Chapter 2

Methods

2.1 General

Buffers were prepared with MilliQ water and filtered (0.22 μm). Chemicals were purchased from Sigma unless stated otherwise. Guanidine hydrochloride (Gdn, Sigma Ultra) concentration was determined by refractive index measurements [50].

2.2 Protein Preparation

2.2.1 Cytochrome cb_{562} Expression and Purification

Mutations were introduced in the cyt cb_{562} plasmid [37] by site-directed mutagenesis using a QuikChange kit (Stratagene) with oligonucleotide primers purchased from Eurofins MWG Operon; e.g., the K83C forward primer was GCTGGCA AATGAAGGT**TGC**GTAAAAGAAGCGC. After the polymerase chain reaction, reactions were digested with the restriction enzyme DpnI to remove

parental methylated DNA. The mutant DNA was amplified by transformation into *E. coli* XL1-blue cells (Stratagene) and expressed overnight at 37°C on LB agar plates containing 100 μg/mL ampicillin. LB medium with 100 μg/mL ampicillin or carbenicillin was inoculated with colonies, and the cells were expressed at 37°C with shaking 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 [37]. 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 (3035 mL) were inoculated with glycerol stocks, shaken for 8 hours at 37°C, and distributed into large cultures (12 L). 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. Yield was higher when cells were lysed within a few days of expression.

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 NaCl gradient on a Mono-S 10/10 GL cation-exchange column (GE Healthcare). Depending on the mutant, the protein was eluted with a final concentration of 130 to 150 mM NaCl. When necessary, the protein was further purified on a Mono-Q 10/10 GL anion-exchange column (GE Healthcare) at pH 8.0 in 10 mM Tris-hydrochloride buffer (pH 7) with a NaCl gradient. Samples were concentrated using an Amicon YM10 membrane (Millipore).

2.2.2 Cytochrome c_{552} Expression and Purification

Cyt cyt c_{552} variants were prepared by Seiji Yamada, a visiting scientist from Sony Corporation. For detailed methods, Supporting Information is free on the

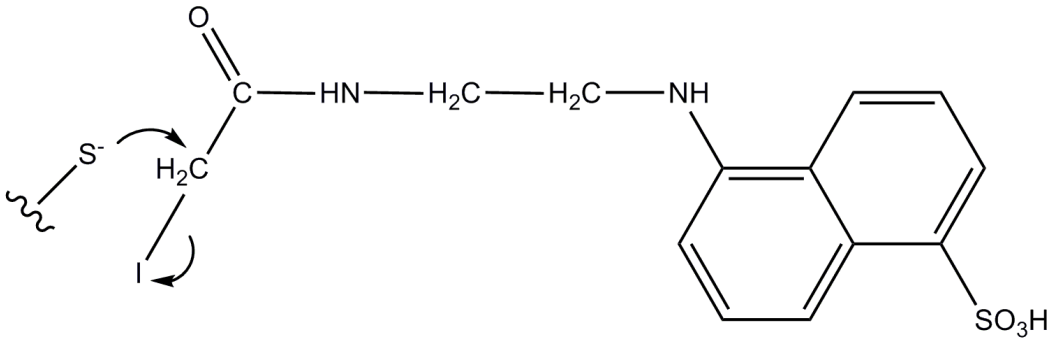

Figure 2.1: Covalent labeling of cysteine residues with dansyl.

web: www.pnas.org/ content/110/5/1606/suppl/DCSupplemental [51].

2.2.3 Covalent Labeling with Photosensitizers

Photosensitizers were covalently attached to cysteine thiols in labeling reactions (Figure 2.1). For trFRET measurements, single-cysteine variants were labeled with a dansyl group (1,5-IAEDANS, 5-((((2iodoacetyl)amino)ethyl)amino) naphthalene-1-sulfonic acid, Invitrogen). For electron transfer luminescence measurements, single-cysteine variants were labeled with $[Ru(bpy)_2(IA\text{-}phen)]^{2+}$ (IA-phen = 5-iodoacetamido-1,10-phenanthroline), synthesized by members of the Gray Group as described previously [31].

Disulfides bonds were reduced with 20 mM dithiothreitol (DTT) at pH 8; concentrated Tris-hydrochloride buffer used to adjust the pH as needed. The protein was exchanged into pH 8.0 buffer on a HiPrep 26/10 desalting column (GE Healthcare) or a PD-10 desalting column (GE Healthcare). Tris-hydrochloride buffer was used for most labeling reactions; however, many dansyl-labeling reactions were carried out in 6 M Gdn with 50 mM HEPES (pH 8.0). Molar excess

of the label (5–10x dansyl label, 2–5x Ru label) was dissolved in dimethyl sulfoxide and slowly added to the Ar-deoxygenated protein. The sample was stirred in the dark for 3–4 h at room temperature or overnight at 4°C.

