Zinc porphyrin: A fluorescent acceptor in studies of Zn-cytochrome *c* unfolding by fluorescence resonance energy transfer

Amy A. Ensign*, Iris Jo*, Ilyas Yildirim[†], Todd D. Krauss*, and Kara L. Bren**

Departments of *Chemistry and [†]Physics, University of Rochester, Rochester, NY 14627

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FRET between the zinc porphyrin (ZnP) chromophore in zinc-substituted cytochrome *c* (Zn-cyt *c*) and an Alexa Fluor dye attached to specific surface sites was used to characterize Zn-cyt *c* unfolding. The use of ZnP as a fluorescent acceptor eliminates the need to doubly label the protein with exogenous dyes to perform FRET experiments in which both donor and acceptor fluorescence is monitored. The requirement for attachment of only one dye also minimizes perturbation to the protein. This sensitive technique allowed for the determination of distances between the label placed at six different sites and ZnP through a range of denaturant concentrations. Fitting of the data to a three-state model provides distances in the unfolding intermediate. The use of ZnP as a fluorescent acceptor of energy in FRET has a significant potential for application to a range of other systems including heme-binding proteins and proteins to which a covalently attached heme tag may be added.

FRET | protein folding | heme

Fluorescence resonance energy transfer (FRET) is a simple and widely used technique for measuring molecular distances. FRET involves the nonradiative energy transfer between donor and acceptor fluorophores, the efficiency of which depends strongly on donor–acceptor separation (1). Structure, dynamics, and conformational changes of biomolecules including proteins, RNA, DNA, and polypeptides are amenable to study using FRET (2). As a biomolecular “ruler,” FRET is most useful when both donor quenching and acceptor enhancement of fluorescence are observed, because the presence of multiple nonradiative pathways for the donor can potentially cause inaccurate distance measurements if only donor fluorescence quenching is monitored.

Because proteins are not readily synthesized chemically, preparing a pure sample of protein site-specifically labeled with two fluorophores appropriate for FRET is technically difficult and time-consuming. The most convenient and widely available chemically specific approach is to label Cys thiols, because Cys is an infrequently occurring amino acid. However, preparing a sample with the donor attached to one particular Cys and acceptor to a second particular Cys requires multiple purification steps, if it can be achieved at all (3, 4). Therefore, the majority of intramolecular FRET studies of proteins make use of an intrinsic fluorophore as one member of the donor–acceptor pair, with tryptophan the most commonly used (2). This approach allows two-color FRET studies on a sample labeled with only one attached extrinsic dye. Although tryptophan is advantageous in that it is naturally occurring and can be introduced (or removed) as needed through site-directed mutagenesis, it is easily quenched by surrounding amino acids, complicating data interpretation (5, 6). Additionally, tryptophan fluoresces in the UV, making it difficult to differentiate tryptophan fluorescence from other parasitic sources of fluorescence; a recent study highlighted the need to separate tryptophan fluorescence from intrinsic porphyrin fluorescence in tryptophan-to-heme energy transfer studies (7).

FRET is a vital technique for use in the exciting and emerging field of protein folding and dynamics on the single-molecule (SM) level. Despite the great potential of this area, there are relatively few published studies of SM protein folding using FRET (8–14). A major roadblock has been the difficulty of preparing a homogeneous sample labeled with both donor and acceptor chromophores that are detectable on the SM level (4, 15). Intrinsic fluorophores such as tryptophan are not acceptable for SM spectroscopy because of small extinction coefficients and poor quantum yields (16); additionally, tryptophan undergoes irreversible photodamage before producing sufficient photons for SM analysis. Compounding the problem of labeled protein preparation is the need to investigate derivatives labeled at a number of different sites to create a comprehensive profile of unfolding. A protein sample that is doubly labeled in a site-specific manner (i.e., donor to site 1 and acceptor to site 2) is tremendously valuable, because it greatly simplifies data interpretation (17, 18). Most SM protein folding studies use samples that contain a mixture of labeled derivatives; the data then must be subjected to complex statistical analysis to interpret the SM fluorescence data (15). Use of samples labeled site-specifically would remove an extra level of complexity in an already challenging experiment.

