Two tryptophans are better than one in accelerating electron flow through a protein

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Supporting Information

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## Section S1: Crystal structures

**Table S1.** X-ray data collection, refinement statistics, and validation for Re-labeled azurins. Data for outermost shells are given in parentheses.

|                          | ReH126W124W122Cu\textsuperscript{II} | ReH126W124F122Cu\textsuperscript{II} | ReH126F124W122Cu\textsuperscript{II} |
|--------------------------|---------------------------------------|---------------------------------------|---------------------------------------|
| **X-ray data collection**| SSRL beamline 12-2                    | In-house rotating anode               | In-house rotating anode               |
| Wavelength (Å)           | 1.0000                               | 1.5418                                | 1.5418                                |
| Unit cell (Å)            | 53.072, 221.563, 89.996               | 97.91, 33.97, 77.26                   | 62.25, 62.26, 133.69                   |
| Space group              | C222\textsubscript{1}                 | C121                                  | P2\textsubscript{1},2,2\textsubscript{1} |
| Resolution range (Å)     | 35.00-1.85 (1.95-1.85)                | 38.4-1.9 (2.0-1.9)                    | 45.95-2.0 (2.06-2.01)                  |
| Unique reflections (#)   | 44003 (6369)                          | 19028 (2625)                          | 34806 (2493)                          |
| Multiplicity             | 3.3 (3.4)                             | 2.4 (2.5)                             | 2.8 (2.6)                             |
| Completeness (%)         | 96.2 (96.3)                           | 93.8 (88.8)                           | 97.6 (96.2)                           |
| <I/\sigma(I)>           | 8.3 (1.4)                             | 9.6 (5.1)                             | 10.5 (5.1)                            |
| R\textsubscript{pim}    | 0.048 (0.534)                         | 0.049 (0.121)                         | 0.055 (0.187)                         |
| Wilson B-value (Å\textsuperscript{2}) | 31.652                              | 27.61                                 | 21.54                                 |
| **Refinement statistics**|                                       |                                       |                                       |
| Resolution range (Å)     | 35.0-1.85                             | 21.0-1.9                              | 23.0-2.0                              |
| Reflections used (#)     | 43951                                 | 19026                                 | 34679                                 |
| Free R reflections (%)   | 5.0                                   | 5.2                                   | 5.0                                   |
| R/R\textsubscript{free} | 0.1687/0.1978                         | 0.2208/0.2446                         | 0.1452/0.1893                         |
| Rmsd bond length (Å)     | 0.012                                 | 0.008                                 | 0.008                                 |
| Rmsd bond angle (°)      | 1.42                                  | 1.31                                  | 1.23                                  |
| <B>                      | 35.3                                  | 37.6                                  | 27.0                                  |
| Ramachandran (%)         |                                       |                                       |                                       |
| No. of residues in Favored regions | 98.2                                 | 96.8                                  | 98.6                                  |
| Outlier regions          | 0                                     | 0                                     | 0                                     |
| PDB entry                | 6MJS                                  | 6MJT                                  | 6MJR                                  |
Figure S1. Structure of Re124W122CuII. From ref.¹ PDB: 2I70. Distances in Å.
Note the near-parallel orientation of the dmp and indole planes that contain an angle of 20.8°.¹

Figure S2. Packing of neighboring chains of Re126WWCuII (left) and Re126FWCuII (right) in the asymmetric unit. Note that the Re126WWCu interface (WW) provides intermolecular pathways for W122’→*Re, W122’↔W124, W122↔W124’, and Re(H124)(CO)₃(dmp**)→Cu(II)’ ET, which may operate together with the intramolecular process. The Re126FWCu interface (FW) enables W122’→*Re intermolecular ET, making this mutant potentially photoactive at high concentrations in solution.
Section S2. Estimating single-step tunneling rates

Hopping acceleration is calculated as a ratio between the measured rate of Cu\textsuperscript{II} formation and the estimated rate of a hypothetical single-step process. Hence, the actual value depends on the way the latter is estimated.