The reaction was quenched with DTT, and the buffer was exchanged with 15 mM sodium acetate buffer (pH 4.5) using the HiPrep 26/10 desalting column. Labeled protein was separated from unlabeled protein on a Mono-S 10/10 GL column (GE Healthcare) equilibrated with 15 mM sodium acetate buffer (pH 4.5) or a Mono-Q 10/10 GL column (GE Healthcare) equilibrated with 10 mM Tris-hydrochloride buffer (pH 8.0) using a NaCl gradient. Samples were concentrated using an Amicon Ultra 3 kDa unit (Millipore). As the photosensitizers are light-sensitive, all labeled samples were kept in the dark.

2.3 Protein Characterization

Purity of the expressed and labeled protein variants was verified by SDS-PAGE on a Pharmacia PhastSystem (Amersham Biosciences). The molecular mass of the expressed protein was confirmed by electrospray ionization mass spectroscopy (Caltech Protein/Peptide Microanalytical Laboratory). Circular dichroism (CD) and fluorescence spectra were collected using an Aviv 62ADS spectropolarimeter (Aviv Associates, Lakewood, NJ) and a Fluorolog2 spectrofluorimeter (Jobin Yvon), respectively.

The purity, concentration, and heme environment of cyt cb_{562} and c_{552} samples were assessed by UV-visible spectroscopy using an Agilent 8453 diode array spec-

trophotometer (Agilent Technologies, Santa Clara, CA). The extinction coefficient for oxidized cyt cb_{562} is 0.148 $\mu M^{-1}cm^{-1}$ at the maximum Soret absorbance (415 nm) [37]; to confirm the purity of each sample, an absorbance ratio (418 nm: 280 nm) of five or greater was verified. The extinction coefficient for reduced cyt c_{552} is 21.1 $mM^{-1}cm^{-1}$ [52].

Though mutant positions were carefully selected at solvent-exposed positions, stability studies were performed to quantify perturbation by the cysteine mutation and the addition of a dansyl group. Denaturant unfolding curves of the proteins were obtained by fitting CD, absorbance, and fluorescence spectroscopy data to a two-state unfolding mechanism [22] using Igor Pro version 5.02 or 6.1 (Wavemetrics). Samples of 2 μM cyt cb_{562} were prepared in 50 mM sodium acetate (pH 5.0) with Gdn (0–6 M); the protein was allowed to equilibrate in denaturing conditions for an hour. The unfolding curves were generated from CD (222 nm), absorption (402 nm: 415 nm), and Trp59 fluorescence (355 nm excitation and integration of emission) data. Samples of 2-20 μM cyt c_{552} were measured in various concentrations of Gdn buffered with 100 mM sodium citrate (pH 3.0) for absorbance and fluorescence measurements and with 10 mM citrate buffer for CD measurements; the unfolding curves were generated from CD (222 nm), absorption (391 nm), and Dns fluorescence (355 and 513 nm for excitation and emission, respectively) data.

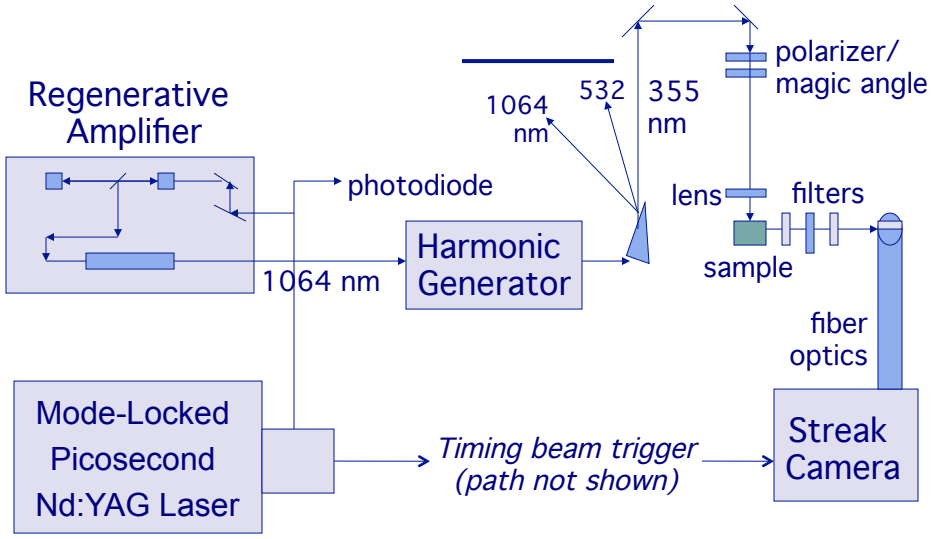

Figure 2.2: Schematic of the picosecond laser system.

