Supplementary Material (ESI) for Chemical Communications

A Small Fluorophore Reporter of Protein Conformation and Redox State

Graham J. Pound, Alexandre A. Pletnev, Xiaomin Fang, and Ekaterina V. Pletneva*

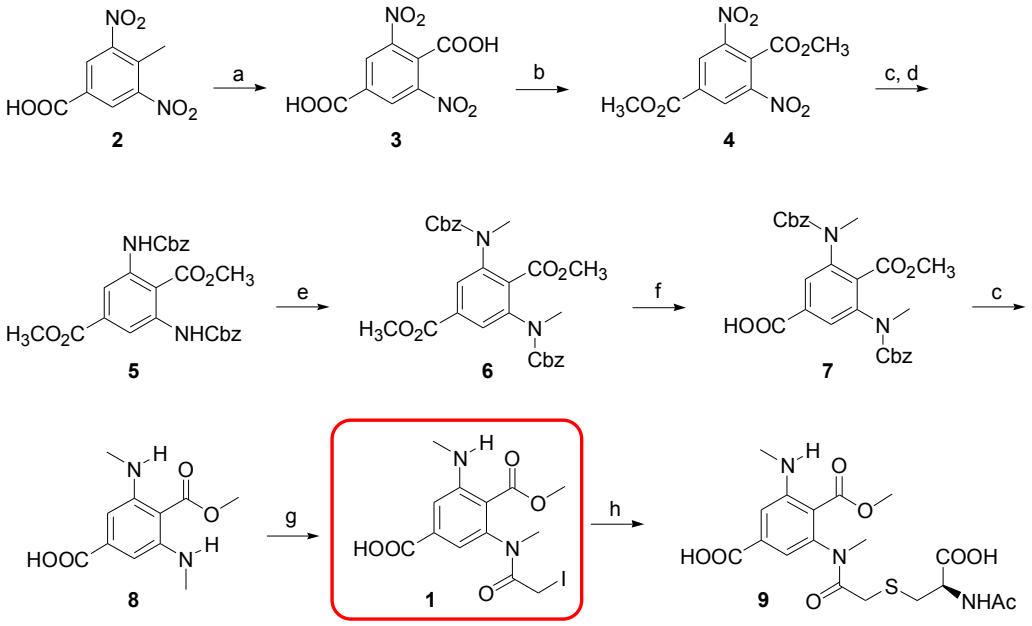
Department of Chemistry, Dartmouth College, Hanover, NH 03755, USA

Supplementary Information

Experimental Methods

Synthesis of Atpt Iodoacetamide

Scheme S1 outlines a synthetic route for **1**. Commercially available 3,5-dinitro-*p*-toluic acid was converted into 2,6-dinitroterephthalic acid, which was fully esterified and reduced to afford the corresponding diamine. The amino groups were then functionalized and the less hindered methyl ester was selectively hydrolyzed with lithium hydroxide. Removal of the two benzyloxycarbonyl protective groups furnished the target blue fluorescent 2,6-bis(methylamino)terephthalic acid monomethyl ester **8**. Reaction of **8** with 2-iodoacetyl chloride yielded the Atpt iodoacetamide **1**.



Reagents: (a) $Na_2Cr_2O_7$, H_2SO_4; (b) $(CH_3)_2SO_4$, K_2CO_3; (c) H_2, cat. Pd/C; (d) CbzCl, Zn; (e) CH_3I, NaH; (f) LiOH, H_2O-MeOH; (g) 2-iodoacetyl chloride, $NaHCO_3$; (h) *N*-acetyl-L-cysteine, Et_3N.

Scheme S1. Synthesis of Atpt Iodoacetamide

Synthetic Procedures

2,6-Dinitroterephthalic acid (**3**) was prepared according to a literature procedure.[1] A stirred solution of 3,5-dinitro-p-toluic acid (**2**, 10 g, 44.2 mmol) in conc. H_2SO_4 (70 mL) was cooled in an ice-water bath and sodium dichromate dihydrate (18.4 g, 61.7 mmol) was added in portions keeping the internal temperature below 25 ^oC. The reaction mixture was allowed to stir at rt overnight, poured onto ice (300 g) and the resulting mixture was extracted with EtOAc. The organic phase was dried (Na_2SO_4) and concentrated to afford 10.55 g (93%) of an off-white solid. M.p. 280 ^oC (decomp). ^1H NMR (dmso-d_6) δ 8.78 (s). ^{13}C NMR (dmso-d_6) δ 128.4, 130.0, 133.8, 146.6, 163.1, 163.6. IR (thin film) 1716, 1699, 1558, 1541, 1341 cm^{-1}. HRMS (ESI, *m/z*) calculated for $[M+23]^+$ ($C_8H_4N_2O_8Na$) 278.9865, found 278.9866.