This work addresses the need for site-specific double labeling of a protein with fluorophores by modifying an intrinsic chromophore in horse heart cytochrome *c* (cyt *c*), a small electron transfer protein that has long served as a model system for protein folding (19). Cyt *c* contains a *c*-type heme that is covalently attached to the polypeptide via the only two cysteine residues in the protein. Replacing the heme iron with Zn(II) creates an intrinsic fluorophore (20), zinc porphyrin (ZnP), and does not significantly alter the structure or stability of the protein (21–23). Using site-directed mutagenesis, single cysteine residues were introduced in six different locations, positions 4, 39, 50, 66, 73, and 99, for attachment of Alexa Fluor 488-maleimide (AF) (Fig. 1). These sites are located on the surface of the protein and were selected based on the work of Gray and coworkers (24, 25). Monitoring AF-labeled Zn-cyt *c* (AF-Zn-cyt *c*) unfolding provides the opportunity to validate the use of ZnP as a fluorescent energy acceptor by comparing the results with previously published work. The results also provide insight into the equilibrium unfolding of cyt *c* by clarifying of the roles of the N- and C-terminal helices and the axial methionine-bearing loop in forming the unfolding intermediate.

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*To whom correspondence should be addressed. E-mail: bren@chem.rochester.edu.

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Fig. 1. Structure of horse cyt *c* (Protein Data Bank ID code 1HRC) (55) showing the heme and the locations of residues mutated to cysteine for labeling indicated in color. The heme is covalently attached to residues 14 and 17 on a loop following helix 1. A maleimide derivative of the AF donor was covalently attached to the introduced Cys on each Zn-cyt *c* variant. Substructures (helices and loops) within the protein are indicated by arrows.

Results and Discussion

Analysis of FRET Data. Representative absorption and fluorescence spectra of AF-Zn-cyt *c* are shown in Fig. 2. AF labeling was achieved in a 1:1 manner for each mutant [supporting information (SI) Fig. S1], and denaturation monitored by CD spectroscopy verified that the mutations and subsequent labeling did not significantly alter the stability and fold of the protein (Figs. S2 and S3). Fluorescence intensities and spectra of the AF donor and the ZnP acceptor were measured at equilibrium for each AF-labeled mutant in variable concentrations of the denaturant guanidine hydrochloride (GuHCl). The increase in AF fluorescence is accompanied by a decrease in ZnP fluorescence as GuHCl concentration is increased (representative data are shown in Fig. 3). These changes in fluorescence of the donor and the acceptor are the signatures of the expected decrease in FRET efficiency resulting from the distance increase between

Fig. 2. Absorption spectra of AF-Zn-K73Cyt *c* (red) and Alexa Fluor 488 (blue). (Inset) Absorption (red) and fluorescence spectra of AF-Zn-K73Cyt *c*. Normalized fluorescence from ZnP and the AF are indicated by the blue and green curves, respectively.

Fig. 3. Fluorescence from AF-Zn-K99Cyt *c* in increasing concentrations of GuHCl (0–7 M). Dependence on [GuHCl] of fluorescence from AF 488 (A) and normalized fluorescence from ZnP (B).

AF and ZnP as the protein unfolds. Control experiments demonstrate that the observed intensity variations are due to changes in AF–ZnP distance. First, unlabeled Zn-E4Cyt *c* in the presence of various concentrations (0–7 M) of GuHCl was directly excited at 550 nm and ZnP fluorescence was found to be unaffected by protein unfolding (Fig. S4). Zn-E4Cyt *c* was also excited at the excitation wavelength used in the FRET experiments (495 nm) to determine whether any ZnP fluorescence observed in FRET studies originates from direct photoexcitation. Minimal fluorescence, <1% of the fluorescence intensity from ZnP seen in the FRET experiments, was observed. Finally, AF in various concentrations (0–6 M) of GuHCl was excited at 495 nm to determine whether the denaturant affects AF fluorescence. The fluorescence intensity of AF remained constant over the range of GuHCl concentrations used (Fig. S5).

Evaluation of Donor–Acceptor Distances. The strong dependence of FRET efficiency on donor–acceptor distance makes FRET an excellent probe of overall protein compactness, but only for

and the ZnP fluorescence intensity of the folded and unfolded proteins fixed based on the experimental data endpoints (Fig. S6). (See *SI Methods* for details of the fitting procedure.) This was the simplest treatment of the data that yielded a robust fit. The need to treat m values as local variables reflects the sensitivity of the probe position to local changes in structure upon denaturation. Indeed, m values have been shown to be related to changes in solvent-exposed surface area for the transition (33), and it is reasonable to expect that this value may depend on probe position in a complex process such as unfolding. In other words, unfolding is more complex than implied by the three-state model. Similarly, hydrogen exchange analysis of cyt *c* unfolding yields m values dependent on local or subglobal structure (30). The best-fit midpoint concentrations were found to be $C_{m1} = 0.99$ M GuHCl and $C_{m2} = 1.7$ M GuHCl, whereas the best-fit values of m_1 and m_2 and the distances between the AF probe and ZnP in the intermediate state can be found in Table 2.

