

## Electronic Supporting Information:

# Photophysical properties of Zn-Cytc investigated by single-molecule and ensemble-averaged spectroscopy.

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## Materials and methods

*Materials.* A horse heart cytochrome *c* (Cytc), guanidine hydrochloride (GdHCl) and PVA (Average M.W. ~ 13000) are purchased from Sigma-Aldrich.

*Preparation of Zn-Cytc.* Zn-substituted cytochrome *c* (Zn-Cytc) was prepared from horse heart cytochrome *c* (purity > 95 %) as previously reported.<sup>1</sup> All preparations were done under minimal light. All glassware was rinsed with concentrated acetic acid, and washed using deionized water to avoid contamination from trace metals. First, 10 ml of HF-pyridine (70% HF) was mixed with 30 mg of lyophilized Cytc in a Teflon beaker, stirred for 10 min, and then 5 ml of 50 mM ammonium acetate (pH 5.0) is added to the mixed solution for quenching of the generation of HF gas. Nitrogen was flowed over the beaker for 2 hours followed by 4 hours of dialysis against 10 mM sodium acetate (pH 5.0) and 10 mM DTT. The protein was applied to a CM Sepharose fast flow ion exchange column (GE Healthcare) equilibrated with 10 mM sodium acetate (pH 5.0). The integrity of the metal-free Cytc (H<sub>2</sub>Cytc) was confirmed by absorption spectroscopy: H<sub>2</sub>Cytc has characteristic peaks at 506, 540, 568, and 620 nm. Their appearance indicates the removal of the iron (III) ion from the heme. H<sub>2</sub>Cytc was concentrated by an Amicon ultrafiltration device with an YM-3 membrane (Millipore) and changed the buffer into 10mM 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 solution, and the resultant solution was incubated at 60°C for 10 min and then was centrifuged. The insertion of Zn was monitored by absorption spectroscopy. Excess zinc was removed by applying the sample to a PD-10 column equilibrated with 10 mM Tris buffer (pH 7.5). Zn-Cytc shows characteristic peaks at 423, 549 and 584 nm in the absorption spectrum.

*Measurement of the fluorescence intensity trajectory (FIT) by TIRFM.* The measurement of FIT of a single-molecule was performed with total internal reflection fluorescence microscopy (TIRFM). Since proteins can readily interact non-specifically with the slide surface and consequently the non-specific interaction between a protein and the slide surface can interfere a protein dynamics, FITs are measured in the vesicle conditions with TIRFM. In TIRFM, one creates an evanescent field of excitation light that extends only ~100-200 nm from the surface to which the sample bound, which

greatly reduces background fluorescence. In addition, encapsulation of single molecules inside surface-tethered phospholipids vesicles mimics cellular entrapment, reduces perturbation to the system and does not require tether attachment to the molecule.