We have used an experimental rate constant value for a single-step Cu\textsuperscript{I}→\textsuperscript{*}Re(His83)(CO)\textsubscript{3}(phen)\textsuperscript{*} ET in Re-labeled WT azurin, $1.3\times10^6$ s\textsuperscript{-1} (phen = 1,10-phenanthroline).\textsuperscript{2} Assuming that the reaction occurs from a \textsuperscript{3}MLCT excited state formally described as \textsuperscript{*}Re\textsuperscript{II}(His126)(CO)\textsubscript{3}(phen\textsuperscript{**})\textsuperscript{+}, the ET distance was taken to be the crystallographically determined Cu-Re distance, 16.8 Å.\textsuperscript{2} The hypothetical single-step rates in \textbf{Re124WCu\textsuperscript{I}} and \textbf{Re126WWCu\textsuperscript{I}} were calculated as $k_{ss} = (1.3\times10^6)\exp(-\beta\Delta r)$, where $\beta = 1.1$ Å\textsuperscript{-1} and $\Delta r$ is the difference between the Cu-Re distances in the investigated hopping mutant and in Re(H83)(WT-azurin), as shown in Table S2a.

\textbf{Table S2a.} Estimating the single-step tunneling rates and acceleration factors in \textbf{Re124WCu\textsuperscript{I}} and \textbf{Re126WWCu\textsuperscript{I}} evaluated by comparison with Re(H83)-azurin.

|        | $r$(Re-Cu), Å | $\Delta r$, Å | $k_{ss}$, s\textsuperscript{-1} | $k_{Cu}$, s\textsuperscript{-1} | Acceleration |
|--------|----------------|----------------|---------------------|----------------|--------------|
| Re126WWCu\textsuperscript{I} | 22.9            | 22.9-16.8=6.1 | (631 $\mu$s)$^{-1}$ | (70 ns)$^{-1}$ | 9 000         |
| Re124WCu\textsuperscript{I} | 19.4            | 19.4-16.8=2.6 | (13.4 $\mu$s)$^{-1}$ | (40 ns)$^{-1}$ | 336          |

An alternative way to estimate ET acceleration in \textbf{Re126WWCu\textsuperscript{I}} relative to \textbf{Re124WCu\textsuperscript{I}} relies on the ratio between measured single-step rate constants in flash-quench generated Ru\textsuperscript{III}(H126)AzurinCu\textsuperscript{I} ($1.3\times10^2$ s\textsuperscript{-1}) and Ru\textsuperscript{III}(H124)AzurinCu\textsuperscript{I} ($2.2\times10^4$ s\textsuperscript{-1}), as shown in Table S2b. Adjusting these values for different Ru-Cu and Re-Cu distances provides estimates of single-step rate constants for \textbf{Re126WWCu\textsuperscript{I}} and \textbf{Re124WCu\textsuperscript{I}} and indicates that single-step ET in \textbf{Re126WWCu\textsuperscript{I}} should be 21-times slower than in \textbf{Re124WCu\textsuperscript{I}}. Experimentally, it is only ~1.5-times slower, demonstrating the larger ET acceleration in \textbf{Re126WWCu\textsuperscript{I}}. It follows that ET acceleration in \textbf{Re126WWCu\textsuperscript{I}} is 15-times larger than in \textbf{Re124WCu\textsuperscript{I}}. Given the approximations involved in these estimates, this result corresponds well with the factor of 32 obtained from Table S2a.

\textbf{Table S2b.} Estimating the single-step tunneling rates and acceleration factors in \textbf{Re124WCu\textsuperscript{I}} and \textbf{Re126WWCu\textsuperscript{I}}, evaluated by comparison with Ru(H124)- and Ru(H126)-azurins. Distances in Å. $k_{s}$ values from ref.\textsuperscript{3-4}

|        | $r$(Re-Cu) | $r$(Ru-Cu) | $\Delta r$ | $k_{ss}$, s\textsuperscript{-1} (Ru) | $k_{ss}$, s\textsuperscript{-1} (Re) | $k_{Cu}$, s\textsuperscript{-1} | Acceleration |
|--------|------------|------------|------------|-----------------------------------|-----------------------------------|----------------|--------------|
| Re126WWCu\textsuperscript{I} | 22.9       | 26.0       | 3.1        | 1.3$\times10^2$                   | 3.93$\times10^3$                  | (70 ns)$^{-1}$ | 3.6$\times10^1$ |
| Re124WCu\textsuperscript{I} | 19.4       | 20.6       | 1.2        | 2.2$\times10^4$                   | 8.24$\times10^4$                  | (40 ns)$^{-1}$ | 3.0$\times10^2$ |
The rate constant for ReH126(CO)3(dmp•–)→CuII back ET was estimated from the experimental value (3.1 μs)−1 of the analogous reaction in Re124W122CuI. Using Cu-Re and Cu-dmp distances (Table 1) produces values of 146 and 440 μs, respectively.