FRET studies of protein unfolding have the significant advantage that distances between the probe chromophores can be directly determined as the protein is unfolded. Here, the distance between AF and ZnP in the intermediate I for each variant provides insight into the structure of the intermediate. For example, analysis of NMR, CD, and absorption data suggests that I retains overall native-like structure while the protein “loosens” up (28), and another NMR analysis has shown that the N-to-I transition has a small overall m_1 value (31). Our results are consistent with this analysis: the AF–ZnP distance changes for almost all AF label positions for the transition from N to I are small in comparison with the distance changes observed in the I-to-U transition (position 4 is the exception, discussed subsequently). Also in accord with the interpretation that I bears more similarity to N than to U, m_2 values are larger than m_1 values, with the exception of data for positions 4 and 73 (see further discussion below).

AF labels at positions 4 and 99 probe the locations of the N- and C-terminal helices (helix 1 and helix 4; Fig. 1), respectively, relative to ZnP. We unexpectedly found that position 4 has $m_1 > m_2$ and that the ZnP–AF distance (for position 4) in I has almost reached the value observed for U. Our interpretation is that during the N-to-I transition, helix 1 extends away from ZnP whereas upon transitioning from I to U there is only a minor additional extension. Notably, residue 4 is only 10 residues from Cys-14, to which ZnP is covalently attached, thus limiting the AF–ZnP distance change for this position. Position 99 has a relatively large m_1 and moves a similar distance away from ZnP as does position 4 in the N-to-I transition despite the large number of residues between position 99 and the closest heme attachment site (His-18). These observations suggest that helices 1 and 4 move away from ZnP together when forming I. This interpretation agrees with the finding that in the proposed common cyt *c* unfolding intermediate the N- and C-terminal helices contact one another (30, 32). The other labeling position that does not display a larger m_2 than m_1 value is 73, which has similar m_1 and m_2 values. This observation is consistent with reports of a cyt *c* equilibrium unfolding intermediate in which the loop bearing the heme axial ligand Met-80 as well as Lys-73 (loop 3) undergoes rearrangement (31, 34, 35). The relatively large m_1 value for position 73 relative to what is observed for other positions is consistent with the movement of position 73 away from ZnP with loop rearrangement upon formation of I.

It is informative to compare these results with an NMR study of cyt *c* denaturation, whereby persistence of a native-like chemical environment for residues on helices 1 and 4 (Asp-2 and Asn-103) in increasing denaturant was observed, relative to probe nuclei on loop 1 (Gly-37), helix 2 (Thr-49), and loop 3 (Met-80), which show changes in local structure at low denaturant concentrations (28). This may seem contradictory to our

result that helix 1, as probed at position 4, experiences its more significant unfolding event in the transition from N to I. However, the present results are complementary to the NMR results, because the NMR data reflect immediate chemical environment and FRET is dependent on a long-range coupling. An altogether consistent picture is that as I is formed helices 1 and 4 maintain native-like structure locally as their distance from the heme increases.

Applications to SM Studies. AF–ZnP is thus demonstrated to be a novel and effective donor–acceptor pair for measuring protein distances by FRET. The use of ZnP overcomes the difficulty of doubly labeling the protein, requiring only a single mutation and labeling site for attachment of the AF donor. In particular, the fluorescence of both donor and acceptor FRET chromophores can be monitored, thereby allowing for a potentially superior characterization of the multistate nature of protein folding. A significant advantage of this method is that the labeling is site-specific, which would ease data interpretation for SM studies (3, 4). Disadvantages of this method include the need to replace iron with zinc in covalently bound heme, and the low quantum yield of ZnP fluorescence, which makes it subject to spectral cross-contamination from brighter dye molecule fluorescence. Nevertheless, SM fluorescence detection of Zn-cyt *c* (36), including SM Zn-cyt *c* fluorescence images achieved in our laboratory (Fig. S7), indicate that ZnP could be used in SM FRET studies.