For the single-molecule experiments, cover glasses ( $22 \times 22$  mm) were purchased from Matsunami Glass and cleaned by sonication in a 20% detergent solution (As One, Cleanace) for 2 hours, followed by repeated washing with running water for 30 min. Finally, the cover glasses were washed with Milli-Q water. An aqueous solution of Zn-Cytc (1 nM, 0.1 M phosphate buffer (pH7)) was coated onto the clean cover glass by spin coating at 2000 rpm for 50 s. The sample was enclosed in a homemade glove box, and the oxygen concentration inside the box was adjusted by an Ar gas purge. The experimental setup for single-molecule experiments is based on an Olympus IX71 inverted fluorescence microscope. Continuous-wave light emitted from a 532-nm diode laser that passed through an objective lens (Olympus, UPlanSApo, 1.40 NA,  $100\times$ ) after reflection at a dichroic mirror (Olympus, DM570) was used to excite the Zn-Cytc. The excitation beam of 532 nm was circularly polarized with quarter-wave plate (CVI Melles Griot, QWPO-532-10-4). The emission from Zn-Cytc encapsulated in the lipid vesicles glass was collected by an oil-immersion microscope objective, magnified by the built-in  $1.6\times$  magnification changer (thus, net magnification was  $160\times$ ), passed through an emission filter (Olympus, BA575IF (longpass filter  $> 575\text{nm}$ )) to remove the undesired scattered light, and imaged by electron-multiplying charge-coupled device (EM-CCD) camera (Rope Scientific, Cascade II:512). The images were recorded at a frame rate of  $10\text{ frames s}^{-1}$  and processed using ImageJ (<http://rsb.info.nih.gov/ij/>) or OriginPro 8.1 (OriginLab). All experimental data were obtained at room temperature. FITs for individual Zn-Cytc were collected by using ImageJ software. The FITs of  $>150$  single molecules was examined to determine  $t_{\text{On}}$  and  $t_{\text{Off}}$  of the folded and unfolded Zn-Cytc, respectively.  $t_{\text{On}}$  and  $t_{\text{Off}}$  from each FIT was determined calculated by the calculation method as shown in Fig. 2b. The calculated times were binned and plotted as a cumulative distribution as depicted in Figure 3a and S2. In order to precisely determine  $t_{\text{On}}$  and  $t_{\text{Off}}$ , these histograms were fitted by a single-exponential decay function. Figure S3 shows histograms of the duration times at on- and off-state of folded and unfolded Zn-Cytc under argon atmosphere and air conditions. The fitting results were summarized in Table 1.

*UV-Visible absorption and fluorescence spectra.* The steady-state UV-Visible absorption and fluorescence spectra in various GdHCl solutions were measured using a Shimadzu UV-3100 and a Hitachi 850, respectively.

*Measurement of the fluorescence lifetime of single Zn-Cytc by confocal laser scanning microscopy.* Confocal laser scanning microscopy is a technique for obtaining high-resolution optical images with depth selectivity. Unlike TIRFM using an evanescent field, the target molecule in the confocal laser scanning microscopy is directly excited. Thus, it is very difficult to measure the fluorescence lifetime of a biomolecule with a low fluorescence quantum yield ( $\Phi_{\text{fl}}$ ) because of the fast photobleaching due to the direct excitation. Substantially Zn-Cytc ( $\Phi_{\text{fl}} \sim 0.055$ ) encapsulated in vesicles is rapidly photobleached by the direct excitation. Therefore, we measured the fluorescence

lifetime of single Zn-Cytc embedded in 1 % polyvinyl alcohol (PVA) film on a glass coverslip without GdHCl. Since PVA is a hydrophilic polymer used in the single-molecule experiment, the photophysical properties and structure of a protein should not be affected by the addition of 1 % PVA by weight. Thin film was prepared by spin casting 40  $\mu\text{L}$  of 1 % PVA solutions onto a microscope cover slip and was dried in air. The samples were excited through an oil objective (Olympus, UAPON 150XOTIRF; 1.45 NA, 150x) with a 405-nm pulsed laser (PicoQuant, full width at half-maximum 120 ps, PicoQuant) controlled by a PDL-800B driver (PicoQuant). The excitation power of  $\sim 0.15 \mu\text{W}$  was used. The emission was collected with the same objective and detected by a single photon avalanche photodiode (Micro Photon Devices, PDM 50CT) through a dichroic beam splitter, bandpass filter (Semrock, FF01-609/152-25), and 50- $\mu\text{m}$  pinhole for spatial filtering to reject out-of-focus signals. The data collected using the PicoHarp 300 TCSPC module (PicoQuant) were stored in the time-tagged time-resolved mode (TTTR), recording every detected photon with its individual timing, which were used for the single-molecule analysis. All of the experimental data were obtained at room temperature.

*Time-resolved fluorescence spectroscopy.* The time-resolved fluorescence spectroscopy were carried out with the single photon counting method using a streak scope (Hamamatsu Photonics, C4334-01) equipped with a polychromator (Acton Research, SpectraPro150). The second harmonic oscillation (415 nm) of the output of the femtosecond laser (Spectra-Physics, Tsunami 3941-M1BB; full width at half-maximum (fwhm), 80 fs; 840 nm) pumped by a diode-pumped solid-state laser (Spectra-Physics, Millennia VIIIs) was used to excite a sample in a quartz cell.