**Section S3. Kinetics fitting and modeling**

**Kinetics fitting.**

Luminescence decay and transient absorption kinetics were fit to multiexponential functions using the lsqnonlin function in Matlab (The Mathworks, Inc.). Luminescence decays from 5- and 50-ns streak camera timebases, and logarithmically spaced data from the 10-μs digitizer timebase of the nanosecond instrument, were spliced together to make single luminescence decay profiles spanning the time range from 50 ps to 10 μs. These data were fit to multiexponential functions by iterative reconvolution with an instrument response function. The data, fits, and residuals for Re126WWCuI, Re126WWCuII, Re126WFCuI, Re126FWCuI, and Re126WWZn2⁺ appear in Figures S3-S7. The fitting parameters are summarized in Table S3. Nanosecond transient absorption data recorded at 632.8 and 500 nm with Re126WWCuI were fit simultaneously to a 4-exponential function. Nanosecond transient absorption data recorded at 500 nm with Re126FWCuI, Re126WFCuI, and Re126WWZn2⁺ were fit to exponential functions. The data, fits, and residuals appear in Figures S8-S11. The fitting parameters are summarized in Table S4.

**Table S3. Amplitudes (Aᵢ) and time constants (τᵢ) from multiexponential fits to Re126-azurin luminescence decay kinetics.**

|          | A₁ (%) | τ₁ /ps | A₂ (%) | τ₂ /ns | A₃ (%) | τ₃ /ns | A₄ (%) | τ₄ /μs |
|----------|--------|--------|--------|--------|--------|--------|--------|-------|
| Re126WWCuI | 61±3   | 270±20 | 15±2   | 3.7±1  | 23±1   | 81±6   |
| Re126WWCuII| 71±1   | 290±10 | 9±1    | 4±2    | 20±2   | 79±7   |
| Re126WFCuI | 73±2   | 200±10 | 14±1   | 2.9±0.6| 13±1   | 71±8   |
| Re126FWCuI | 36±6   | 340±100| 14±6   | 3±2    | 5±4    | 120±100| 45±5  | 1.16±0.15|
| Re126WWZn2⁺| 45±2   | 430±40 | 18±2   | 9.5±3  | 36±2   | 100±10 |

**Table S4. Amplitudes (Aᵢ) and time constants (τᵢ) from multiexponential fits to Re126-azurin nanosecond transient absorption kinetics.**

|          | A₁       | τ₁       | A₂       | τ₂       | A₃       | τ₃       | A₄       | τ₄       |
|----------|----------|----------|----------|----------|----------|----------|----------|----------|
| Re126WWCuI | -0.011  | 68±3 ns  | 0.005    | 1.2±0.1 μs | 0.020    | 123±2 μs | 0.002    | 4.6±0.5 ms |
| TA 632.8 nm | 0.004    | 0.003    | 0.005    | 0.008    | 0.008    | 0.0008   | 0.0008   | 0.0008   |
| TA 500 nm  |          |          |          |          |          |          |          |          |
| Re126FWCuI | 0.005    | 1.1±0.15 μs | 0.001    | 20±10 μs | 0.0008   | 0.0008   | 0.0008   | 0.0008   |
| TA 500 nm  | 0.004    | 0.9±0.1 μs | 0.0007   | 0.0007   | 0.0007   | 0.0007   | 0.0007   | 0.0007   |
| Re126WWZn2⁺|          |          |          |          |          |          |          |          |
| TA 500 nm  | 0.008    | 125±30 ns | 0.001    | 4±3 μs   | 0.0005   | 0.0005   | 0.0005   | 0.0005   |
Figure S3. Luminescence decay kinetics of Re126WWCuI (B, red), iterative reconvolution fit to 3-exponential decay function (B, black), and residuals (calculated–observed) (A).
Figure S4. Luminescence decay kinetics of Re126WWCuII (B, red), iterative reconvolution fit to 3-exponential decay function (B, black), and residuals (calculated–observed) (A).
Figure S5. Luminescence decay kinetics of Re126WFCuI (B, red), iterative reconvolution fit to 3-exponential decay function (B, black), and residuals (calculated−observed) (A).
Figure S6. Luminescence decay kinetics of Re126FWCuI (B, red), iterative reconvolution fit to 4-exponential decay function (B, black), and residuals (calculated−observed) (A).
Figure S7. Luminescence decay kinetics of Re126WWZn2+ (B, red), iterative reconvolution fit to 3-exponential decay function (B, black), and residuals (calculated−observed) (A).
Figure S8. Nanosecond transient absorption kinetics of Re126WWCuI recorded at 632.8 nm (B, red) and 500 nm (B, green), simultaneous fit to 4-exponential decay function (B, black), and residuals (calculated–observed) (A).
Figure S9. Nanosecond transient absorption kinetics of Re126FW-CuI recorded at 500 nm (B, red), fit to 2-exponential decay function (B, black), and residuals (observed–calculated) (A).
Figure S10. Nanosecond transient absorption kinetics of Re126WFCu recorded at 500 nm (B, red), fit to single-exponential decay function (B, black), and residuals (calculated–observed) (A).
Figure S11. Nanosecond transient absorption kinetics of Re$_{126}$WWZn$^{2+}$ recorded at 500 nm (B, red), fit to 2-exponential decay function (B, black), and residuals (calculated−observed) (A).
Kinetics Modeling

