APPENDIX B

Fluorescent Dye Labels for Energy Transfer

B.1 INTRODUCTION

A wide variety of experimental techniques have been employed to study proteins and their structure, the most common being Nuclear Magnetic Resonance (NMR), X-ray crystallography, circular dichroism (CD), fluorescence and absorbance. While NMR and X-ray crystallography provide a great deal of information on equilibrium protein structure, fluorescence and transient absorbance are effective for studying of the dynamics of folding reactions. Transient absorbance requires the presence of an optical probe that is sensitive to protein structure, such as the metal ion found in azurin or the porphyrin group found in cytochromes. While azurin and cytochromes have other characteristics useful for protein studies, such as their stability, they are a small subset of proteins. Since it is not always easy to introduce an optical probe such as a metal ion into a non-metalloprotein, fluorescence is a more general technique for probing the dynamics of the structural changes in a protein. Amino-acid fluorophores such as tryptophan and tyrosine are either naturally available within proteins, or they can be introduced into the desired locations relatively simply.

B.1.2 Tryptophan

Tryptophan is a great natural fluorophore due to its high quantum yield (compared to other amino acids such as tyrosine), long excited-state lifetime, and the sensitivity of its emission maximum to the environment. Due to this sensitivity to environment, the tryptophan emission alone can be used to determine the general environment of proteins, and when used as a member of a *D-A* FRET pair, can provide great structural detail.
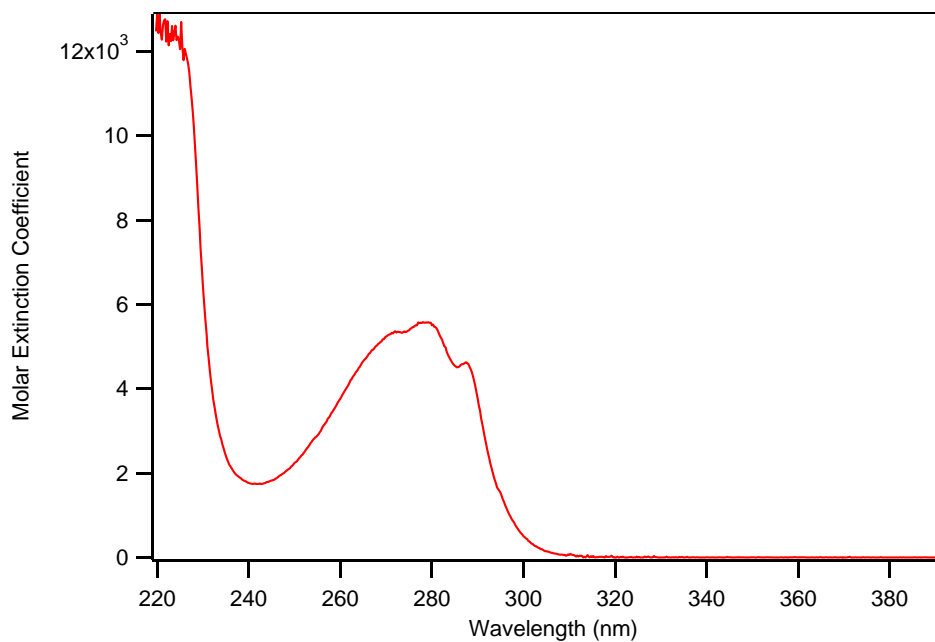
Figure B.1. Absorption spectrum of tryptophan
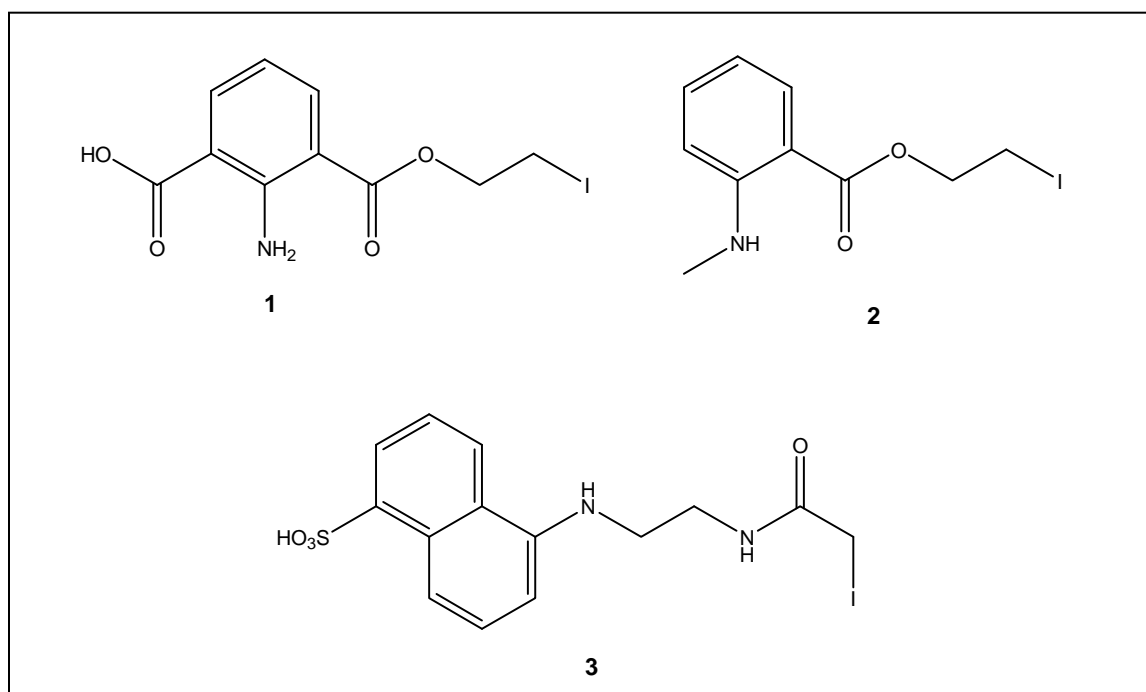
Figure B.2. Structures of the cysteine reactive
dyes I-Shark (1), PhI-Shark (2), and Dansyl (3)

Tryptophan absorbs and emits near the ultraviolet range (see Figure B.1). The indole group of tryptophan contains two low-lying singlet excited states (^1L_a and ^1L_b): the ^1L_a state fluorescence blue-shifts in nonpolar environments, such as membranes, while fluorescence from the ^1L_b state is insensitive to the environment. Interconversions between these two overlapping electronic transitions as well as interconversion between conformers result in multiexponential fluorescence decays.

