Supplementary Information

EXPERIMENTAL METHODS

Protein Expression and Purification. Mutations were introduced in the cyt cb_{562} plasmid[1] by site-directed mutagenesis using a QuikChange kit (Stratagene) with oligonucleotide primers purchased from Eurofins MWG Operon. LB medium with 100 μg/mL ampicillin was inoculated with colonies, and the cells were expressed at 37 °C for 14-16 h. Plasmids were then isolated using a Qiagen Miniprep kit, and the mutations were confirmed by DNA sequencing (Laragen, Inc.).

The cyt cb_{562} plasmid was cotransformed with a cytochrome *c* maturation gene cassette pEC86 in BL21 Star (DE3) One Shot *E. coli* (Invitrogen), as previously described.[1] Transformants were grown overnight at 37 °C on LB agar plates containing 100 μg/mL ampicillin and 34 μg/mL chloramiphenicol. Single colonies were selected to inoculate LB medium with the same concentration of antibiotics and shaken for 6-8 h. Glycerol stocks of the cultures were prepared by flash freezing a 3:1 mixture of culture to 80% glycerol using liquid nitrogen and stored at −80 °C. The remaining test cultures were incubated for an additional 10-12 h, and then the cells were harvested by centrifugation. Bright red colonies were indicative of cyt cb_{562} overexpression; the corresponding glycerol stocks were selected for subsequent protein expressions.

Proteins were expressed by *E. coli* in LB medium containing 100 μg/mL ampicillin and 34 μg/mL chloramphenicol. Small cultures (30 mL) were inoculated with glycerol stocks, shaken for 8 hours at 37 °C, and distributed into large cultures. After expressing for 16 h at 37°C with rotary shaking, cells were harvested and stored at −80 °C. Protein was extracted from cells by sonicating for 5 s pulses at 50% duty cycle for 30 min in Tris-HCl buffer (pH 8.0) containing 2

mM ethylenediaminetetraacetic acid, 13 mM dithiothreitol (DTT), 1 mg deoxyribonuclease I, 6 mM sodium deoxycholate, and 7 mM phenylmethanesulfonyl fluoride, predissolved in isopropyl alcohol with dimethyl sulfoxide (Sigma).

Following centrifugation, the supernatant containing the crude protein was purified on a Q-Sepharose Fast Flow column (GE Healthcare) in 10 mM Tris-HCl (pH 8.0) with 1 mM DTT and a typical gradient of 0 to 110-150 mM NaCl. Cyt cb_{562} was subsequently purified by fast-protein liquid chromatography on a Pharmacia AKTA Purifier system. Disulfide bonds were reduced with 5-20 mM DTT, and the buffer was exchanged with 15 mM sodium acetate buffer (pH 4.5) on a HiPrep 26/10 desalting column (GE Healthcare). Purified protein was eluted with a sodium chloride gradient on a Mono-S 10/10 GL cation-exchange column (GE Healthcare). Samples were concentrated using an Amicon YM10 membrane or an Amicon Ultra 3 kDa unit (Millipore).

RESULTS AND DISCUSSION

Calculation of Diffusion Coefficients. We calculate the diffusion coefficients of Ru-variants of cyt cb_{562} using equations developed by Hagen, Hofrichter, and Eaton[2] based on an approximate solution to the Smoluchowski equation by Szabo, Schulten, and Schulten (SSS).[3] The bimolecular equilibrium constant for the encounter complex is estimated as $K^{bi} \approx (4/3)\pi a^3$, where a is the reaction radius, estimated as 4 Å. With assumptions described in reference 3, the unimolecular equilibrium constant K^{uni} relates to K^{bi} with the ratio $K^{bi}/K^{uni} \approx (2\pi\langle r^2\rangle/3)^{3/2}$, where $\langle r^2\rangle$ is the mean-squared end-to-end distance. SSS theory[3] predicts that the encounter complex associates at a rate $k_{D+}{}^{uni} \approx 3DK^{uni}/a^2$, where D is the intrachain diffusion coefficient. We solved for D through rearrangement of the equations describing the equilibrium properties and the dynamics of the polypeptide chain: $D \approx k_{D+}{}^{uni} (2\pi\langle r^2\rangle/3)^{3/2} / 4\pi a$.

The mean-squared end-to-end distance predicted by polymer theory is $\langle r^2 \rangle = C_n n^{2\nu} l^2$, where C_n is Flory's characteristic ratio, n is the number of residues in the polypeptide chain, ν is equal to ½ in θ-solvents where chain-solvent interactions compensate for intrachain interactions,[2] l = 3.8 Å is the distance between C_α atoms on the chain, and a value of 8 was used for C_n.