2.4 Picosecond Laser System

For trFET measurements, samples with 1-2 μM protein were prepared with 0–8 M Gdn in buffer (cyt cb_{562}: 50 mM sodium acetate (pH 4–5) or 100 mM sodium phosphate buffer (pH 7); cyt c_{552}: 100 mM sodium citrate (pH 3.0)). Samples were deoxygenated by 30 pump-back-fill cycles with argon. Samples in denaturing conditions were allowed to equilibrate for at least an hour before data collection.

A schematic of the laser system is shown in Figure 2.2. Samples were excited at 355 nm with the third harmonic (10 ps, 0.5 mJ) from a regeneratively amplified (Continuum), mode-locked picosecond Nd:YAG laser (Vanguard 2000-HM532; Spectra-Physics). A long-pass cutoff filter (>430 nm) selected for dansyl fluorescence. Luminescence was collected using a picosecond streak camera (C5680; Hamamatsu Photonics) in the photon-counting and analog integration modes for equilibrium unfolding and refolding kinetics experiments, respectively.

The decay data were collected on short (5 ns) and long (20 or 50 ns) time scales. Single-shot streak camera traces (10,000 exposures for 5 ns and 5,000 exposures for 50 ns timescales) were averaged. Samples were stirred continuously. Data were collected at ambient temperature ($\sim$18°C).

The resulting short- and long-time scale data were spliced together; the point of maximum laser pulse intensity was defined as t=0. The combined traces were compressed logarithmically before fitting (70 points per decade); this compression does not alter the interpretation of data [29]. Fluorescence energy transfer analysis involves the numerical inversion of a Laplace transform [$I(t) = \sum_k P(k)\exp(-kt)$] [53] [54]. The trFET data were fit using MATLAB (Mathworks) with a Tikhonov regularization (TR) and a maximum-entropy (ME) fitting algorithm with regularization methods that impose additional constraints on the properties of $P(k) \geq 0$.

TR fitting minimizes the sum of the squares of the deviations between calculated and observed fluorescence intensities (χ^2), as well as the gradient of the $P(k)$ distribution ($\bigtriangledown P(k)$). ME fitting minimizes the sum of the squares of the deviations between calculated and observed fluorescence intensities (χ^2), while maximizing the entropy of the rate-constant distribution [$S = -\sum_k P(k)\ln[P(k)]$]. TR and ME fitting yield stable and reproducible numerical inversions of the kinetics data. The balance between minimization of χ^2 and $\bigtriangledown P(k)$ minimization or S maximization was determined by L-curve analysis [55].

The probability distributions of rate constant, $P(k)$, were converted to prob-

ability distributions of distance, P(r_{DA}), using the Förster equation [k = k_0 (1 + $(R_0/r_{DA})^6$)] [56]. The decay rate constant of the unquenched Dns fluorophore, k_0, is 1.0 x 10^8 s^{-1}, determined using Dns-modified cysteines. The Förster critical length, R_0, for the Dns-heme pair is 39 Å under both native and denatured conditions [21]. At distances longer than $1.5r_0$, energy transfer quenching of D is not competitive with excited-state decay; D-A distances cannot be obtained reliably, and different structures in the protein ensemble with $r \geq 59$ Å cannot be resolved.

2.5 Continuous Flow Mixer

For refolding kinetics experiments, Gdn-denatured protein was mixed with buffer (volume ratio of 1:5) in a T-shaped continuous flow mixer to initiate folding (Figure 1.6) [57]. In Chapter 5, denatured cyt cb_{562} in 6 M Gdn, 50 mM sodium phosphate (pH 4.0) was mixed with 50 mM sodium phosphate (pH 4.0). In Chapter 7, denatured cyt c_{552} in 6 M Gdn, 100 mM sodium citrate (pH 3.0) was mixed with 100 mM sodium citrate (pH 3.0). Final protein concentrations were 12-15 μM.