Relative to other biomolecules, there are few examples of FRET studies on doubly labeled proteins, and even fewer studies of proteins for which each of the two labels is attached to a defined position. There have, however, been a number of advances in protein labeling technology that address this issue (37). Attachment of a biarsenical dye to a tetracysteine motif incorporated into a protein allows for site-specific protein labeling, which has proved to be a valuable technique for imaging *in vivo*. However, there have been no studies on purified protein, likely because of the instability of the biarsenical dye-bound protein (38). Studies of specifically labeled proteins prepared synthetically have been performed (9). This approach offers the advantage of great flexibility in choice of fluorophores and means of attachment but works only for the restrictive case of proteins small enough to be synthesized. Other noteworthy methods make use of the action of an enzyme on an appended recognition substrate. In one example, a substrate recognition tag was developed for transglutaminase that facilitates acyl transfer between a dye molecule and the glutamine of the tag; this method allowed site-specific double labeling of a protein that was suitable for SM studies but requires a single solvent-exposed glutamine be present for specific labeling (3). An 11-residue tag for protein labeling with small-molecule probes by Sfp phosphopantetheinyl transferase has been reported, which also is a promising approach (39). In addition, a 6-aa aldehyde tag prepared by the action of formylglycine-generating enzyme on a Cys residue has been reported; the aldehyde serves as a reactive labeling site. This approach has great potential for allowing site-specific double labeling of a range of proteins (40). Labeled protein samples for FRET studies of folding must display robustness of the tag and of the label and high sample purity. An advantage of the method reported here is that a highly purified sample of protein is readily prepared with covalently bound ZnP, and there is no chance for nonspecific or incorrect attachment of the ZnP chromophore to protein. Use of ZnP as a fluorescent acceptor in FRET also may be particularly useful for studies of heme proteins as described below. Finally, the present work shows a high dynamic range for changes in fluorescence intensity for the AF–ZnP pair, which makes them extremely sensitive to structure changes.

Extension to Other Systems. The use of ZnP as an acceptor in FRET studies of proteins has exciting potential for more general use; i.e., it is not limited to studies of cyt *c*. Indeed, ZnP binds site-specifically to heme-binding sites in cytochrome P450cam (41), myoglobin (42), horseradish peroxidase (43), and hemoglobin (44). ZnP is expected to bind specifically to most heme-binding proteins for which the AF–ZnP pair may serve as a valuable probe of both intramolecular and intermolecular distances. For example, flavin fluorescence is often quenched by the heme in flavin-binding heme proteins as a result of efficient energy transfer (45, 46). Substituting heme with ZnP may allow sensitization of ZnP fluorescence by energy transfer from flavin, facilitating studies of flavohemoglobins and heme enzymes with flavin-binding reductases. This method also may be extended to proteins that normally do not bind heme: Any protein that can be expressed in *Escherichia coli* may be prepared with a covalently bound ZnP by expressing the protein with an appended heme-attachment sequence recognized by the promiscuous *c*-heme attachment apparatus of *E. coli* (47, 48), followed by substituting zinc for iron. Employing this approach will facilitate preparation of proteins specifically labeled with two fluorophores appropriate for detection on the SM level. Because preparation of such samples has hindered FRET studies of single proteins, this methodology is expected to aid the advance of this exciting field of study.

Materials and Methods

Site-Directed Mutagenesis and Expression/Purification of Cyts *c*. The pBTR-1 (Amp^r) plasmid (49) containing the horse cyt *c* gene and the yeast cytochrome *c* heme lyase gene (kindly provided by A. Grant Mauk of the University of British Columbia, Vancouver, Canada) was used as a template for site-directed mutagenesis and for protein expression. E4C, D50C, and K39C mutants of horse cyt *c* were prepared using the QuikChange II method (Stratagene). E66C, K73C, and K99C mutants were prepared by using the MegaWhop procedure (50). Details are provided in *SI Methods*. Protein expression and purification were as described (51), except that all steps were taken in the presence of 10 mM DTT.

Preparation of Zn-Cyts *c*. Lyophilized cyts *c* were demetalated by using a procedure adapted from Vanderkooi *et al.* (20). All preparations were done in minimal light. Ten milliliters of HF-pyridine (70% HF) for every 30 mg of lyophilized cyt *c* were placed in a Teflon beaker, stirred for 10 min, and quenched by addition of 5 ml of 50 mM ammonium acetate (pH 5.0). Nitrogen was flowed over the beaker for 2 h followed by 4 h of dialysis against 10 mM sodium acetate (pH 5.0) and 10 mM DTT. The protein was then applied to a CM Sepharose fast flow ion exchange column (GE Healthcare) equilibrated with 10 mM sodium acetate (pH 5.0). The protein was eluted using a 0–0.5 M NaCl gradient; ferricyt *c* eluted at 0.25 M NaCl, and the demetalated porphyrin cyt *c* (P-cyt *c*) eluted at 0.35–0.5 M NaCl. The integrity of the P-cyt *c* was confirmed by absorption measurements (UV-2041PC Shimadzu photometer) (20). P-cyt *c* was concentrated by using an Amicon ultrafiltration device with a YM-3 membrane (Millipore) and exchanged into 10 mM sodium acetate (pH 5.0) by using a PD-10 Sephadex G-25 column (GE Healthcare). A 100-fold excess of zinc acetate was added to the protein and incubated at 70°C for 10 min. The insertion was monitored by absorption and fluorescence (SpectraPro 300i; Acton Research) spectroscopy. Quantitative insertion was observed as the expected sharp Soret band at 423 nm, and Q-bands at 549 and 584 nm appeared (20). Excess zinc was removed by applying the sample to a PD-10 column equilibrated with 10 mM Tris buffer (pH 7.5).