The Gray Group has long used the tryptophan emission as a probe of protein environment. In particular, the cytochromes c, c' and cb_{562} each have a single tryptophan.
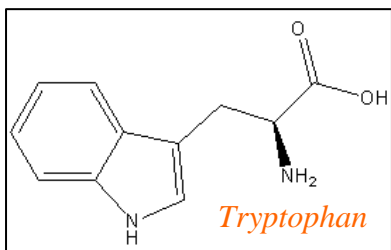
With the heme cofactor as a natural fluorescence acceptor that quenches tryptophan emission in a distance dependent manner, we have been able to study the protein folding dynamics with great ease. However, in more complicated systems requiring precise analysis, the multiexponential decay of tryptophan and its low inherent quantum yield become prohibitive.

B.1.3 Synthetic Dyes

Dye labels allow us to better investigate our protein systems because of their greater quantum yield and the ability to tune emission and excitation wavelengths. We use chemical modification to add fluorescent donors or electrochemical handles to protein systems of interest. In our group, we have done extensive research using synthetic dyes as fluorescence donors in FRET donor-acceptor pairs (also as acceptors!). These dyes enable us to determine **D-A** distances and, therefore, significant information regarding protein dynamics.

Fluorescent dyes must meet certain requirements including low cost, accessibility and easy, specific labeling, monoexponential fluorescence decays, desirable excitation wavelengths, and minimal perturbation of the protein structure. If they are part of a donor-acceptor pair, they must have appropriate spectral overlap with fluorescence acceptors.

B.2 RESULTS AND DISCUSSION

B.2.2 Tryptophan

Figure 5.1 shows the absorbance spectrum of tryptophan. The maximum fluorescence emission of NATAis 350 nm in 20 mM NaPi, pH 7 but can blue-shift tens of nanometers in a nonpolar environment like vesicles or micelles. The quantum yield of NATA is 0.13 in buffer, but changes slightly in GuHCl to 0.15 and, to a greater degree, in urea to 0.23. The quantum yield is also sensitive to temperature. The quantum yield and the emission spectrum are important in determining the overlap integral if tryptophan is used as a donor in a ***D-A*** pair. Table 5.1 lists the quantum yield of tryptophan and corresponding R_o of the tryptophan-heme pair in the Zn-cyt *c* FRET system.

	Förster Distance (W-Zn)	**Quantum Yield (W)**
Buffer	29	0.15
Urea	33.8	0.17
Guanidine hydrochloride	39	0.25

**Table B.1.** The quantum yield of tryptophan and the corresponding Förster distances for tryptophan to Zn-cyt c under different conditions.

B.2.3 Dansyl

The dansyl fluorophore has thus far proven a very robust, dependable, accessible dye label. Typically, a surface exposed cysteine can be easily labeled with the dansyl label with little structural disruption. One method of ensuring the stability of a protein with a dye label is through CD temperature unfolding curves. In the case of DNS(C102)-cyt c, the midpoint is shifted to 51 $^{\circ}$C from a midpoint of 59 $^{\circ}$C in the dansyl-free (C102S)-cyt c case. Since this is not a large shift, it is assumed that the dye does not disturb the structure significantly. In addition, the UV-Vis spectra overlap of DNS-cyt c and unlabeled cyt c shows no disruption of the heme microenvironment. Steady-state equilibrium comparisons of DNS-cyt c and DNS-cb_{562} to the unlabeled proteins are discussed in greater detail in section 3.2.3.

B.2.4 Steady-State Equilibrium Unfolded States of DNS-labeled Cyt c and Cyt cb_{562}

Fluorescence energy transfer is a useful tool in monitoring the dynamics of proteins. Since cytochromes have the heme as a built-in native energy acceptor, labeling of a native or engineered cysteine or histidine with a fluorescence donor gives rise to a ***D-A*** pair. Tryptophan may also be used as the fluorophore, and though it is a natural amino which disrupts protein structure minimally, it has some inherent disadvantages. One of the most convenient fluorophores is the cysteine-reactive Dansyl group (DNS). Although small, this dye has the potential to disrupt protein structure, particularly if the cysteine is more buried (less surface-exposed). After successful labeling and purification, each DNS-labeled protein is characterized to check for stability.
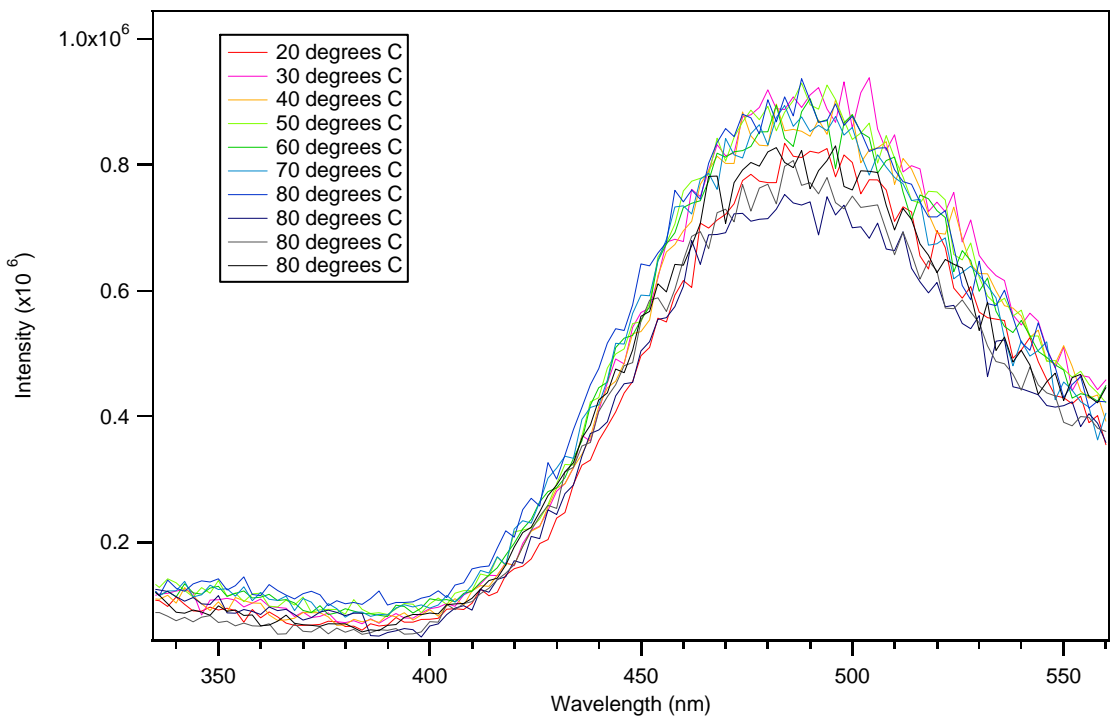
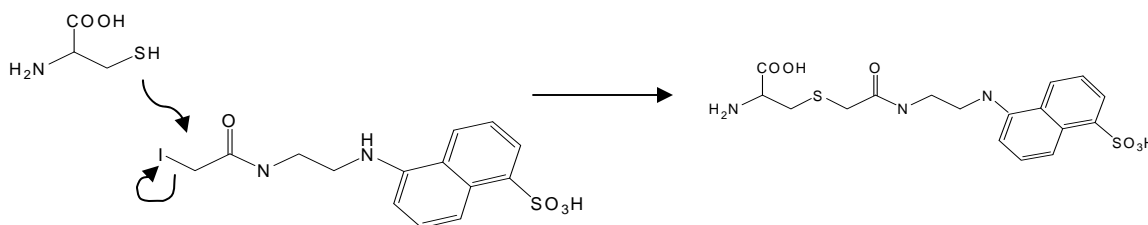**Figure B.3.** Emission of Dansyl model complex at various temperatures.
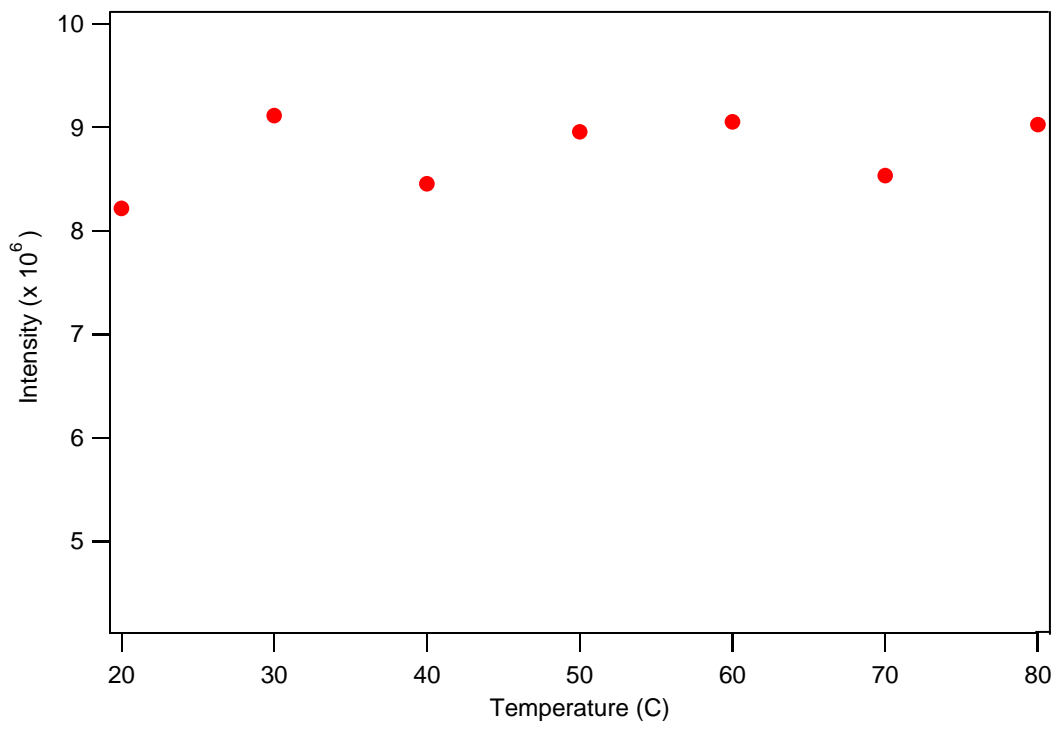
Figure B.4. The adjusted emission maximum of Dansyl model complex as a function of temperature.