A general mechanistic model for electron transport in Re126-azurins is outlined in Scheme S1. The limited number of experimental observations precludes unique determination of the 9 elementary rate constants indicated in the model.

The general solution to the five coupled first-order differential equations in Scheme S1 is a 4-exponential function. One of the empirical rate constants in the solution is equal to the elementary rate constant $k_9$. The remaining empirical rate constants are functions of $k_{1-8}$, given...
by the roots of a third-order polynomial. Some of the elementary rate constants can be
determined by comparisons to model compounds as outlined below.

1. The value of the rate constant $k_4$ was fixed equal to the $\text{Re126FWCu}^1$ luminescence
decay rate constant: $k_4^{-1} = 1.15$ µs.

2. Luminescence decay in $\text{Re126WFCu}^1$ corresponds to Scheme S1 with $k_{5-9} = 0$. This rate
law can be solved analytically and, using the experimental luminescence amplitudes and
rate constants, the following assignments can be made: $k_1^{-1} = 234$ ns; $K_1 = 5.7$; $k_3^{-1} = 61$
ns.

To model the kinetics in $\text{Re126WWCu}^1$, 3 elementary rate constants were fixed ($k_3^{-1} = 60$ ns,
$k_4^{-1} = 1.15$ µs, $k_9^{-1} = 120$ µs). The remaining 6 rate constants were varied systematically and used
to provide numerical solutions to the Scheme S1 differential equations with the initial condition
$[A]_0 = 1$; $[B]_0 = [C]_0 = [D]_0 = [E]_0 = 0$. The set of rate parameters used in solving the differential
equations was:

\[
\begin{align*}
    k_1^{-1} &= 200 - 750 \text{ ps} \\
    K_1 &= 1 - 5 \\
    k_5^{-1} &= 1 - 25 \text{ ns} \\
    K_2 &= 0.1 - 0.75 \\
    k_7^{-1} &= 100 - 750 \text{ ns} \\
    k_8^{-1} &= 40 - 100 \text{ ns}.
\end{align*}
\]

The observations used to test the solutions were the rate constants extracted from the
luminescence and transient absorption data, the relative amplitudes of the luminescence kinetics
phases, and the yield of Cu$^{II}$ formed in the hopping process. The relative Cu$^{II}$ yield was estimated
from the experiment shown in Figure 2B: Kinetics profiles of transient absorption of
\text{Re124W122Cu}^1 and $\text{Re126WWCu}^1$ at 633 nm were measured on the same day at virtually
identical experimental conditions and normalized for the absorbance at the 355 nm excitation
wavelength. The relative yield was determined as the ratio of the amplitude of the respective
decay kinetics components. Whereas the $\text{Re124W122Cu}^1$ 633 nm TA decays with single-
exponential ~3.4 µs kinetics (3.1 µs in ref.1), $\text{Re126WWCu}^1$ shows biexponential 1.2 and 123 µs
decay (Table 2) due to inter- and intramolecular reactions, respectively. Considering only the 123
µs decay, the Re124/Re126 relative yield was estimated as 1.7, without knowing the partial light
absorption that triggers intramolecular reactivity. The total relative yield (calculated from both
1.2 and 123 µs amplitudes) is 2.4. The actual value corresponding to the intramolecular process
lies in the 1.7-2.4 range.
Systematic variation of the six rate parameters required solution of $4.8 \times 10^7$ systems of differential equations. The following criteria were employed to screen for acceptable solutions:

- Cu$^{II}$ yield: 2.0 – 2.2
- $\tau_1$: 260 – 280 ps
- $\tau_2$: 3.5 – 4 ns
- $\tau_3$: 70 – 90 ns
- $\tau_4$: 120 $\mu$s

The time constants $\tau_i$ are reciprocals for the four empirical rate constants in the solution to the differential equations. The resulting elementary rate constants are listed in Table 3 of the main text.

**Summary of mechanistic aspects:**

(i) The CT $\Leftrightarrow$ CS1 equilibrium and the W124$\rightarrow$*Re forward ET rate constants in Re126WWCu$^I$ are close to those estimated$^1$ for Re124W122Cu$^I$ (2.8, 500 ps), despite different dmp/indole orientations.

(ii) W124 and W122 are distinct hopping intermediates, as opposed to a single highly delocalized {W124,W122} unit. (For the latter possibility, the simulations predicted too high Cu$^{II}$ relative yields, unless the recombination rate constant ($k_3 = k_7$) was unrealistically large.)

(iii) The CS1 $\Leftrightarrow$ CS2 equilibrium is shifted to the left ($K_2 \approx 0.55 – 0.75$), i.e., the hole-localization on W124 proximal to Re is thermodynamically preferred.

(iv) The W122$\rightarrow$W124** (CS1 $\rightarrow$ CS2, $k_3$) and Re'(H126)(CO)$_3$(dmp**) $\rightarrow$ W124** (CS2 $\rightarrow$ GS) steps are much faster and slower, respectively, than Cu$^I$$\rightarrow$W124** ET (CS2$\rightarrow$RP, $k_8^{-1}$ $\approx$ 60-90 ns). Hence, they do not affect the luminescence decay and Cu$^{II}$ formation kinetics and their rate constants cannot be determined from the present experiments.

(v) Comparison with parameters estimated for Re126WWCu$^II$ ($k_1^{-1}$ = 375-425 ps$^{-1}$, $K_1 = 2.25 - 3.25$, $k_3^{-1}$ = 60 ns, $k_4^{-1}$ = 1.15 $\mu$s, $k_5^{-1}$ = 9-21 ns, $K_2 = 0.25 - 0.75$, $k_7^{-1}$ = 30 ns) indicate that the kinetics and thermodynamics of the $^3$CT $\Leftrightarrow$ CS1 $\Leftrightarrow$ CS2 redox processes are nearly independent of the Cu oxidation state (and, presumably, also of replacing Cu$^{II}$ by Zn$^{II}$).

(vi) Parameters estimated for Re126WFCu$^I$ ($k_1^{-1}$ = 234 ps, $K_1 = 5.7$, $k_3^{-1}$ = 61 ns) show that initial $^3$CT $\Leftrightarrow$ CS1 equilibrium (i.e., W124$\rightarrow$*Re ET) is only little affected by the 122 residue.
Section S4. QM/MM dynamics simulations

