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Zinc Porphyrin as a Donor for FRET in Zn(II)cytochrome *c*

Andrea J. Lee, Amy A. Ensign, Todd D. Krauss,* and Kara L. Bren*

Department of Chemistry, University of Rochester, Rochester, New York 14627

Received October 29, 2009; E-mail: krauss@chem.rochester.edu; bren@chem.rochester.edu

Fluorescence resonance energy transfer (FRET) is a powerful technique for the study of the structure and function of biomolecules in that it allows for the direct measurement of distances on the nanometer scale. FRET is particularly useful for applications such as single-molecule (SM) spectroscopy,[1] and the need for fluorescently labeled molecules for ensemble and SM FRET has stimulated the development of new techniques for site-specific labeling of proteins,[2] as well as improvements to individual fluorophore photostability.[3] Even with these innovations, it is still difficult to site-specifically label a single amino acid chain with two fluorophores suitable for FRET.[2,4] Recently, it was demonstrated that a zinc porphyrin (ZnP) cofactor in Zn(II)-substituted horse heart cytochrome *c* (Zn cyt *c*) can serve as a FRET acceptor in protein folding studies.[5] Since ZnP is an intrinsic fluorophore, FRET studies of Zn cyt *c* require attachment of only one dye molecule, simplifying the labeling procedure while minimizing perturbation to the protein structure. However, the applicability of ZnP as an acceptor for SM FRET studies is inherently limited by the low fluorescence quantum yield (QY) of the cofactor.[5] Here we show that ZnP is an efficient FRET donor to an Alexa 660 (A660) dye acceptor in Zn cyt *c*. The large separation of donor (590 nm) and acceptor (690 nm) fluorescence energies allows for a simplified analysis of acceptor-sensitized emission, which results in more accurate FRET efficiency values than reported previously.[5]

To observe a range of donor−acceptor distances, we studied unfolding of Zn cyt *c* labeled with A660 at several different positions. The sites chosen for labeling were K99, E66, D50, K39, and E4 and are shown in Figure 1a.[6] Five variants with cysteine substituted for the above residues were constructed and purified (see Supporting Information). Zinc was substituted for iron in the cyt *c* variants using the hydrofluoric acid procedure previously reported,[5,7] and the protein was labeled with A660-maleimide using standard techniques (see Supporting Information). The absorption and fluorescence spectra of the ZnP donor and A660 acceptor are plotted in Figure 1b. For unfolding studies, A660-labeled variants ($\sim 3.0 \times 10^{-7}$ M) were prepared in 10 mM Tris, 50 mM NaCl, pH 7.5 in 0−5 M guanidine hydrochloride (GuHCl). The fluorescence spectrum of each sample was collected with excitation at the respective Soret peak maximum. Fluorescence spectra of A660-E66C-Zn cyt *c* as a function of GuHCl concentration are shown in Figure 2. The absorbance of each sample was also measured (see Supporting Information).

The folded form of A660-E66C-Zn cyt *c* shows emission at 690 nm when ZnP is excited at 420 nm, indicating FRET. In contrast, when A660 alone is excited by 420-nm light, no A660 fluorescence is observed (Supporting Information). As A660- labeled Zn cyt *c* is titrated with increasing concentrations of GuHCl, the fluorescence at 690 nm decreases with a concomitant increase of fluorescence at 590 and 640 nm (data for A660-E66C-Zn cyt *c* are shown in Figure 2a). The emission of free A660 and unlabeled Zn cyt *c* do not change significantly in concentrations of GuHCl between 0.25 and 5 M (Supporting Information); thus, we conclude that the

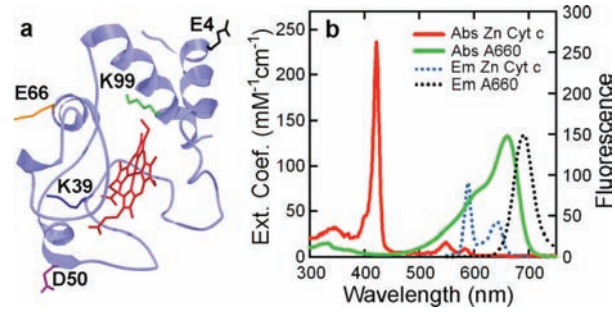

Figure 1. (a) Crystal structure of cyt *c* with labeling positions highlighted (PDB 1HRC). (b) Absorption and fluorescence spectra of unlabeled Zn cyt *c* and free A660 dye.