	Quantum Yield	Förster Distance
W	0.13	*29* (3.7/1.8 ns)
DNS	0.38	39 (10.2 ns)
I-SHark	0.32	50 (13.5 ns)
PhI-SHark	0.48	60

Table B.2. The quantum yield of fluorophores and the corresponding Förster distances for dye to Fe-cyt c in 20 mM NaPi.

In order to better understand the protein environment, a model compound was synthesized, consisting of free N-acetyl cysteine coupled to the dansyl moiety, mimicking the protein system (Scheme B.1).
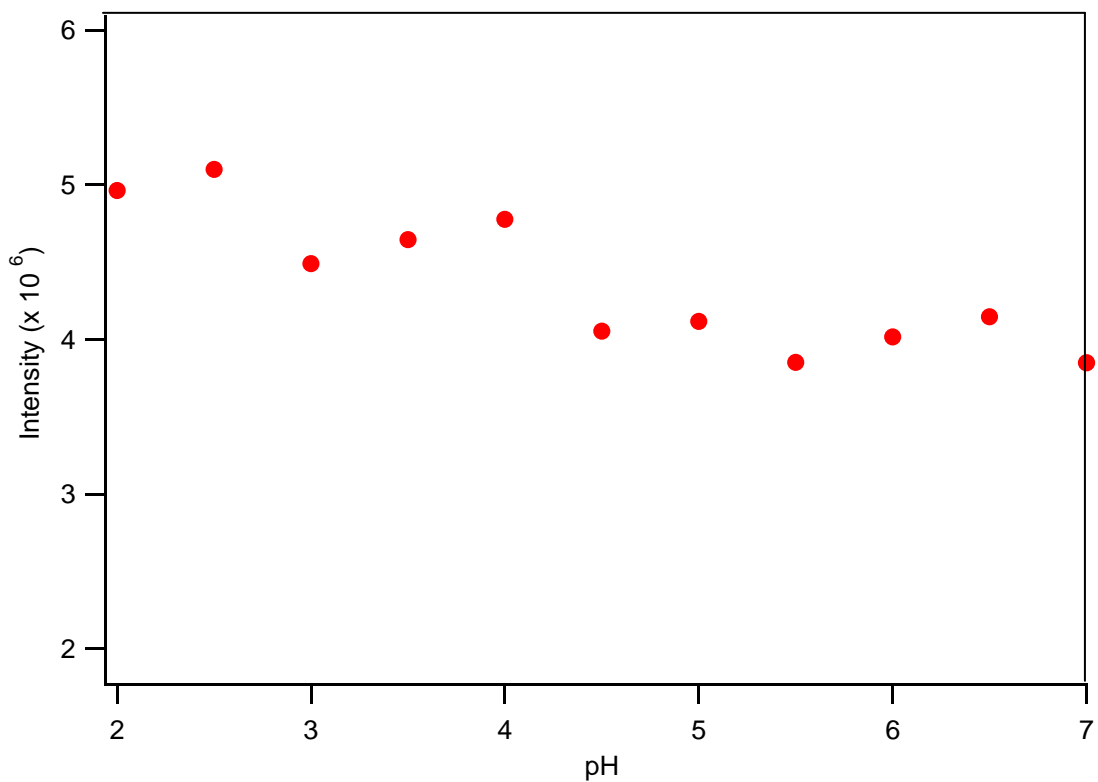

