovered, except for a very small component due to multi-photon absorption as discussed above.

When deoxy-Mb is excited at 290 nm, a sum of three exponentials is not sufficient. The minimum number of exponentials necessary to best-fit the selected kinetic traces is six, whose associated time-scales are: $230 \pm 60$ fs, $1.5 \pm 0.2$ ps, $4.4 \pm 0.4$ ps, $18 \pm 2$ ps, $106 \pm 10$ ps and 5 ns. The last time-constant was fixed and used to account for the unknown lifetime of the LL photo-product, which is longer than our measurement window of 1 ns. Moreover a careful analysis of the kinetic traces shows that, at $\sim20$ ps (Trp$^{14}$ relaxation), a small growth of the transient signal can be seen.

![Figure S3: Selected kinetic traces upon 315 nm excitation (A) and 290 nm excitation (B), along with the global fit traces (solid lines) and their residuals in the upper part.](image)
Similar results were obtained by an SVD analysis, which is presented in Figure S4. The most important DAS are shown in the main paper, here we report the entire set of DAS. As already mentioned in the paper, the 230 fs DAS is not fully reliable since this time-constant is very close to the IRF of the measurement (~300 fs). However it can be seen that a qualitative comparison between the 280 fs DAS (315 nm excitation) and 230 fs DAS (290 nm excitation) shows good agreement. Good agreement is also found for the ~1.6 ps DAS and the ~4 ps DAS’s of the two measurements (Figure S4).

To calculate the quantum yield for the photo-product generation we have taken into account an indirect estimation of the total number of molecules undergoing reduction (\( \text{Molec}_{\text{RED}} \)), the total number of excited molecules (\( \text{Molec}_{\text{EXC}} \)) and the excited Trp14 percentage (\( \text{Exc}_{\text{Trp14}} \)). With these three parameters we can obtain the quantum yield for the generation of the photo-reduced species with respect to the total number of excited molecules (\( \text{QY}_{\text{RED}} \)) and with respect to the Trp14 (\( \text{QY}_{\text{ET}} \)).

\[
\text{QY}_{\text{RED}} = \frac{\text{Molec}_{\text{RED}}}{\text{Molec}_{\text{EXC}}} \quad \text{QY}_{\text{ET}} = \frac{\text{QY}_{\text{RED}}}{\text{Exc}_{\text{Trp14}}} \quad \text{(S5)}
\]

The total number of excited molecules was obtained by summing up all the DAS’s and rescaling the absorption spectrum (in the same experimental condition of the measurement) to

---

**Figure S4**: Comparison of the deoxy-Mb DAS’s upon 315 nm excitation (A) and 290 nm excitation (B). The color code for the haem dynamics is the same for both the excitation wavelengths.
the Ground State Bleach (GSB) amplitude. A similar procedure was followed to account for the number of molecules undergoing photo-reduction: the spectrum was rescaled to the GSB amplitude of the transient signal at 900 ps. The scaling values obtained for the total number of excited molecules and the number of reduced molecules were found to be: Molec$^{\text{EXC}}_{\text{GSB}} = 0.0257$ and Molec$^{\text{RED}}_{\text{GSB}} = 0.00112$. To estimate the number of excited Trp$^{14}$ molecules we have compared the molar extinction coefficient of haem and Trp at 290 nm, finding that the haem accounts for ~70% of the total absorption and the two Trp account for ~30%. This implies that ~15% of the excited molecules are Trp$^{14}$, leading to Exc$^{\text{Trp14}}_{\text{GSB}} = 0.15$. Inserting the numbers in Eq. 5 we can obtain the quantum yield for the total reduction and for the electron transfer process, finding:

$$QY^{\text{RED}} = 4.4\% \quad QY^{\text{ET}} = 29\%.$$  

SII.3 Porphyrin radical anions

Scheme S1 was reproduced from the work of Yamaguchi and Morishima (1) and shows a qualitative representation of the relative energies of the porphyrin and metal orbitals for Fe$^{\text{II}}$-Octaethylporphyrin (OEP) and –Tetraphenylporphyrin (TPP). In their work, Yamaguchi and Morishima (1) reported the influence of the meso- and β-substitution of the porphyrin ring on the energy of its e$_8$ orbitals. The latter is then related to the energy of the d orbitals of the metals, leading to the definition of different types of products obtained upon reduction. They synthesized several Fe$^{\text{II}}$-OEP and -TPP derivatives and performed one and two electron reductions for each of them. The latter were characterized by UV-Vis, NMR and ESR spectroscopy, allowing them to gather information on the absorption spectrum related to each species together with the spin state and the molecular structure. With all this information, they could generalize their results, obtaining four categories: 1) four-coordinated Fe$^{\text{I}}$ low-spin porphyrin (Type I); 2) five-coordinated Fe$^{\text{I}}$ low-spin porphyrin (Type II); 3) four-coordinated Fe$^{\text{II}}$ low-spin π-anion radical porphyrin (Type III); 4) five-coordinated Fe$^{\text{II}}$ high-spin π-anion radical porphyrin (Type IV).

Scheme S1 shows the orbital energies for the Type II and Type IV, which are relevant to our case. Indeed, a Type I or Type III photo-product, in the Mb case, would imply the detachment of the haem from the protein backbone leading to deterioration of the protein itself.

In Scheme S1A, the e$_8$ orbitals of the porphyrin lie at higher energy than the metal d orbitals, such that one-electron reduction of [Fe$^{\text{II}}$ (CN-OEP)] generates [Fe$^{\text{I}}$ (CN-OEP)]. On the other
hand, Scheme S1B represents an electronic configuration in which the \( E_g \) orbitals lie at lower energies than the \( d_{x^2-y^2} \) orbital such that one-electron reduction of \([\text{Fe}^{II} (\text{NO}_2\text{-OEP})]\) generates \([\text{Fe}^{II} (\text{NO}_2\text{-OEP})]^*\). These two examples show that the modulation of the energy levels on the porphyrin ring, performed by the substituent, can lead to two completely different products. In their work, Yamaguchi and Morishima (1) underlined the importance of the relative energy between the porphyrin \( e_g \) orbitals and the metal \( d \) orbitals. Furthermore, their UV-Vis results can be applied to our results although the type of porphyrin and the solvent used are different (see main paper). As already mentioned in the main paper, in deoxy-Mb the energy difference of the \( e_g \) and \( d \) orbitals is very small, leading to the formation of the \( \text{Fe}^{II} \pi \)-anion radical porphyrin.

**Scheme S1**: Schematic representation of relative metal and porphyrin orbital energies for penta-coordinated \( \text{Fe}^{I} \) (A) and penta-coordinated \( \text{Fe}^{II} \) porphyrin \( \pi \)-anion radical (B). The Fe \( d \)-orbitals are in black while the \( e_g \) orbitals of the porphyrin ring are in red. Reproduced from ref. (1)
SII.4 Additional Figures

Here we present a larger set of TA spectra (Figures S5 and S6) than in the main text.

**Figure S5:** Transient absorption spectra of deoxy-Mb at selected time delays after 315 nm excitation.

**Figure S6:** Transient absorption spectra of deoxy-Mb at selected time delays after 290 nm excitation.
Figure S7 (A) shows the structure of the myoglobin (backbone in green) reported from the Protein Data Bank. Here the most important residues for the electron transfer pathway, namely Trp, Leucine (Leu), Valine (Val) and Histidine (His), and the Haem are highlighted. (B) zooms into the most interesting region of Mb, where the Trp$^{14}$ and the Haem are seen along with the two residues through which electron tunneling could occur, namely Leu$^{69}$ and Val$^{68}$.

Figure S7: (A) Structure of myoglobin. The backbone is in green, the haem is highlighted by the used of different colors for each atom (namely C, O, N and Fe) and the most important residues for the electron transfer are labeled (Trp in red, Val in blue, Leu in violet and His in yellow). (B) Zoom of the region most relevant for electron transfer showing the residues between Trp and Haem.

Figure S8: Protein structure of the Cytochrome c in its ferric (A) and ferrous (B) state, displaying distances between the indol-moiety and the haem in both cases. In blue is Nitrogen, in red Oxygen, in gray Carbon and in ochre the Fe.
Figure S8 shows the structures of ferric (A) and ferrous (B) Cytochrome c (Cyt c), with the relevant distances.(15, 16)

References