Dimethyl 2,6-dinitroterephthalate (**4**).[1] Sodium hydrocarbonate (8.45 g, 100.58 mmol) was added to a solution of **3** (10.3 g, 40.23 mmol) in acetone (200 mL) followed by dimethyl sulfate (7.61 mL, 80.46 mmol). The resulting mixture was stirred at reflux overnight, allowed to cool to rt and filtered. The filtrate was poured into 350 mL of water and the resulting white precipitate was collected, washed with water and dried in air. Yield – 8.2 g (72%). An analytically pure sample was obtained after column chromatography (5:1 hexanes-EtOAc). M.p. 147-148 ^oC. ^1H NMR ($CDCl_3$) δ 4.07 (two s, 6H), 9.07 (s, 2H). ^{13}C NMR ($CDCl_3$) δ 54.0, 54.6, 128.7, 130.4, 133.6, 147.1, 162.4, 162.5. IR (thin film) 3097, 2961, 1740, 1551, 1345, 1276 cm^{-1}. HRMS (ESI, *m/z*) calculated for $[M+23]^+$ ($C_{10}H_8N_2O_8Na$) 307.0178, found 307.0183.

Dimethyl 2,6-bis(benzyloxycarbonylamino)terephthalate (**5**). Compound **4** (2 g, 7.04 mmol) was dissolved in EtOAc (50 mL) and hydrogenated using 10% Pd/C (0.3 g, 4 mol %) under 1 atm of hydrogen for 4.5 h. Filtered through Celite and concentrated to afford a yellow solid that was dissolved immediately in EtOAc (45 mL). This solution was added to a solution

of benzyl chloroformate (2.2 mL, 15.49 mmol) in EtOAc (20 mL) containing Zn dust (1.01 g, 15.49 mmol). The resulting suspension was stirred at rt for 2.5 h and filtered through a glass frit. The precipitate (containing the poorly soluble product **5**) was washed with EtOAc until no more UV-active material went into solution. The combined filtrate was washed with water, dried (Na_2SO_4) and concentrated to yield an amber oil. The oil was triturated with toluene to afford a beige precipitate that was removed by filtration. The filtrate was concentrated to afford 2.88 g (83%) of compound **5**. M.p. 120-121 ^oC. ^1H NMR ($CDCl_3$) δ 3.92 (s, 3H), 3.97 (s, 3H), 5.23 (s, 4H), 7.35-7.44 (m, 10H), 8.57 (s, 2H), 9.20 (br s, 2H). ^{13}C NMR ($CDCl_3$) δ 52.7, 53.4, 67.4, 115.9, 128.5, 128.6, 128.7, 134.7, 136.0, 140.1, 153.3, 165.9, 167.1. IR (thin film) 3403, 3323, 3034, 2954, 1741, 1697, 1582, 1264, 1199 cm^{-1}. HRMS (ESI, *m/z*) calculated for $[M+23]^+$ ($C_{26}H_{24}N_2O_8Na$) 515.1430, found 515.1434.

Dimethyl *N,N'*-dimethyl-2,6-bis(benzyloxycarbonylamino)terephthalate (**6**) was prepared according to a published procedure[2]. Sodium hydride (0.88 g of 60% suspension in oil, 22.07 mmol) was washed with hexanes and suspended in DMF (5 mL) at 0 ^oC under inert atmosphere. A solution of **5** (2.68 g, 5.45 mmol) in DMF (20 mL) was then added dropwise and the reaction mixture was stirred at 0 ^oC for 1 h. Methyl iodide (1.7 mL, 27.25 mmol) was then added and stirring continued for 4 h at rt. The reaction was quenched by addition of ice and the resulting mixture was extracted with EtOAc. The organic phase was washed with brine, dried (Na_2SO_4) and concentrated. The crude product was purified by column chromatography (2:1 hexanes-EtOAc) to yield 1.6 g (56%) of compound **6** as a colorless oil. ^1H NMR ($CDCl_3$) δ 3.27 and 3.33 (two br s, 6H), 3.57 (br s, 3H), 3.94 (s, 3H), 5.03 and 5.19 (two br s, 4H), 7.18-7.41 (br m, 10H), 7.85 (br s, 2H). ^{13}C NMR ($CDCl_3$) δ 38.4, 52.7, 52.8, 67.7, 128.0, 128.4, 128.7, 133.5, 136.3, 142.3, 143.2, 155.0, 165.1 (two signals missing due to overlap). IR (thin film) 3033, 2952,

1716, 1570, 1434, 1339, 1252, 1154 cm^{-1}. HRMS (ESI, *m/z*) calculated for $[M+1]^+$ ($C_{28}H_{29}N_2O_8$) 521.1924, found 521.1931.

***N,N'*-dimethyl-2,6-bis(benzyloxycarbonylamino)terephthalic acid 1-methyl ester** (**7**). Lithium hydroxide monohydrate (0.2 g, 4.73 mmol) was added to a solution of **6** (1.54 g, 2.96 mmol) in 4:1 methanol-water mixture (25 mL). The resulting solution was stirred at rt for 4 h, acidified with 2N HCl to pH 1, diluted with brine and extracted with EtOAc. The organic phase was dried (Na_2SO_4) and concentrated to afford 1.54 g (100%) of compound **7** as a colorless oil. ^1H NMR ($CDCl_3$) δ 3.28 and 3.34 (two br s, 6H), 3.59 (br s, 3H), 5.05 and 5.21 (two br s, 4H), 7.20-7.42 (br m, 10H), 7.92 (br s, 2H), 8.90 (br s, 1H). ^{13}C NMR ($CDCl_3$) δ 38.4, 52.8, 67.9, 128.0, 128.3, 128.5, 128.7, 129.2, 132.9, 136.2, 136.4, 142.4, 143.1, 155.1, 155.8, 165.2, 169.0. IR (thin film) 3065, 2952, 1717, 1433, 1342, 1265, 1157 cm^{-1}. HRMS (ESI, *m/z*) calculated for $[M+23]^+$ ($C_{27}H_{26}N_2O_8Na$) 529.1587, found 529.1584.