Scheme B.1. SN2 reaction of N-acetyl-cysteine with

The dansyl model can be excited with 355 nm light and has an emission maximum of ~500 nm. The emission maximum does not appear to be sensitive to polarity like tryptophan. However, it is also not sensitive to temperature and is only
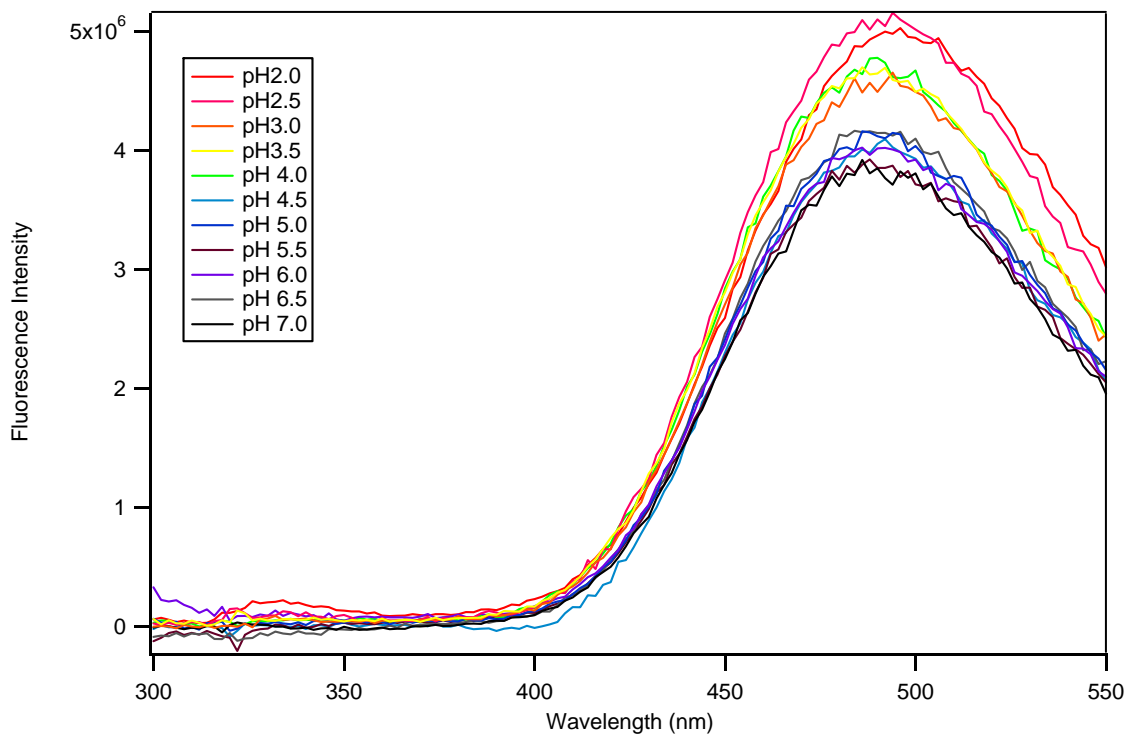
Figure B.5. Emission of Dansyl model complex at various pH.

Figure B.6. The adjusted emission maximum of Dansyl model complex as a function of pH.

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slightly sensitive to pH. The quantum yields of the dansyl model under different

conditions are listed in Table B.3.

|	Quantum Yield (RT)	Quantum Yield (70 C)
pH 7	**3.82588E-01**	**3.83009E-01**
pH 4	**3.88127E-01**	**3.83900E-01**

Table B.3. The quantum yield of the dansyl fluorophore at various temperature and pH.

The FET kinetics of the dansyl model reveal a monoexponential 10.2 ns decay.

When coupled to the C102 thiolate sulfur of *Saccharomyces cerevisiae* cyt *c*, the

emission is quenched significantly by the heme group, which is in close proximity to the

dye label.

B.2.4 Shark Dyes

Two highly-emissive, cysteine-reactive, synthetic dyes have recently been developed in the Gray group. The structures of these dyes, I-Shark and PhI-Shark, are shown in Figure B.2, alongside the structure of dansyl label, and in Figure B.7. Unlike the dansyl fluorophore, the model complexes of the Shark dyes show some temperature dependence (Figures B.8 and B.9). The dyes' emission does not appear to be very sensitive to urea or pH although I-SHark might have a slightly higher quantum yield in GuHCl (Figures B.11 and B.12).

Bert Lai previously labeled cytochrome *c* with the Shark dyes and demonstrated the effectiveness of these systems as *D-A* pairs (Figure B.7). However, unlike DNS-cyt *c*, the FET kinetics rate distributions are not a single distribution for either Shark dye. Since the FETks of the model complexes are a single distribution, it is possible that the protein is not fully folded but is disrupted by the dye.

I-Shark (Gray/Peters Group)

(a)

Phi-Shark (Gray/Peters Group)