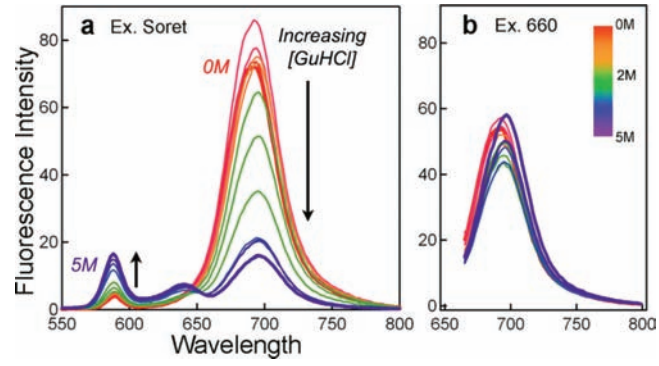

Figure 2. Fluorescence spectra of A660-E66C-Zn cyt *c* in various concentrations of GuHCl denaturant (0−5 M) with excitation at (a) the Soret (418−422 nm) and (b) 660 nm. As [GuHCl] increases, the fluorescence of A660 (660 nm) decreases and the fluorescence of ZnP (590, 640 nm) increases.

fluorescence changes that occur are due to changes in FRET efficiency. GuHCl-dependent changes were observed in fluorescence spectra of all labeled Zn cyt *c* variants (Supporting Information).

The FRET efficiency (*E*) was calculated using the following equation for sensitized emission of the acceptor:[8]

$$E = \left(\frac{F^{FRET}_{Soret}}{F^{A}_{660}}\right) * \left(\frac{\varepsilon^{A}_{660}}{\varepsilon^{D}_{420}}\right) * \frac{1}{d^{+}} \qquad (1)$$

where F^{FRET} is the fluorescence of A660-Zn cyt *c* measured at 694 nm with excitation at the Soret (418−420 nm), F^{A} is fluorescence at 694 nm with excitation at 660 nm, ε^{A}_{660} is the extinction coefficient of A660 at 660 nm (132 000 M^{-1} cm^{-1}, Invitrogen), ε^{D}_{420} is the extinction coefficient of A660-Zn cyt *c* at 420 nm (234 000 M^{-1} cm^{-1}),[7] and d^{+} is the fraction of donor labeled protein (assumed to be 1). This equation ignores the contribution of A660 absorption at 420 nm, as it is insignificant (Figure 1b).[8] The extinction coefficients of the dye and ZnP do not change significantly as a result of the addition of GuHCl (see Supporting

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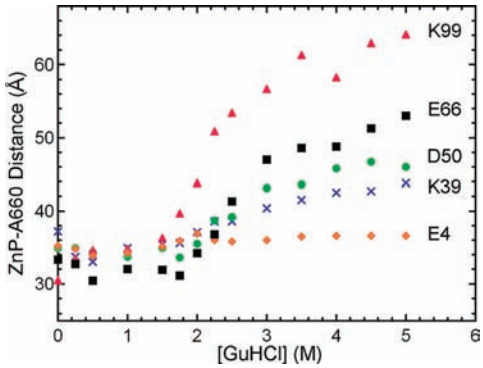

Figure 3. Measured distances between donor and acceptor in each dye-labeled Zn cyt *c* variant: K99C (red triangles), E66C (black squares), D50C (green circles), K39C (blue ×'s), and E4C (orange diamonds).