2,6-Bis(methylamino)terephthalic acid 1-methyl ester (**8**). 10% Pd/C (0.2 g, 0.19 mmol) was added to a solution of **7** (1.5 g, 2.96 mmol) in methanol (30 mL) and the reaction mixture was stirred under 1 atm of H_2 for 3.5 h at rt, filtered through Celite and concentrated to afford 0.67 g (95%) of compound **8** as a yellow solid. Decomp. above 175 ^oC without melting. ^1H NMR (acetone-d_6) δ 2.88 (s, 6H), 3.86 (s, 3H), 6.57 (s, 2H), 7.50 (br s, 1H). ^{13}C NMR (acetone-d_6) δ 51.1, 97.7, 98.7, 136.0, 153.1, 167.9, 169.5 (one signal missing due to overlap). IR (thin film) 3465, 3361, 1692, 1665, 1583, 1453, 1410, 1259, 1229, 1180, 1073 cm^{-1}. HRMS (ESI, *m/z*) calculated for $[M+1]^+$ ($C_{11}H_{15}N_2O_4$) 239.1032, found 239.1033.

2-(2-Iodoacetyl)-2,6-bis(methylamino)terephthalic acid 1-methyl ester (**Atpt-iodoacetamide, 1**) was prepared according to a literature procedure.[3] Compound **8** (0.2 g, 0.84 mmol) was dissolved in a solution of sodium bicarbonate (0.54 g, 6.42 mmol) in water (20 mL).

The resulting solution was cooled to 0 ^{o}C and treated with 2-iodoacetyl chloride (79 μL, 0.88 mmol). The reaction mixture was stirred in the dark for 1 h at 0 ^{o}C and for 1 h more at rt, acidified with 2N HCl to pH 2 and extracted with EtOAc. The organic phase was dried (Na_2SO_4), concentrated and the residue purified by column chromatography (EtOAc) to afford 87 mg (24%) of compound **1** as a yellow solid. Decomp. above 140 ^{o}C without melting. ^{1}H NMR ($CDCl_3$) δ 3.14 (s, 3H), 3.33 (s, 3H), 3.75-3.82 (m, 2H), 4.05 (s, 3H), 7.42 (s, 1H), 7.62 (s, 1H), 9.20 (br s, 1H). ^{13}C NMR ($CDCl_3$) δ -2.8, 30.4, 38.4, 53.3, 112.3, 113.5, 116.4, 134.6, 144.1, 152.4, 167.5, 168.6, 169.5. IR (thin film) 3373, 2921, 1717, 1698, 1685, 1616, 1572, 1419, 1243 cm^{-1}. HRMS (ESI, *m/z*) calculated for $[M+1]^+$ ($C_{13}H_{16}N_2O_5I$) 407.0104, found 407.0110. The solubility of **1** in water is 1.4±0.1 mmol/L, which is more than sufficient for high-efficiency labeling of proteins.

***N*-Acetyl-*S*-Atpt-cysteine** (**9**). A solution of triethylamine (15 μL, 108 μmol) and *N*-acetyl-L-cysteine (6 mg, 37 μmol) in dichloromethane (0.5 mL) was added to a solution of compound **1** (7 mg, 17 μmol) in dichloromethane (0.5 mL) at 0 ^{o}C. The resulting solution was stirred at rt in the dark for 3 h, concentrated *in vacuo*, dissolved in water (3 mL), acidified to pH 2 with conc. HCl and extracted with EtOAc. The organic extracts were concentrated and the product purified on the GE Healthcare Source 15RPC column HPLC (Buffer A: 0.1% TFA in water, Buffer B: 10% Buffer A(v/v) and 90% acetonitrile; 0-100 % gradient of buffer B, eluted at 20% B) to afford 5 mg of the target compound **9** as a yellow solid. HRMS (ESI, *m/z*) calculated for $[M+1]^+$ ($C_{18}H_{24}N_3O_8S$) 442.1284, found 442.1285.

Site-Directed Mutagenesis, Protein Expression and Purification

Plasmids for *iso*-1 yeast cyt *c* bacterial expression have been prepared in our previous work.[4] Expression and purification of K72A/C102S (WT*) and E66C/K72A/C102S cyt *c* were done as described.[4]

Mutations W253F and C85S/D121C/C138S/W253F were introduced in the pET3 vinculin D1 plasmid[5] using the QuickChange method. DNA sequencing at Dartmouth Molecular Biology Core confirmed the mutations. Vinculin plasmids were transformed into *E. coli* BL21 Star™ cells (Invitrogen) and the protein variants were expressed as described.[5] Harvested cells were broken by French Press and the solution was clarified from cell debris by centrifugation. Protein purification followed a published protocol[5] using GE Healthcare HisTrap and HiTrap Q prepacked columns connected to an Akta FPLC system.