(b)
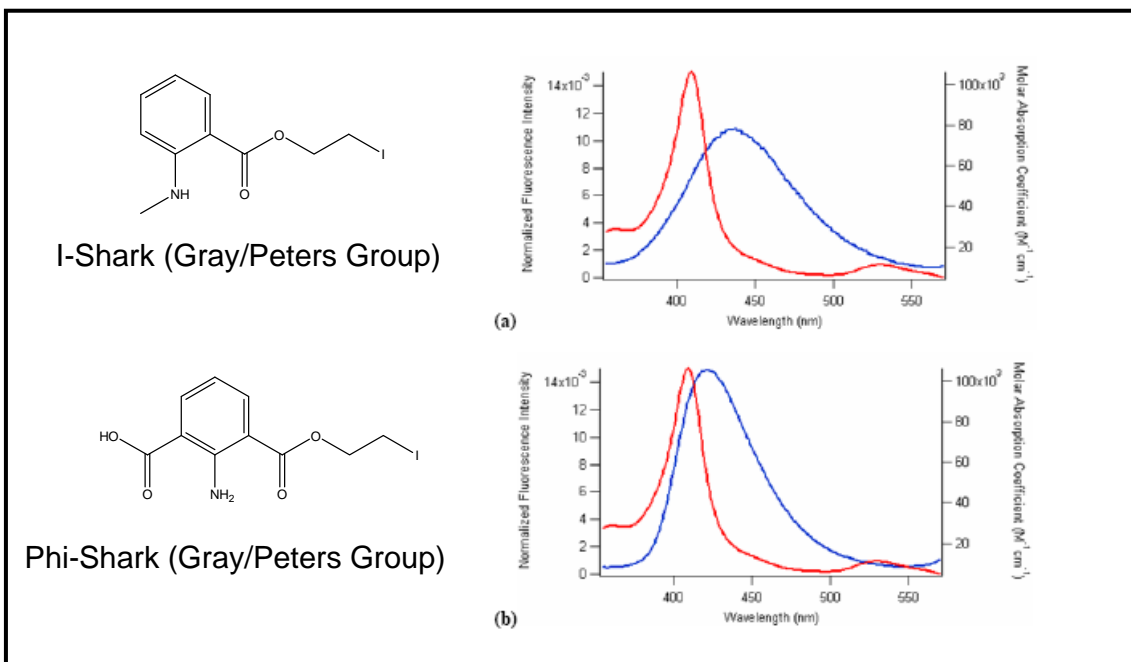
Figure B.7. The spectral overlap of cyt *c* absorption and emission of I-Shark (top) and PhI-Shark (bottom). Adapted from the thesis of Bert Lai.
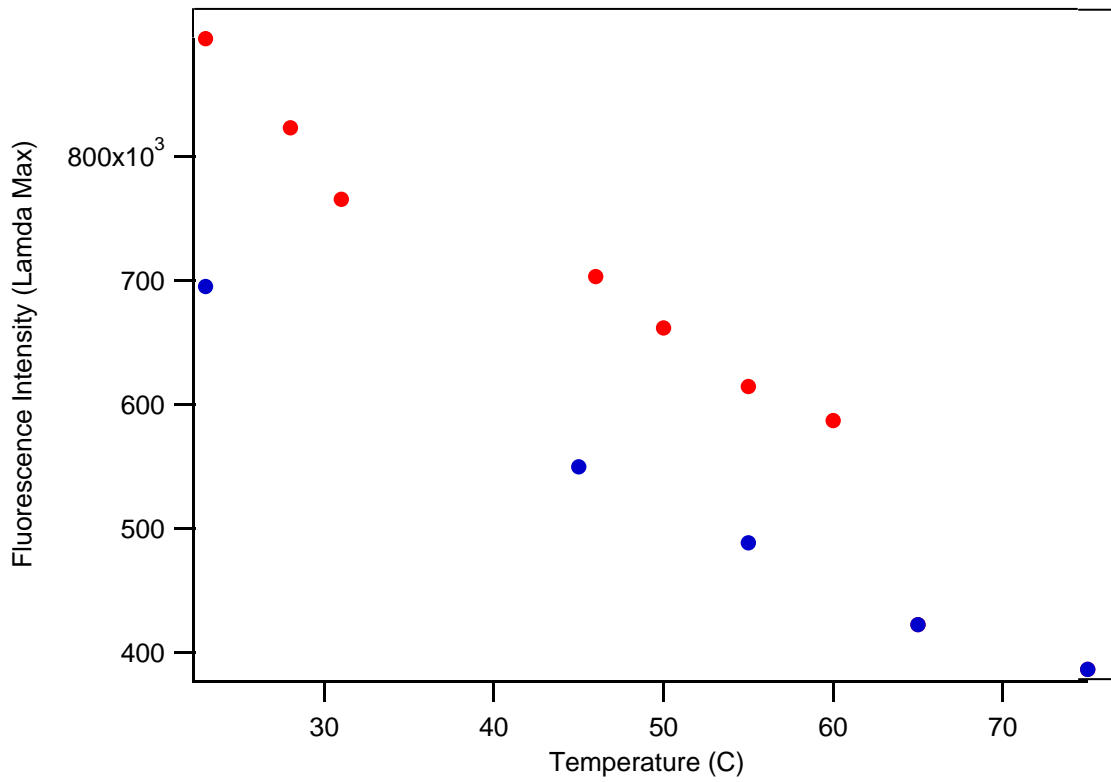
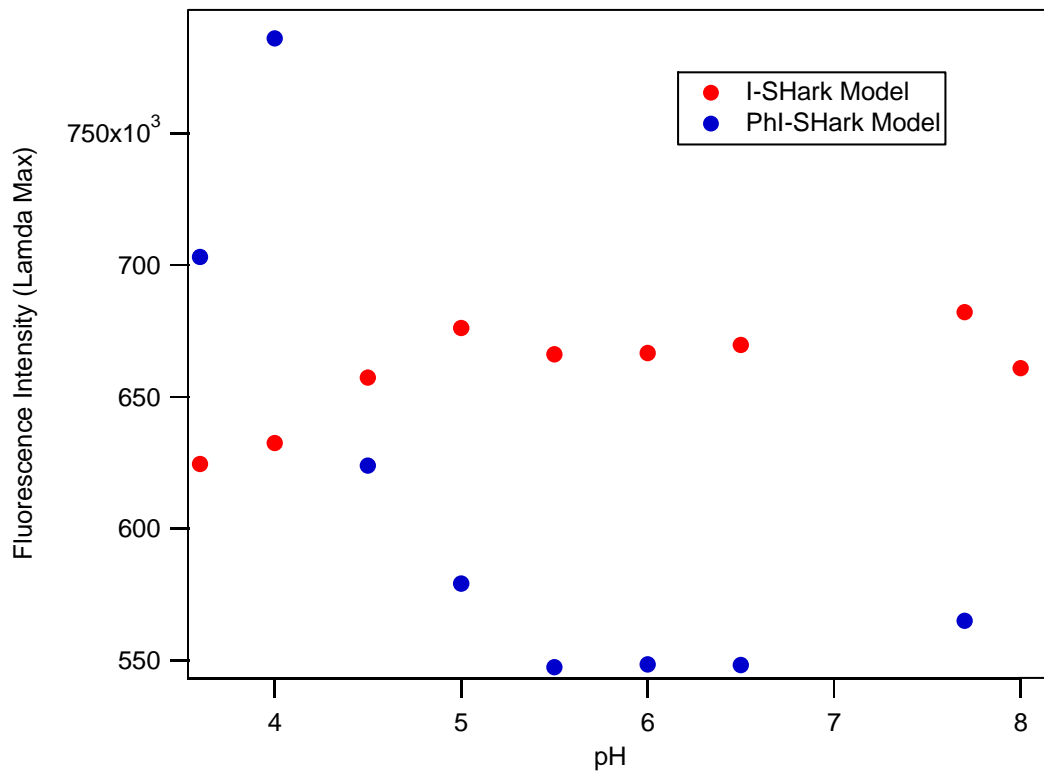**Figure B.8.** The adjusted emission maximum of the I-Shark model complex as a function of temperature.
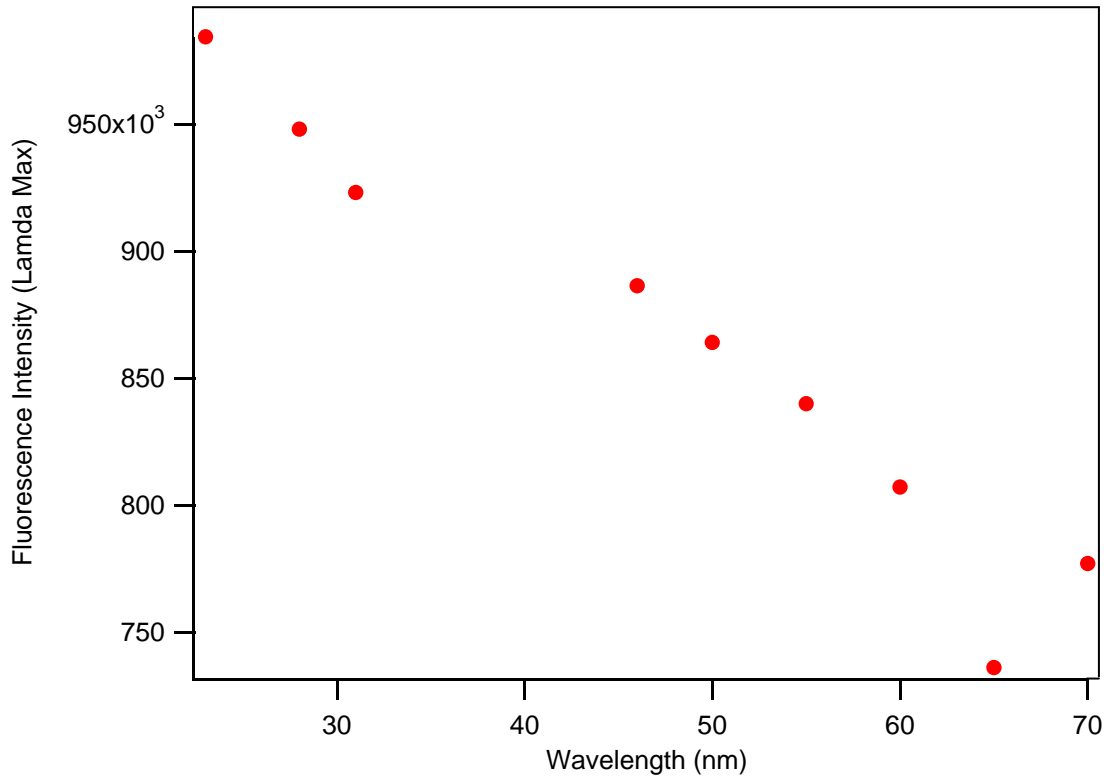
Figure B.9. The adjusted emission maximum of the PhI-Shark model complex as a function of temperature.