The mixing cell, based on a design by Fujisawa and coworkers [58], was given to the Gray Group by former group member Dr. Tetsunari Kimura. The device has a mixing dead time of $\sim$150 μs [57]. A 200-μm-thick stainless steel plate (Toray Precision, Shiga, Japan) was etched with two flow channels (35 μm width, 100 μm depth) and an observation channel (200 μm width and depth). The plate was placed between two synthetic fused silica windows and into a stainless steel holder. The layers were held in place by screw-tightened Teflon pieces.

The mixer was carefully assembled to avoid introduction of dust particles. Pieces were sonicated, rinsed with water, and dried with Kimwipes or pressurized air. The quartz can be further cleaned by soaking in dilute hydrochloric acid. Following assembly, the device was tested for leaking. Uninterrupted flow through the mixer was confirmed with a microscope. After use, the mixer and tubing were dried by injecting air. An online filter prevented particles from entering the mixer.

Two solutions were injected into the mixer by a syringe pump at a combined flow rate of 3 mL/min. The picosecond excitation beam (see section 2.4) was focused into a strip along the observation channel using a combination of spherical and cylindrical lenses. Luminescence from the mixing chamber was focused onto a fiber-optic bundle with optical fibers in a linear array, allowing for simultaneous data collection at twenty-five different points along the mixing chamber. At this flow rate (3 mL/min = 125 cm/s in mixer), the folding time resolution was 200 μs, calculated using a fiber-fiber distance of 250 μm. The streak camera was operated in the analog integration mode, and the background signal of buffer with the corresponding concentration of Gdn was collected and subtracted prior to analysis.

We performed control experiments by flowing protein in 1 M or 6 M Gdn through the mixer. We confirmed that the fluorescence decay for each fiber was consistent with that of the folded and unfolded proteins, respectively. Twenty fibers in the center provided the best signal.

The moments (M_n) of the P(r) distributions were calculated according to the equation [$M_n = \sum \mathrm{P}(r)r^n \,/\, \sum \mathrm{P}(r)$]. The variances ($V$), which reflect the breadth of the P(r) distributions, were calculated according to the equation [$V = M_2 - (M_1)^2$].

2.6 Nanosecond Laser System

For contact quenching studies (Chapter 4), luminescence decays were collected for Ru-labeled cyt cb_{562} (1.5–6 μM) denatured with 6 or 8 M Gdn in 50 mM sodium acetate buffer (pH 4–5) or 100 mM sodium phosphate buffer (pH 7). For bimolecular measurements, $Ru(bpy)_3Cl_2$ (1 μM) was combined with cyt cb_{562} (0–175 μM) in 6 M Gdn, 50 mM sodium acetate buffer (pH 4). For electron tunneling studies (Chapter 9), luminescence and transient absorption data were collected for Ru-labeled cyt cb_{562} (6 μM) in 100 mM sodium phosphate buffer (pH 7). Samples were deoxygenated by 30 pump-back-fill cycles with argon.

Luminescence and transient absorption data were collected with the setup illustrated in Figure 2.3. Samples were excited at 480 nm with 10 ns laser pulses from an optical parametric oscillator (Spectra-Physics Quanta-Ray MOPO-700), pumped by the third harmonic of a Q-switched Nd: YAG laser (Spectra-Physics Quanta-Ray PRO-Series) at a rate of 10 Hz. Transmitted light (luminescence or probe light) was detected by a photomultiplier, amplified, and digitized. The laser system was controlled by software written in LabVIEW (National Instruments).

For luminescence measurements, a long-pass cutoff filter (>600 nm) and a monochromator with 1 mm slits selected for Ru luminescence (630 nm). Lumi-

nescence decays comprised of three hundred averaged laser shots were collected on 2 μs or 10 μs timescales, as appropriate. Samples were stirred continuously.

For transient absorption measurements, probe light (75-W arc lamp, PTI model A 1010) was aligned with the excitation beam at the sample. Appropriate short-pass and long-pass filters and the monochromator selected for the wavelength. For measurements collected with the fast amplifier (<msec), the probe light was pulsed. Samples were stirred continuously, and six hundred traces were typically averaged. For measurements collected with the slow amplifier, samples were stirred between 20–40 cycles of one shot each.

Data were fit to exponential functions using the Curve Fitting Tool (cftool) in MATLAB version R2010b (Mathworks). The point of maximum laser pulse intensity was defined as t=0. Data in the first 10-15 ns that overlapped with the laser pulse were excluded from fits. At high protein concentrations, such as in the bimolecular experiments, a high intensity spike was observed in the luminescence at time zero with deviations in the signal for up to 30 ns; these data points were excluded from the fits. A fitting range of 3e3 to 3e8 was set for the rate constant.