Preparation of AF-Zn-cyts *c*. Mutant Zn-cyts *c* were equilibrated overnight in a 10-fold molar excess of DTT, which was removed before labeling by gel

filtration. Alexa Fluor 488 maleimide (Invitrogen) was dissolved in 10 mM Tris (pH 7.8) to a concentration of 1 mM. The protein and 10-fold molar excess AF were stirred for 2 h in the dark at room temperature. The labeled protein was applied to a DEAE Sepharose fast-flow column (GE Healthcare) and eluted with 10 mM Tris (pH 7.8). Dye labeling was confirmed by using absorption and fluorescence spectroscopy. AF labeling of mutant cyts *c* also was performed for control experiments. Labeling of the protein in a 1:1 fashion was verified by observation of the expected dye and heme (or ZnP) absorption band intensity ratios (Fig. S1).

FRET Studies of Denaturation. AF-labeled Zn cyts *c* were prepared in 10 mM Tris (pH 7.8) in the presence of various concentrations of GuHCl, yielding a total labeled protein concentration from 1.0×10^{-7} to 2.0×10^{-7} M and GuHCl concentrations ranging from 0 to 7 M. Small (microliter) amounts of sodium hydroxide were used to bring the pH of each sample to 7.0. Typical values for [GuHCl] for an unfolding experiment were 0, 0.18, 0.33, 0.57, 0.75, 0.98, 1.19, 1.38, 1.57, 1.8, 1.98, 2.22, 2.4, 2.54, 2.78, 3.03, 3.25, 3.48, 3.79, 3.98, 4.47, 4.96, 5.48, 5.95, 6.45, and 7.08 M. For the control studies, 1.0×10^{-7} M Alexa Fluor samples were prepared with several GuHCl concentrations (Fig. S5). The concentration of GuHCl in each sample was measured by refractive index (52). For fluorescence measurements all samples were excited at 495 nm and fluorescence spectra were collected with an integration time of 100 ms. Samples of Zn-E4Cyt *c* blocked with maleimide were directly excited with an excitation wavelength of 550 nm in a control study.

Analysis of FRET Data. The efficiency of the nonradiative FRET process (*E*) is highly dependent on the donor–acceptor separation and is given by (53):

$$E = \frac{R_0^6}{R^6 + R_0^6}, \quad [2]$$

where *R* is the donor–acceptor distance and *R*₀ is the Förster radius, defined to be the distance at which the energy transfer is 50% efficient (see *SI Methods* for calculation).

To relate fluorescence intensities of the donor and acceptor to FRET efficiencies, the following expression was used (54):

$$E = \frac{F_A}{F_A + \gamma F_D}, \quad [3]$$

*F*_A and *F*_D correspond to the acceptor and donor fluorescence intensities, respectively, and

$$\gamma = \frac{\eta_A \Phi_A}{\eta_D \Phi_D}, \quad [4]$$

where $\eta_{A(D)}$ corresponds to the detector efficiency at the emission wavelength of the acceptor (donor) and $\Phi_{A(D)}$ corresponds to the fluorescence quantum yield of the acceptor (donor) (see *SI Methods*).

By equating expressions 2 and 3, the donor–acceptor distance can be related to measured fluorescence intensities. To obtain the acceptor fluorescence accurately the donor emission was normalized to 1 at the emission maximum. The donor emission was then subtracted from each spectrum to obtain only the acceptor emission. To obtain the donor fluorescence accurately the acceptor spectra were subtracted from each normalized donor spectrum. Additionally, a Gaussian curve was used to fit the blue side of the emission spectrum to obtain the total integrated counts of the donor. The error in distances measured by FRET was largely due to the error in determining *R*₀ and the error in determining the ZnP integrated fluorescence intensity. Global fitting of data were done to a three-state model (Eq. 1). Details of the fitting procedure are provided in *SI Methods*.

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