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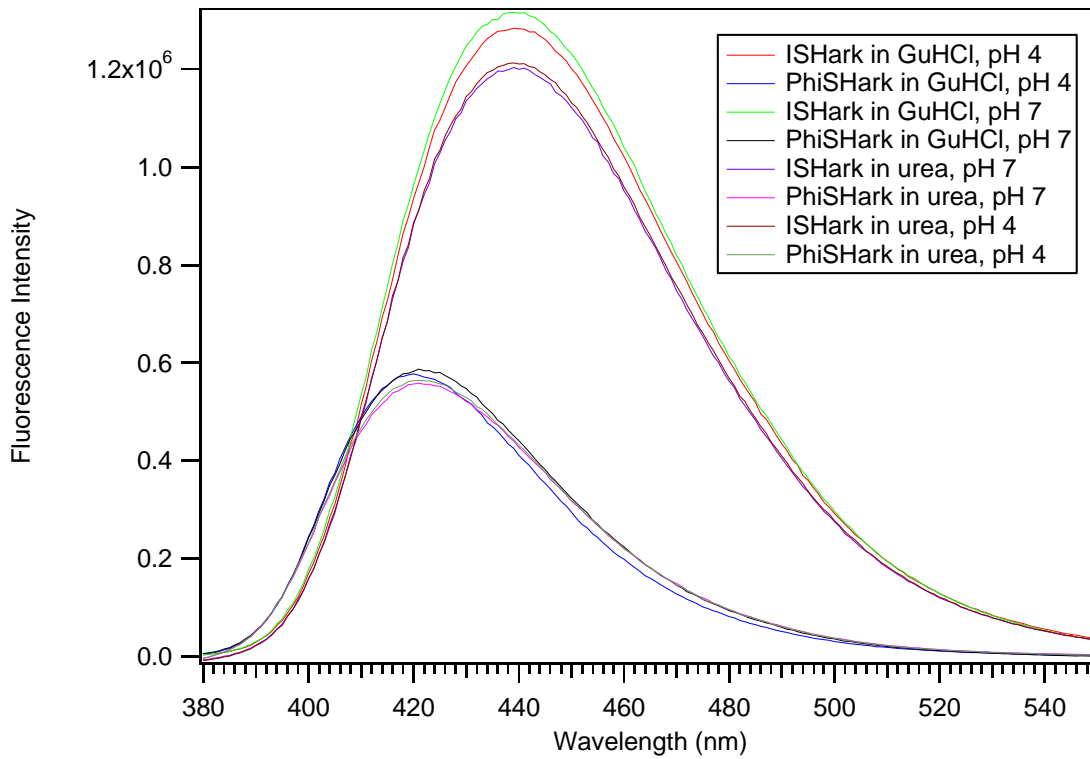
Figure B.10. Emission of Shark model complexes in GuHCl and urea, at both pH 4 and pH 7.


Figure B.11. The adjusted emission maximum of the SHark model complexes as a function of pH.