Natalie T. Burkhard and Dr. D. M. Indika Bandara have prepared some of the E66C/K72A/C102S cyt *c* mutant for this work.

Atpt Labeling

The purified protein (300-500 μM) was pretreated with 5-10 mM DTT to break disulfide protein adducts. DTT was removed and the buffer exchanged to a 100 mM NaPi buffer pH 7.4 using an FPLC desalting column. The protein was then diluted to a concentration of 50-100 μM with a 100 mM NaPi buffer pH 7.4 buffer. A tenfold molar excess of Atpt-iodoacetamide **1** was dissolved in 0.2-0.4 mL of dimethyl sulfoxide (DMSO) and added dropwise in the dark to the stirring solution of the protein. The reaction proceeded for 5 hours, shielded from light. Upon completion of the reaction, an excess of DTT was added to consume non-reacted labeling reagent.

The buffer of the reaction mixture was exchanged by overnight dialysis to a 10 mM NaPi at pH 7.0 (cyt *c*) and 10 mM Tris at pH 8.0 or 10 mM NaPi at pH 7.7 (vinculin). The same buffer (Buffer A) was used to equilibrate an SP (cyt *c*) or Q (vinculin) column for purification of the labeled product. Before applying onto a column, cyt *c* was reoxidized with an excess of $K_3[Fe(CN)_6]$. The proteins were eluted with a shallow gradient from 0 M to 0.5 M NaCl in buffer A, a procedure that resulted in separation of labeled and unlabeled proteins (Figure S2). Formation of a monolabeled adduct was confirmed by ESI-MS done at the W.M. Keck Foundation Biotechnology Resource Laboratory at Yale University School of Medicine.

Spectroscopic Measurements

All the experiments were done at 21±1 °C. Absorption and CD spectra were recorded with an Agilent 8453 diode-array spectrophotometer and a Jasco J-715 spectropolarimeter, respectively. Fluorescence spectra were recorded with a Horiba Jobin Yvon Fluorolog-3 spectrofluorimeter.

Fluorescence lifetimes were measured by time-correlated single photon counting (TCSPC) using NanoLED-375L diode laser (λ_{ex}=375 nm, <70 ps pulsewidth) as the excitation source and a fast TBX-04 detector. The dye emission was observed at 460 nm. The measurements were done under magic angle conditions. The fluorescence decay traces were analyzed with the commercial DAS6 software from Horiba Jobin Yvon and previously described routines in MATLAB.[4, 6]

Quantum Yield Calculations

The quantum yield Φ of Atpt-Cys was measured using quinine sulfate (Aldrich) in 0.1 M H_2SO_4 (Φ=0.58)[7] as the reference *R* with the following equation:[8]

$$\Phi = \Phi_R \frac{A_R}{A} \frac{I}{I_R} \frac{n^2}{n_R^2} \qquad (1)$$

In eq 1, A is the absorbance at the excitation wavelength, I is the integrated emission, and n is the solution refractive index. Corrected emission spectra were collected with 375 nm excitation. Solution refractive indexes were determined with an AO Scientific Instrument ABBE Mark II digital refractometer.

Critical Distance R_0 Calculations

The critical distances, R_0, for the Atpt-heme and Trp-Atpt donor-acceptor pairs were calculated according to eq 2,[8] where the value of the orientation parameter κ^2 was taken as 2/3, Φ_D is the donor fluorescence quantum yield (0.13[8] for Trp and 0.28 for Atpt), n is the refractive index of the solution, F_D is the normalized fluorescence spectrum of the donor, and ε_A is the molar absorbance spectrum of the acceptor:

$$R_0^6 = 8.785 \times 10^{-5} \left(\frac{\kappa^2 \Phi_D}{n^4} \right) \int F_D(\lambda) \varepsilon_A(\lambda) \lambda^4 d\lambda \qquad (2)$$

The overlap integrals $J(\lambda) = \int F_D(\lambda) \varepsilon_A(\lambda) \lambda^4 d\lambda$ (Figure S4) were calculated using the *area* function in SigmaPlot 10.0 from Systat Software, Inc.