Figure S12. Re126WWCu\textsuperscript{i} used QM/MM MD simulations of complexes. The QM (DFT/PBE0-D3) part is shown in a licorice representation, the rest of the protein is treated by MM. Water molecules included in the MM part are omitted for simplicity.
Figure S13. Character and dynamics of the $^3$CT excited state of Re126WWCu. A: Time-dependent spin density distribution of the lowest triplet state ($^3$CT) from UKS QM/MM/MD simulation of the complete system, starting from the optimized $^3$CT geometry. B: UKS-calculated spin density distribution at 0.3 ps where the lowest triplet is predominantly MLCT. C: UKS-calculated spin density distribution at 1.2 ps, where the lowest triplet is predominantly IL. Spin density distributions in B and C were calculated on the QM part of the system solvated with nearby 40 water molecules and embedded in bulk water described as a polarizable continuum.
Figure S14. Distances between redox cofactors in solvated Re126WWWCu4 along UKS/MD trajectories of 3CT, CS1, and CS2 states.
Figure S15. Radial water distribution function around the W124 (left) and W122 (right) indole-NH hydrogen atoms in various electronic states of Re126WWCu1. Solvation tightening is manifested by shifting the strong peak around 2 Å to shorter distances and increasing its height. NH-O distance distribution for individual water molecules is shown in Figure 4. It follows that oxidation of either tryptophan is accompanied by H-bonding and tighter solvation. In particular, W124 solvation is about the same in the ground- and 3CT states but tightens upon oxidation to W124** in the CS1 state where the NH---OH2 distance shortens by about 0.1 Å. Restoring the W124 neutral charge on going to CS2 results in a shift of solvating water toward W122**. This shift is accompanied by the emergence of a second solvation layer between 3.0-3.5 Å around W124. Opposite behavior was found for W122, whose solvation is similar in the ground-, 3CT and CS1 states but becomes tighter in CS2, where the NH group is strongly bound to a single water molecule.
Section S5. Materials and methods

Protein expression and labeling. Site-directed mutagenesis K122W/T124W/T126H, K122F/T124W/T126H, and K122W/T124F/T126H of plasmid *Pseudomonas aeruginosa* Az All-Phe H83Q/Y72F/Y108F/W48F (All-Phe refers to replacement of all native W or Y with F) was carried out using a QuickChange kit (Stratagene). Primers were purchased from Operon. Mutant azurins were expressed in BL21(DE3) *E. coli* cells, purified, and labeled with the Re complex according to a literature procedure:5 [Re(H2O)(CO)3(dmp)](OTf) and protein were incubated in HEPES buffer at 37°C for 4-7 days in a 1.5:1.0 ratio before being exchanged into NaPi buffer. Labeled protein was isolated from unlabeled protein using an IMAC copper-chelating column, followed by purification on a Mono S cation exchange column. Final products were characterized by electrospray ionization mass spectrometry and UV/vis absorption spectroscopy.

X-ray crystallography. Crystals were obtained by sitting-drop vapor diffusion. For Re126WWCuII, 8 mg/mL in solution of 40 mM imidazole buffer at pH 7.2 with 2 mM NaCl were mixed with well solution (100 mM imidazole, 100 mM LiNO3, 6.25 mM CuCl2, 28% PEG 4000) in a 1:1 ratio (vol/vol). For Re126WFCuII and Re126FWCuII, 14.5 mg/mL in solution of 20 mM NaPi buffer at pH 7.6 with 1 M NaCl were mixed with well solutions (0.1 M NaOAc, 120 mM/150 mM Li2SO4, and 48.6% PEG 400/27.7% PEG 8000 at pH 4.5) in 1:1 ratios (vol/vol) respectively. Crystals were harvested after 4d-2wk and cryogenically stored. X-ray diffraction data were collected on the Stanford Synchrotron Radiation Laboratory (SSRL) Beamline 12-2 for Re126WWCuII and on an in-house instrument (Rigaku Micromax-007 HF & R-axis IV++) for the other two mutants. Diffraction data were processed with XDS/iMosflm/Scala,6-7 while the structures were solved using Phaser, Coot, and Phenix.8-10 Statistics for data collection and refinement are shown in Table S1. Atomic coordinates and structure factors were deposited in the Protein Data Bank under entries: 6MJR, 6MJS, 6MJT