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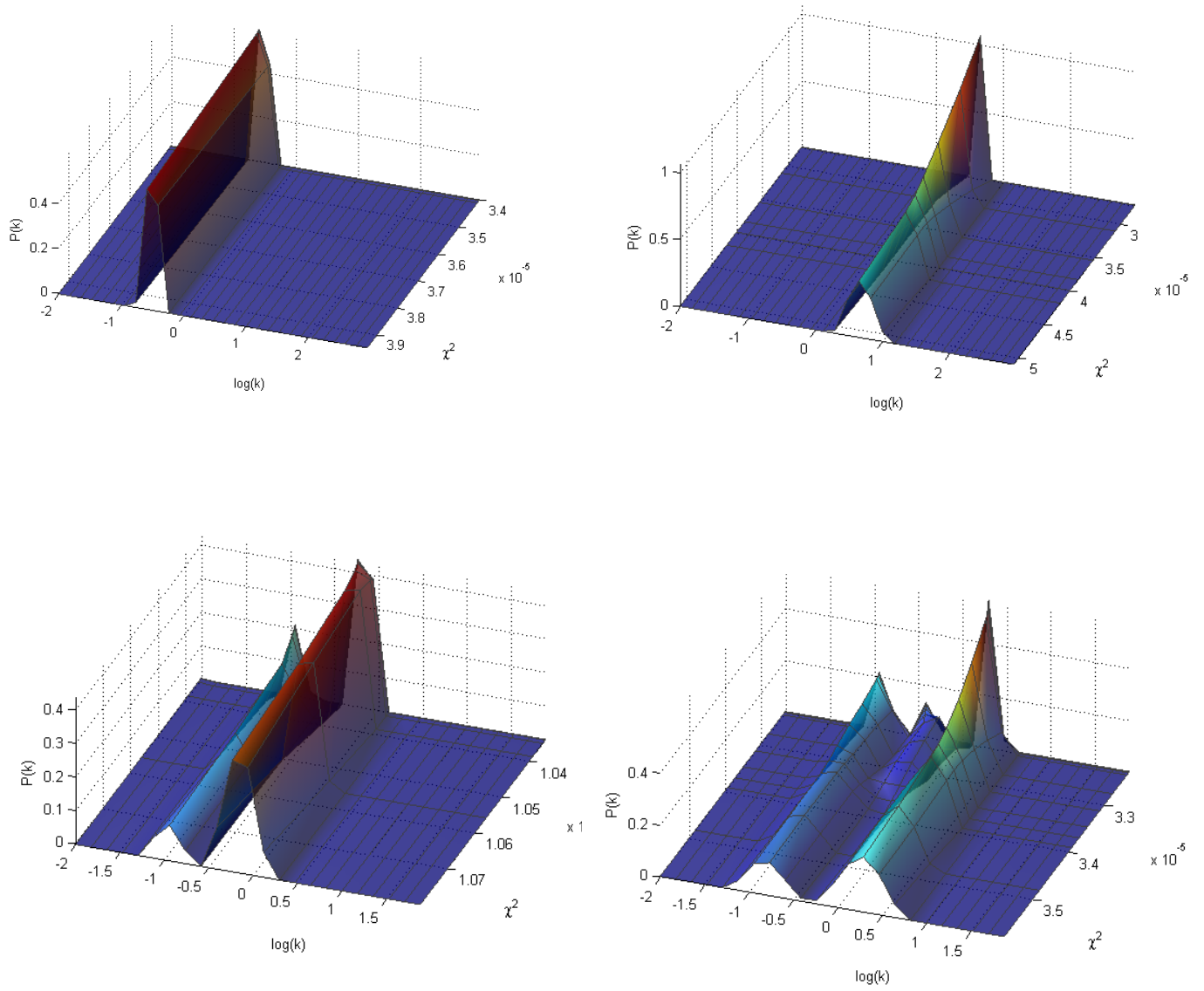
Figure B.12. Rate distributions of NATA (top left), DNS(C102)-cyt c (top right), I-Shark(C102)-cyt c (bottom left), and PhI-Shark(C102)-cyt c (bottom right)..

B.2.5 PyPMPO

Finally, the C102 thiolate sulfur was labeled with 1-(2-maleimideylethyl)-4-(5-(4-methoxyphenyl)oxazol-2-yl) pyridinium methansulfonate (PyPMPO maleimide). The structure of this new dye label is shown in Figure B.14 and its absorption and emission spectra are shown in Figure B.13. The emission of the labeled protein was measured under folded and unfolded (8 M GuHCl) conditions.
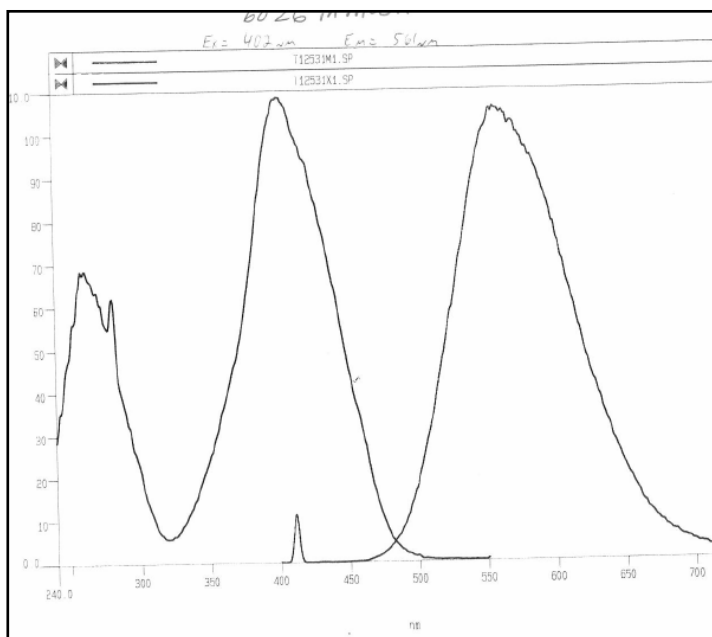
Figure B.13. Absorption and emission spectra of PyPMPO maleimide provided by Invitrogen.
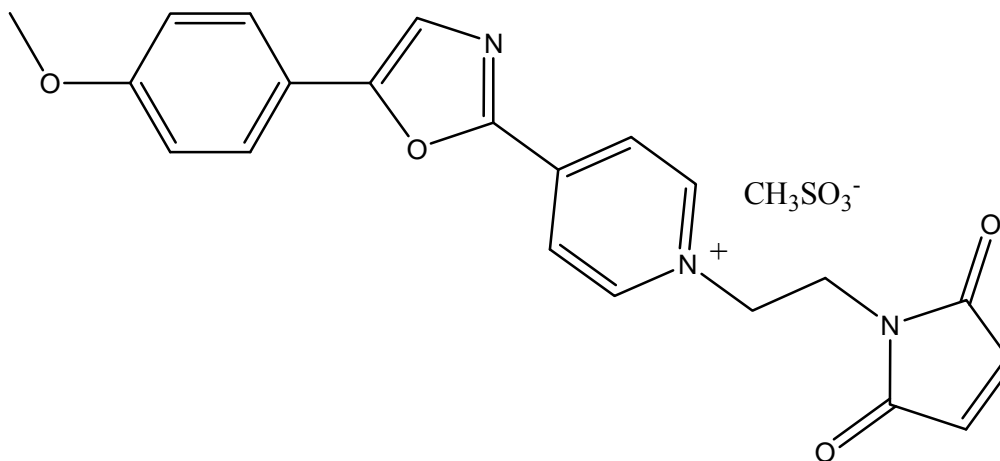
Figure B.14. Structure of PyPMPO maleimide.