Unfolding Curves

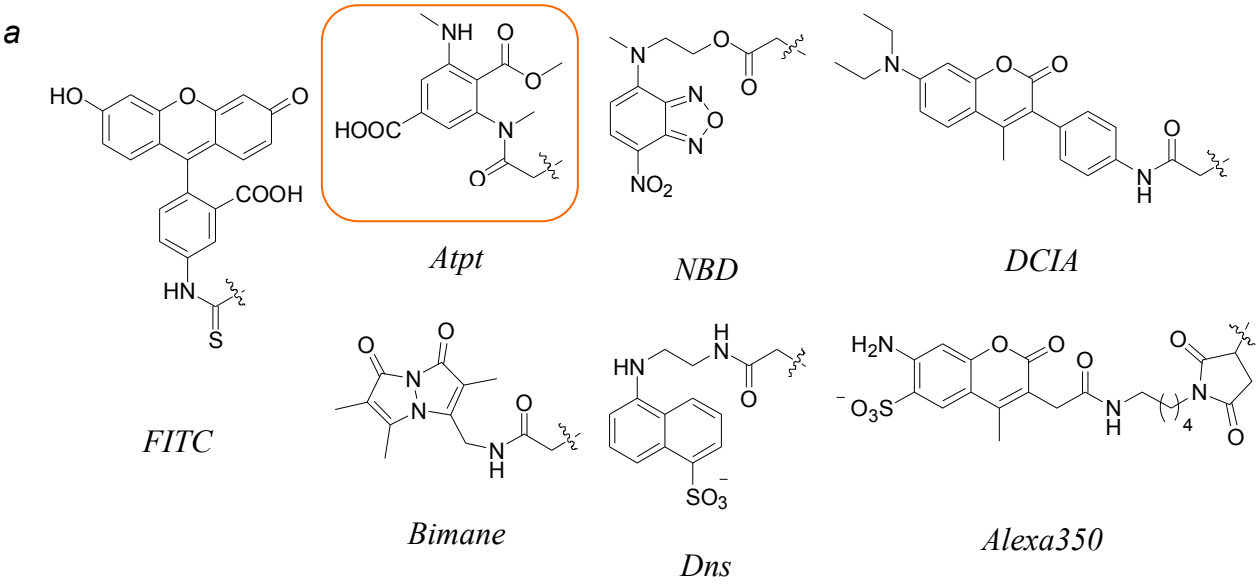
The unfolding curves were obtained from CD and heme absorption measurements. To create a series of GuHCl solutions with the same concentration of protein, aliquots of concentrated protein were added to pH-adjusted GuHCl solutions via gas-tight Hamilton syringes. The samples were incubated at room temperature for 15 minutes prior to measurements. GuHCl concentrations were monitored for accuracy with refractive index measurements. The protein concentrations were between 3 and 10 μM. Analyses of unfolding curves were performed as previously described.[4]

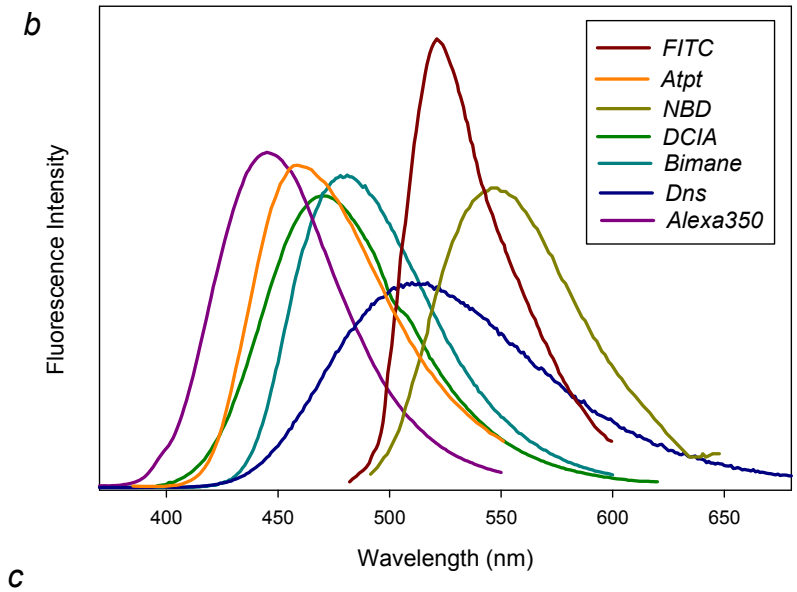
Stopped-Flow Kinetics Measurements

Protein refolding and cyt *c* redox reactions were triggered by a Bio-Logic SFM-300/S mixer. The excitation source was a 150 XeHg lamp, whose wavelengths were selected with a Horiba Jobin Yvon H10-61 UV monochromator. Kinetics were recorded by a Bio-Logic MOS-200 system. The absorption or emission signal was selected with either an Oriel 77250 monochromator or a combination of filters. A 10 mm zigzag (TC-100) and a 0.8 mm (FC-08) cuvettes were used for absorption and fluorescence experiments, respectively.

In refolding experiments, unfolded proteins were diluted 10-fold with a refolding buffer. The final protein concentrations were 10-12 μM. Prior to the experiments, the pH of GuHCl solutions was adjusted to 7.0 (cyt *c*) and 7.4 (vinculin). A freshly made 100 mM HEPES buffer was used as a refolding buffer.

Cobalt (III) phenanthroline chloride $Co(phen)_3Cl_3$ was synthesized from $[Co(NH_3)_5Cl]Cl_2$ and 1,10-phenanthroline according to the published procedure.[9] Concentrations of $Co(phen)_3^{3+}$ in working solutions were determined spectrophotometrically.[10] The buffer for $Co(phen)_3^{3+}$ experiments was deoxygenated by repeated pump-purge cycles on the nitrogen Schlenk line. Prior to stopped-flow experiments, WT* and Atpt66-cyt *c* were reduced with DTT and then separated from the reductant on a FPLC desalting column. The samples were immediately loaded into a stopped-flow syringe and kinetic traces collected within 30 minutes. The reaction was triggered by mixing one part of the cyt *c* solution with five parts of the $Co(phen)_3^{3+}$ solution. Reactions were done under pseudo-first-order conditions with the ratio $[Co(phen)_3^{3+}]/[cyt(II)] \geq 100$. The final protein concentrations in these experiments were 2-7 μM.