Table 1. FRET Distances for Folding of Zn cyt *c*

	ZnP−Dye Distance (Å)					
	Calculated[a]		ZnP Acceptor[a]		ZnP Donor[b]	
Position	Nat.[c]	Unf.[d]	Nat.	Unf.	Nat.	Unf.
E4	29 ± 4	22	29 ± 4	37 ± 6	35 ± 4	36 ± 4
K39	22 ± 2	34	28 ± 4	41 ± 6	37 ± 4	44 ± 5
D50	27 ± 5	44	30 ± 4	44 ± 7	35 ± 4	46 ± 5
E66	24 ± 2	55	32 ± 5	45 ± 7	33 ± 4	53 ± 6
K99	29 ± 3	74	30 ± 4	47 ± 7	30 ± 3	64 ± 7

[a] Taken from ref 5. [b] This work; see Supporting Information for calculation of distances and errors. [c] Determined by MD simulations. [d] Estimated for random coil and does not account for length of dye and tether.

Information). The FRET efficiency was used to calculate donor−acceptor distances using a Förster radius (R_0) of 39.7 Å (see Supporting Information),[9] which allows for observation of separations between ∼20 and ∼60 Å. The distance changes observed in the unfolding of the dye-labeled Zn cyt *c* variants were calculated from *E* in the standard manner (see Supporting Information),[5,9] and are plotted in Figure 3 and summarized in Table 1.

The distance changes during folding of Zn cyt *c* measured using ZnP as a donor show similar trends to those reported previously using FRET between Alexa 488 (A488) as a donor and ZnP as an acceptor (Table 1).[5] Differences in the specific distance values measured may stem from several sources. First, the physical properties of the dye used may affect the distance between the ZnP and the dye in Zn cyt *c*. The structure of A660 is not available, but it is possible that it is larger than A488. Second, the cyt *c* variants in the current study contain a six-histidine tag at the N-terminus of the protein. Though it appears that the overall trends for distance changes in the Zn cyt *c* variants are not greatly affected by this tag, it is possible that the relative position and folding of the N-terminus, as reflected by E4 and K39, might be altered. Finally, the improved signal-to-noise of the acceptor fluorescence, made possible by the use of the high QY A660 acceptor as opposed to the low QY ZnP, allows for detection of greater distances between the fluorophores.[8] Importantly, A660-K99C-Zn cyt *c* unfolding data were analyzed using both the method of sensitized emission (eq 1) and the method described in ref 5, and the same range of distances was derived (data not shown). One distinct advantage of the method of sensitized acceptor emission is a large reduction in precision error for measurement of *E*.[8]

The distances reported in Table 1 are necessarily the average values of a distribution of distances that occur in the ensemble of protein molecules. A study of the folding of individual protein molecules would probe the heterogeneity of the ensemble. The development of ZnP as a FRET donor may allow for SM studies of Zn cyt *c*. When ZnP was used as a FRET acceptor with the donor Alexa 488, there was significant spectral overlap between donor and acceptor fluorescence,[5] preventing independent detection of acceptor fluorescence in ensemble FRET studies. Conversely, the use of ZnP-A660 as a donor−acceptor pair not only allows for observation of high QY acceptor fluorescence but also yields a large separation of donor (590 nm) and acceptor (690 nm) emission peaks that would help to minimize crosstalk in single-molecule FRET experiments.

The use of ZnP as a FRET donor has several possible applications. The large extinction coefficient of ZnP results in enhanced A660 acceptor emission intensity, which permits sensitization of dyes that absorb in the red region of the spectrum (590 to 640 nm). ZnP may be used as a FRET donor in any protein system that can bind specifically to a heme cofactor. It may also be possible to engineer ZnP tags that could be attached to proteins,[10] allowing for FRET studies in systems that lack intrinsic fluorescence. Each of these applications has the potential to acquire FRET efficiency information based on sensitized emission of the acceptor (eq 1), eliminating many sources of measurement error, including incomplete donor or acceptor labeling and low QY of the donor.

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Supporting Information Available: Methods: construction and purification of Zn cyt *c* variants; labeling of Zn cyt *c* and calculation of QY of Zn cyt *c*, R_0, and *R*. Figures: emission spectra of unfolding of Zn cyt *c* variants; effect of GuHCl on the Alexa 660 dye emission; effect of GuHCl on A660-labeled Zn cyt *c* absorption spectra; effect of GuHCl on emission of unlabeled Zn cyt *c*. This material is available free of charge via the Internet at http://pubs.acs.org.

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