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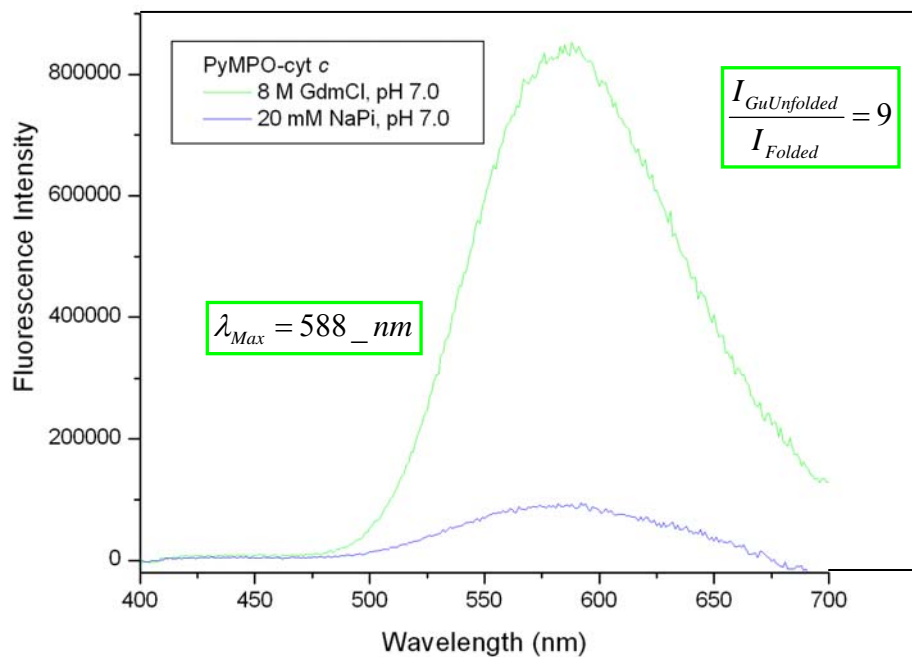
Figure B.15. Emission of folded (blue) and unfolded (green) PyMPO-cyt *c*.

B.3 CONCLUSIONS

The Shark dyes show promise for labeling proteins with a fluorophore. However, the unusual rate distributions seen in the FET kinetics indicate that these dyes might disrupt the protein structure to a greater extent than the dansyl dye.

It is interesting to note that the difference in fluorescence intensity observed for the folded and unfolded PyPMPO-cyt *c* is actually very high when compared to DNS-cyt *c*. This indicates a very large dynamic range. In addition, there is very little overlap between the emission of PhI-Shark and PyPMPo. With a detector that has spectral resolution, these two fluorophores could be used at the same time.

B.4 METHODS AND MATERIALS

B.4.2 Labeling of *Saccharomyces cerevisiae* iso-1 cytochrome *c*

Saccharomyces cerevisiae iso-1 cytochrome *c* (Sigma, St. Louis, MO) was dissolved in 100 mM NaPi, pH 7.4 and stirred under argon. The protein was denatured with 3 M of urea. The fluorophore was dissolved in 1 mL of dimethyl sulfoxide (DMSO) and delivered dropwise into the protein solution. The labeling reaction at the sulfhydryl C102 group was allowed to proceed under argon in the dark until the labeling reaction was complete. In general, the Shark dyes required longer reaction time (~2 days) and the addition of ~10x Tris(2-carboxy-ethyl)phosphine hydrochloride (TCEP) to break up the disulfide bonds.

Abbreviation	Chemical name	ID	Source
DNS	5-((((2-iodoacetyl)amino)ethyl)amino) naphthalene-1-sulfonic acid	1,5-I-AEDANS	Invitrogen
I-SHark	2-iodoethyl 2-(methylamino)benzoate	I-SHark	synthesis*
PhI-SHark	(2-amino-3-(2-iodoethoxy)carbonyl)benzoic acid	PhI-SHark	Synthesis*
PyPMPO	1-(2-maleimideylethyl)-4-(5-(4-methoxyphenyl)oxazol-2-yl) pyridinium methansulfonate	PyPMPO maleimide	Invitrogen

*I-Shark and PhI-Shark and models were synthesized with minor modifications following the protocols outlined in the thesis of Bert Lai.

B.4.3 Purification and Characterization of Labeled *Saccharomyces cerevisiae* iso-1 cytochrome *c*

The excess dye was removed from the protein by gel filtration (PD-10; GE Healthcare, Piscataway, NJ). The labeled protein was further purified using an Akta FPLC system equipped with an ion-exchange column (Mono S; HR 10/10, Pharmacia, New York, NY) and repurified after a week.

B.4.4 Dansyl Model Preparation and Characterization

The typical protocol for the dansyl model synthesis is to add a 2 mL DMSO solution of 1,5 IAEDANS (molecular probes, x mg) to a 4 mL slurry of 400 mg N-actyl-cysteine in 20 mM NaPi, pH 7.0. The solution was stirred in the dark for ~1 hour, at which point the pH was adjusted to 7.0 for 2 hours. A final adjustment brought the pH to 2.0 and the solution was stored in the cold room to promote crystallization.

B.4.5 Steady-state Equilibrium Experiments

Absorption spectra were recorded on a Hewlett-Packard 8453 diode-array spectrophotometer (Santa Clara, CA). Protein concentrations were estimated by using ε_{410} = 106 000 M^{-1} cm^{-1} for oxidized, folded, Fe(III)-cyt *c*. Circular dichroism data were acquired using an Aviv Model 62ADS spectropolarimeter (Aviv Associates, Lakewood, NJ) equipped with a thermostated sample holder. Scans were recorded from 210 to 260 nm at 1-nm intervals with an integration time of 5 s and a bandwidth of 1.5 nm. Spectra were collected at 20 °C and in a 1-mm fused-silica cuvette unless otherwise specified. Thermal unfolding curves were generated from the ellipticity at 222 nm, recorded from 20° C to 80° C at 1° C intervals. All spectra were background subtracted.

Steady-state fluorescence measurements were performed with a Jobin Yvon/SPEX Fluorolog spectrofluorometer (HORIBA Jobin Yvon, Model FL3-11, Edison, NJ) equipped with a Hamamatsu R928 PMT (Hamamatsu Photonics, Bridgewater, NJ). Samples (~1 μM) were excited at 355 nm (2-nm band-pass) and luminescence was observed from 420 to 650 nm at 1-nm intervals with 0.5 s integration times at room temperature or as otherwise specified. Background spectra of buffer only were also recorded and subtracted from DNS(C102)-cyt *c* spectra.

B.4.6 Fluorescence Energy Transfer Kinetics

Fluorescence decay measurements were performed using the third harmonic of a regeneratively amplified Nd-YAG laser (355 nm, 50 ps, <0.5 mJ) for fluorophore excitation and a picosecond streak camera (Hamamatsu C5680) in photon counting mode for detection. Magic-angle polarization was used for both excitation and collection. Fluorescence was selected with a 420-nm long-pass cutoff filter.

Temperature was measured with a thermocouple placed in close contact with the cuvette. The wire thermocouple (K-type) and meter (HH-51) were obtained from Omega (Stamford, CT).

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