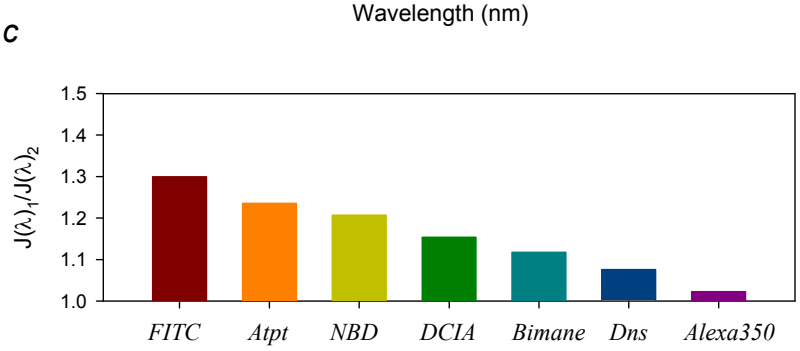


Figure S1. (*a*) Structures, (*b*) emission spectra,[11-14] and (*c*) ratios of the overlap integrals of the fluorophore emission spectrum with the cyt *c* absorption spectra in the two protein redox states $J(\lambda)_{red}/J(\lambda)_{ox}$ or $J(\lambda)_{ox}/J(\lambda)_{red}$ for thiol adducts of Atpt and common commercial dyes. The Atpt-labeled protein is predicted to show the strongest heme redox response among the less bulky dyes.

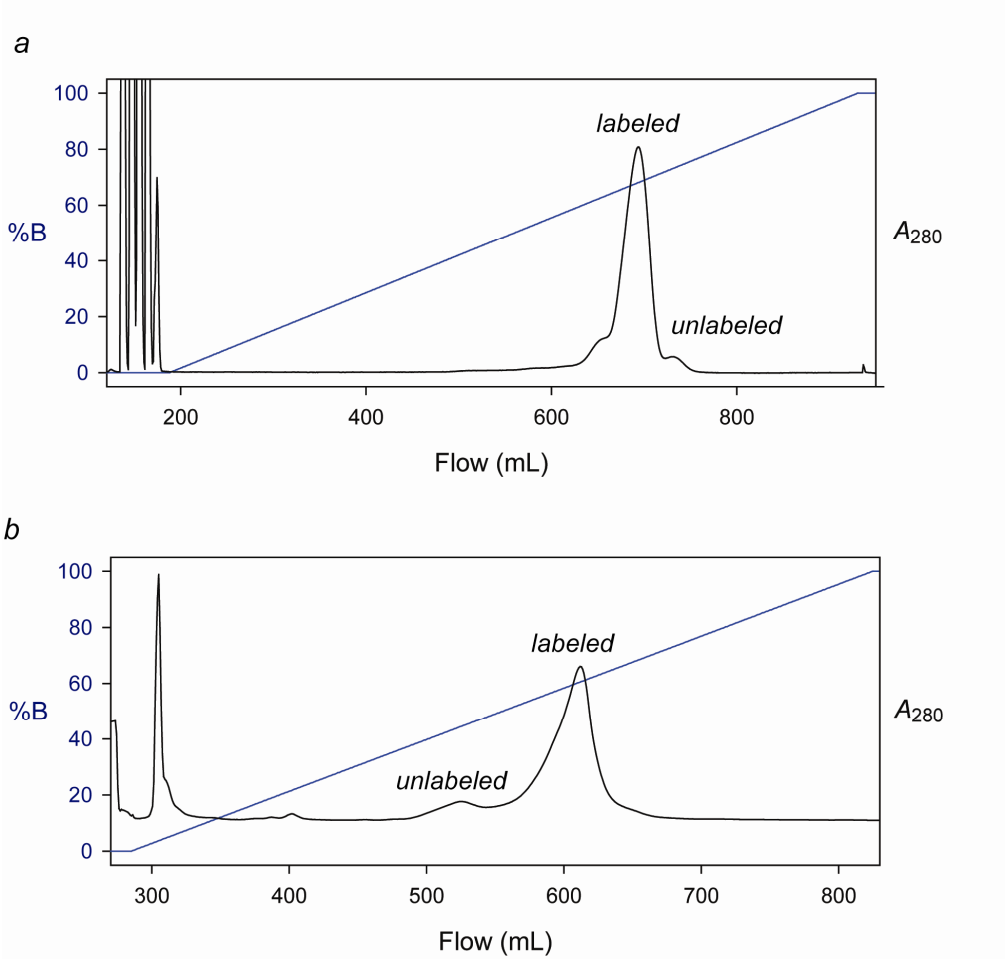


Figure S2. FPLC chromatograms of cyt *c* (*a*) and vinculin D1 (*b*) after Atpt-labeling showing efficient separation of labeled and unlabeled proteins on ion exchange SP and Q resins, respectively. For cyt *c*, Buffer A was 10 mM NaPi at pH 7.0, Buffer B was 10 mM NaPi at pH 7.0 containing 0.5 M NaCl. For vinculin D1, Buffer A was 10 mM Tris at pH 8.0, Buffer B was 10 mM Tris at pH 8.0 containing 0.5 M NaCl.