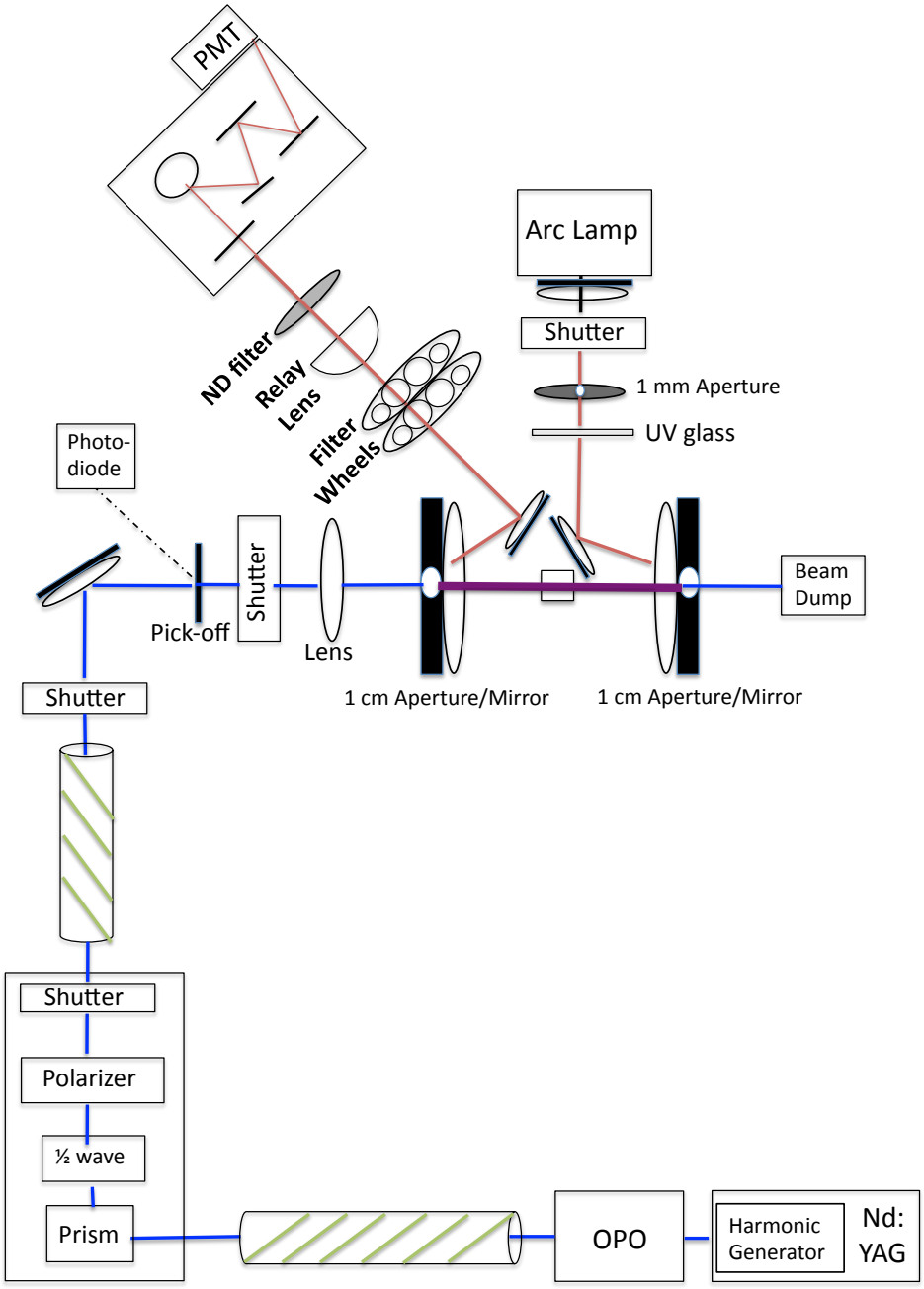

Figure 2.3: Schematic of the nanosecond laser system. The laser beam (blue) excites the sample. During transient absorption measurements, the difference in the absorbance of the probe light (red) is measured over time. While measuring the luminescence of the sample, the shutter for the probe light is closed. The wavelength is selected for using filters and a monochromator. Image courtesy of Gretchen Keller.