Time-resolved luminescence and TA spectroscopy. Laser experiments were conducted at the Beckman Institute Laser Resource Center at Caltech. Samples (15-40 µM) were reduced using either sodium dithionite or ascorbic acid and desalted using PD-10 columns into 25 mM NaPi buffer at pH 7.2. After deoxygenation by pump-backfill cycles with argon, samples were excited by the third-harmonic of either a Q-switched Nd:YAG laser (Spectra Physics, Quanta-Ray PRO-Series, 8 ns pulse width) or a regeneratively amplified passively mode-locked Nd:YAG laser (Spectra Physics Vanguard 2000 and Continuum Regenerative Amplifier RGA60, 10 ps pulse width) for ns/µs and ps/νs-timescale measurements, respectively. For TA measurements, probe light was provided by either a current-pulsed Xe arc lamp (500/630 nm) or a HeNe laser (632.8 nm). For TA and luminescence experiments on the ns/µs-timescale, light was detected by a photomultiplier tube. For faster timescale measurements, a streak camera (Hamamatsu C5680) was used in photon-counting mode. Time-resolved IR experiments were performed at the Lasers for Science Facility, STFC Rutherford Appleton Laboratory (UK) using the ULTRA instrument.11 TRIR spectra in the ps and ns range were measured using 400 (~50 fs fwhm) and 355 nm (~0.7 ps fwhm) excitation pulses, respectively, and probed with difference-frequency generated ~400 cm⁻¹ broad IR probe pulses, recorded on two 128-pixel detectors with ~2 cm⁻¹ spectral resolution. Samples were held in CaF₂ cells (Hellma) with a 50 µm path length.
**Computational details.** Molecular dynamics (MD) simulations of lowest triplet states of Re126WWCu were performed at quantum mechanical/molecular mechanical (QM/MM) level in Terachem 1.912-13 – Amber 1414 framework. Simultaneous calculations of several low-lying triplet states utilized time-dependent DFT (TDDFT) with a closed shell reference state. Individual triplet states were calculated by an unrestricted Kohn-Sham (KS) procedure (UKS).15 Snapshot calculations at individual points of MD trajectories were performed using Terachem 1.9 or Gaussian 1616 (G16) program packages. Calculations of the QM part utilized LAN2DZ quasirelativistic effective core pseudopotentials and a corresponding optimized set of basis functions for Re17 and 6-31g(d) polarized double-ζ basis sets18 for remaining atoms. DFT employed the PBE0 hybrid functional,19-20 together with an empirical dispersion correction D3.21 Test calculations with long-range functionals CAM-B3LYP and wB97XD led to unrealistically large CT-CS energy separations.

The initial modeled complex structure containing a single protein chain with the Re-complex was based on the crystal structure (PDB ID 6MJS). The QM region contained residues 122-126 (W-G-W-L-H) and the Re-complex; the rest of the system is the MM region. The QM region was terminated by CH3 groups that were linked with the MM part through H atoms. This system was solvated by 3342 SPC/E22 water molecules, resulting in minimum 6 Å water shell; 2 Na+ cations23 were added to compensate the charge of the protein chain. We noted that QM/MM does not support periodic-boundary-conditions (PBC) and therefore a harmonic potential for all water molecules was employed to provide a spherical water “cap” around the protein. The whole solvated system was simulated as a large cluster in a gas phase.24 Electrostatics were accounted for by a very large Coulomb cut-off. The solvent continuum within G16 was described by a polarizable continuum model (PCM).25

In the preparatory steps and equilibration of the MM part of the system, strict position restraints were employed on all atoms in the QM region, and the system was treated with PBC; particle mesh Ewald accounted for long range electrostatics.24,26 Minimization of the MM part was performed in two steps. First, all protein atoms were restrained and only solvent was minimized, next, only protein heavy atoms were restrained and solvent molecules and hydrogen atoms were minimized. With the position restraints on heavy atoms, the system was gradually heated up to 300 K during 200 ps in the NVT simulation (at a density 0.75 kg/dm^3); and finally the water was equilibrated. The density reached ~1.04 kg/dm^3 during 500 ps of the NpT simulation at ambient conditions, employing a 2 fs time step. The SHAKE algorithm27 was employed to all bonds that involve hydrogen atoms. The final system, with equilibrated solvent and relaxed protein MM part, was again minimized, with weak restraints applied only on atoms of the QM region. During the all-MM equilibration period, CO and dmp groups were parameterized using the recommended AMBER approach.28 The RESP charges were calculated in G16 and obtained by the antechamber tool in Amber. The Cu atom was modeled on an MM level. To account for the Cu-coordination in the active site, bond-length constraints to all ligands with optimal QM-distances according the literature were employed.29

QM/MM started by minimization, in order to optimize the geometry of the QM-region. QM/MM-dynamic simulations were performed with a 1 fs time step (SHAKE algorithm was used.) During 1 ps equilibration, the system was heated from 50 to 300 K. This step was performed...
separately for KS-singlet and UKS-triplet states and the final geometry served as initial geometry and velocities for the production phase. Production runs were performed at 300 K employing a simulation time up to 10 ps for each of the KS and UKS simulations, while it was 1-4 ps for more computationally demanding TD-DFT simulations.

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