REFERENCES

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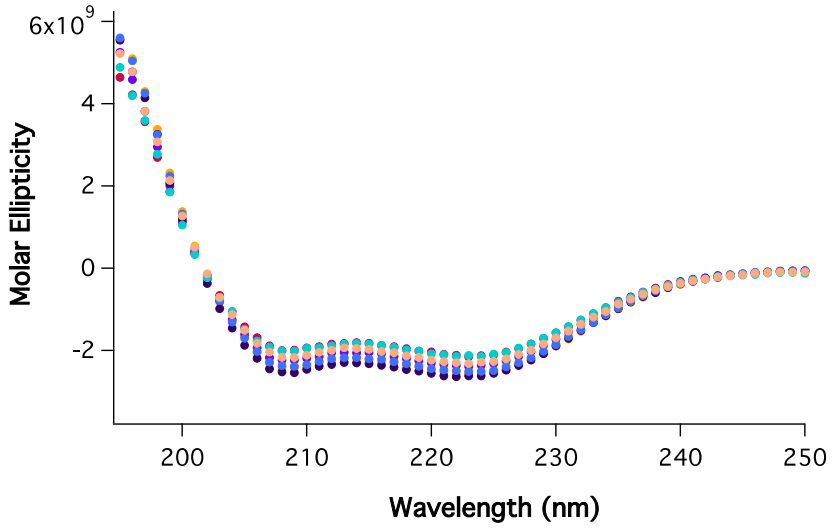
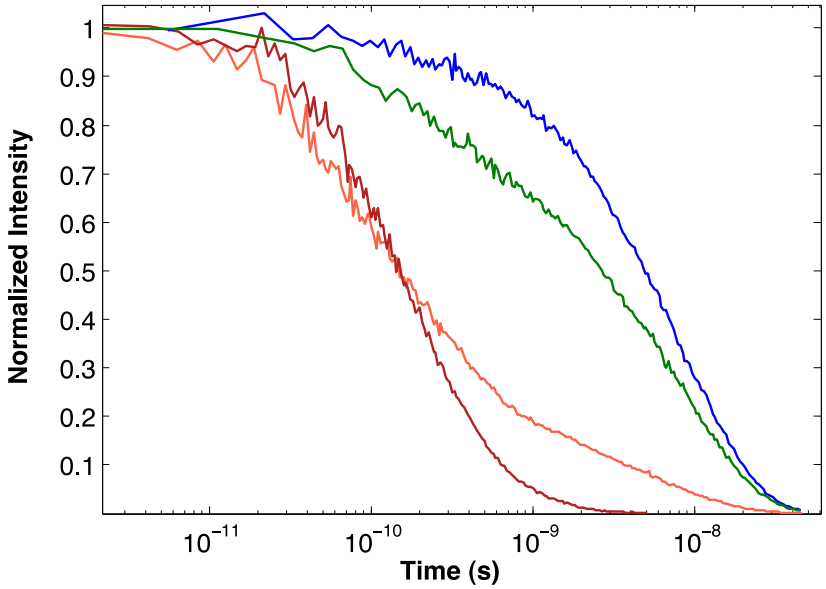
(2) Hagen, S. J.; Hofrichter, J.; Eaton, W. A. Rate of Intrachain Diffusion of Unfolded Cytochrome c. *J. Phys. Chem. B* **1997**, *101*, 2352–2365.

(3) Szabo, A.; Schulten, K.; Schulten, Z. First Passage Time Approach to Diffusion Controlled Reactions. *J. Chem. Phys.* **1980**, *72*, 4350–4357.



SI Figure 1. CD spectra of Ru-cyt cb_{562} variants at pH 7: Ru19 (yellow), Ru32 (red), Ru51 (purple), Ru66 (deep purple), Ru77 (blue), Ru83 (teal), and Ru92 (coral).



SI Figure 2. Fluorescence decays of Dns66-cyt cb_{562}: folded at pH 4 (red) and unfolded in 6 M Gdn at pH 4 (blue), pH 5 (green), and pH 7 (orange).

SI Table 1. Fitted rate constants for luminescence data collected at pH 4 in 6 M Gdn and the corresponding electron transfer rate constants.

Variant	Distance (*n*)	k_{obs} (s^{-1})	k_{ET} (s^{-1})
Free label	N/A	$1.09(\pm0.03) \times 10^6$	N/A
Ru19	79	$1.06(\pm0.02) \times 10^6$	Not observed[1]
Ru32	66	$1.14(\pm0.04) \times 10^6$	Not observed[1]
Ru51	47	$1.53(\pm0.03) \times 10^6$	$4.4(\pm0.4) \times 10^5$
Ru66	32	$1.75(\pm0.04) \times 10^6$	$6.6(\pm0.5) \times 10^5$
Ru77	21	$2.46(\pm0.07) \times 10^6$	$1.37(\pm0.07) \times 10^6$
Ru83	15	$3.1(\pm0.2) \times 10^6$	$2.0(\pm0.2) \times 10^6$
Ru92	6	$9.4(\pm0.6) \times 10^6$	$8.3(\pm0.6) \times 10^6$

[1]Electron transfer is slow and not competitive with excited state deactivation of the Ru-label.