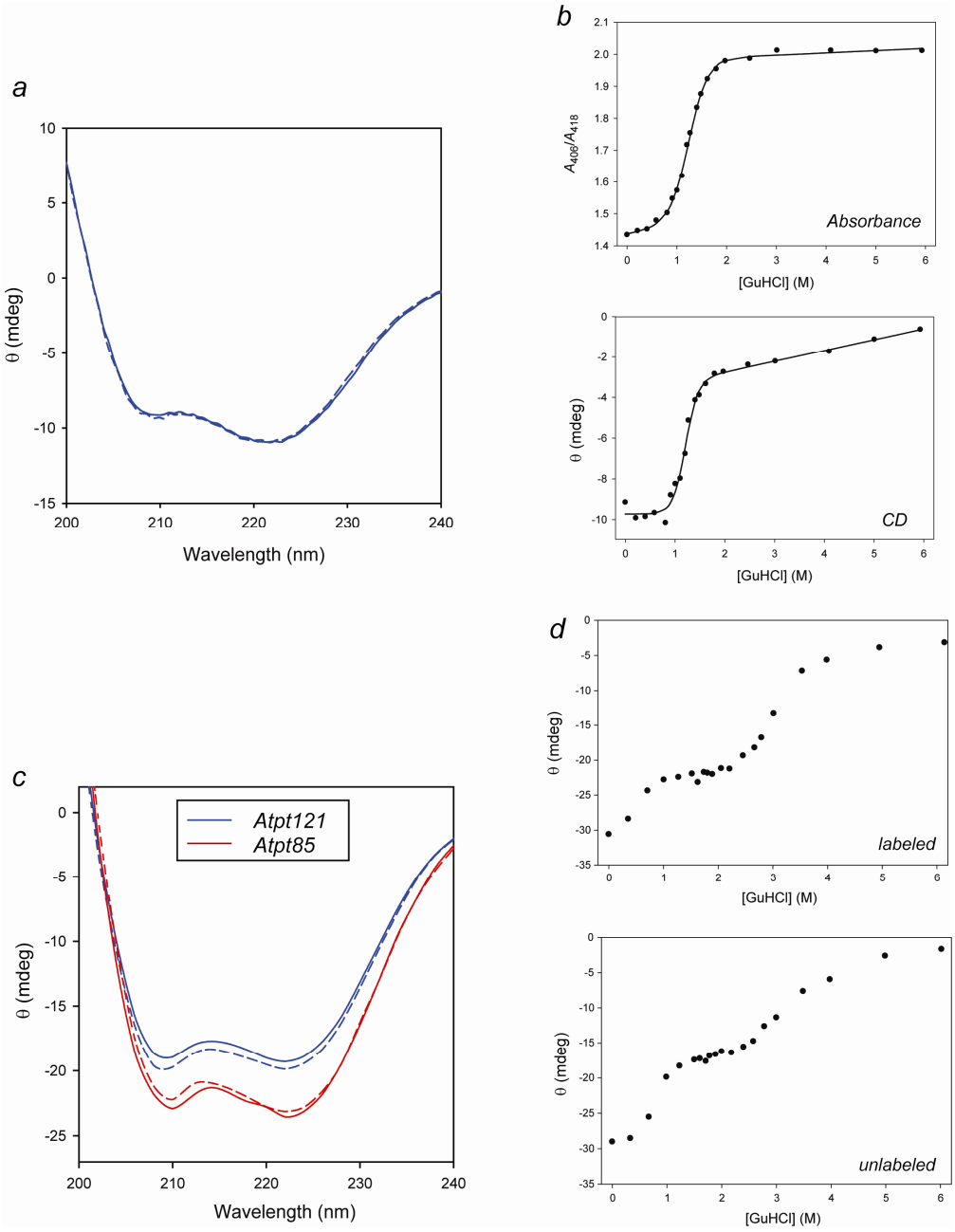


Figure S3. (*a*) Circular dichroism (CD) spectra of Atpt66-cyt *c* and its parent (*dashed* line) yeast *iso*-1 K72A/C102S (WT*) variant in a 100 mM NaPi buffer at pH 7.0. The protein concentrations *c* are 10.0 μM, the pathlength *l* is 1.0 mm. (*b*) Heme absorption and CD signals of ferric Atpt66-cyt *c* as a function of GuHCl concentration at pH 7.0. (*c*) CD spectra of Atpt121-(*blue*) and Atpt85-(*red*) labeled vinculin D1 and their parent (*dashed* lines) variants in a 100 mM NaPi buffer at pH 7.4. The protein concentrations *c* are 5.0 μM, the pathlength *l* is 1.0 mm. (*d*) CD signals of Atpt-labeled and unlabeled vinculin D1 as a function of GuHCl concentration at pH 7.4. The curves suggest similar stepwise unfolding of the protein.

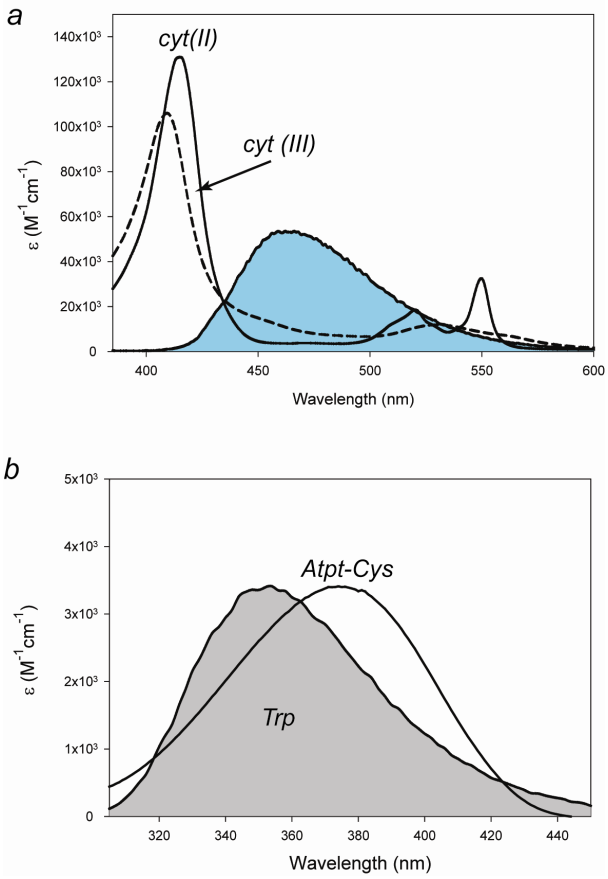


Figure S4. Overlap of Atpt-Cys absorption and emission spectra with (*a*) Trp emission and (*b*) cyt *c* heme absorption spectra, respectively. The calculated R_0 values for Trp-Atpt, Atpt-heme(III), and Atpt-heme(II) donor (***D***)-acceptor (***A***) pairs are 22, 38, and 37 Å, respectively. The isotropic value of $\kappa^2=⅔$ and Trp quantum yield $\Phi=0.13$ were used in these calculations.

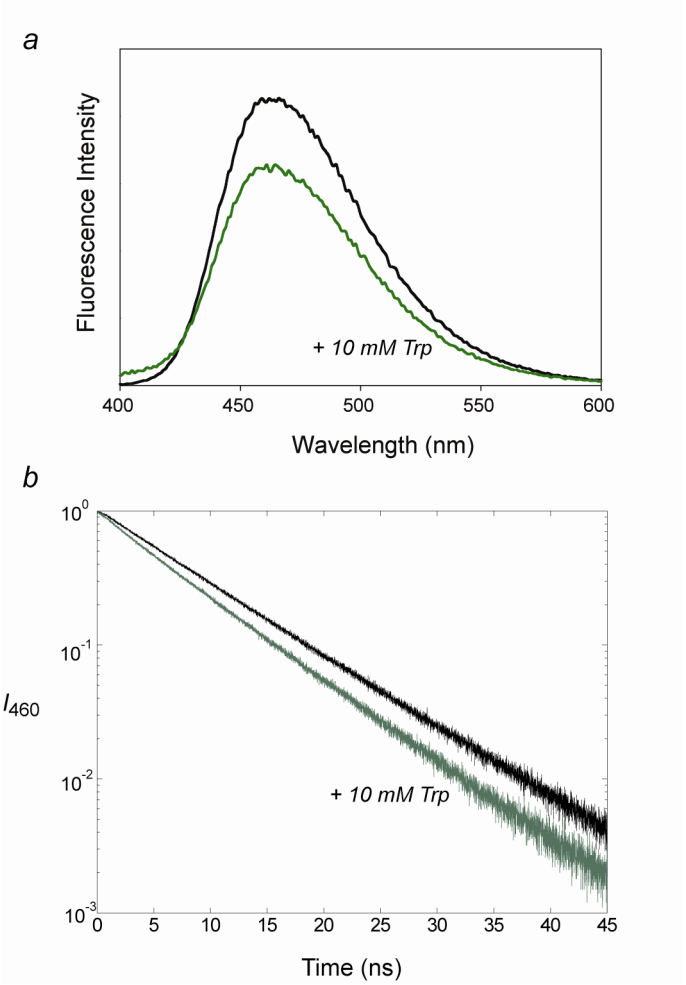


Figure S5. (*a*) Steady-state spectra and (*b*) fluorescence decays of Atpt-Cys in a 100 mM NaPi buffer at pH 7.4 in the absence (*black*) and presence (*green*) of 10 mM Trp. In both cases, the decays are best described by biexponential functions. The time constants are τ_1=6.1±0.2 ns (23%) and τ_2=8.6±0.1 ns (77%) in the absence of Trp and τ_1=2.7±0.1 ns (6%) and τ_2=7.2±0.1 (94%) with 10 mM Trp.

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