### **Protocol of nanovesicle trapping for single-molecule experiments**

Nanovesicle was prepared in slight modification of literature methods.<sup>2</sup>

*Fabrication of flow cell.* A flow cell, formed by double-sided tape placed between two cover glasses (or cover glass and slide glass), was used to hold aqueous sample solutions for single-molecule fluorescence measurements. The chamber is thoroughly washed with the buffer (100  $\mu\text{L} \times 5$ ) and then filled with the buffer.

*Modification of biotinylated BSA and streptavidin on the cover glass.* A biotinylated BSA solution (1 mg/ml) (Invitrogen) in 150 mM NaCl, 10 mM HEPES buffer (pH 7.4) was first introduced into the channel. After incubation for 10 min, the channel was flushed with the buffer (100  $\mu\text{L} \times 3$ ), and streptavidin solution (0.25 mg/ml, 100  $\mu\text{L} \times 2$ ) was incubated for 10 min.

*Preparation of Nanovesicles.* A mixture of L- $\alpha$ -phosphatidylcholine (eggPC) (2.5 mg) and 1% 1,2-dipalmitoyl-sn-glycero-3-phosphoethanolamine-N-(cap biotinyl) (16:0 biotinyl cap PE) (0.25 mg) (Avanti Lipids) in chloroform (500  $\mu\text{L}$ ) (Wako,  $\infty$ pure) was dried under a constant flow of argon gas. For preparing the bilayer support, lipids were hydrated with the buffer (150 mM NaCl, 10 mM HEPES, pH 7.4) (500  $\mu\text{L}$ ) to form multilamellar vesicles at a concentration of 5 mg/mL total lipids. The folded or unfolded protein loaded vesicles were prepared by hydrating the lipid film with solutions containing 1  $\mu\text{M}$  folded or unfolded protein in the buffer. To increase the encapsulation efficiency, the resulting solution was subjected to 10 freeze-thaw-sonicate-vortex cycles, where

“freeze” indicates 3 minutes in a freezer, “thaw” indicates 1 minute resting at 40 °C, “sonicate” indicates 5 minutes of sonication at 40 °C, and “vortex” indicates 10 seconds of vortexing after the sonication step. Throughout this step, the solutions transformed from heterogeneous to opaque, and finally became translucent, indicating a reduction in vesicle size. The solution is then repeatedly extruded through a PTFE membrane with 100 nm pores to form 100-nm diameter unilamellar vesicles encapsulating proteins or dyes. Loaded vesicles should be used for experiments immediately or within 48 hours from preparation.

*Modification of Nanovesicles on the cover glass.* The as-prepared nanovesicle solution is applied (50  $\mu\text{l} \times 2$ ) and incubated for 20 min in a humid environment. The chamber is thoroughly washed with the buffer (100  $\mu\text{l} \times 5$ ) and then filled with the buffer.

### Electron transfer in a protein moiety

Kiefhaber and coworkers reported that the time constants for contact formation over short distances are almost independent of chain length, with a maximum value of about 5 ns for flexible glyceric chains and of 12 ns for stiffer chains, whereas the rates of contact formation over longer distances decrease with increasing chain length.<sup>3</sup> This result implies that the rate of the electron transfer should be slower in the denatured state. However, Chang et al reported that denaturation of Ru(His-33)-Zn-Cytc produces species in which intramolecular ET is faster than the bond-mediated tunneling process in the folded molecule.<sup>4</sup> They explained this behavior in terms of the intrachain diffusion in the denatured polypeptide brings \*ZnP and  $\text{Ru}(\text{NH}_3)_5(\text{His-33})^{3+}$  into close contact before ET. This interpretation is consistent with our results. In main text, we explain that the shorter on-state duration time observed for the denatured Zn-Cytc can be interpreted by the faster intramolecular ET reaction compared with that in the folded protein matrix. Since the denatured protein has a more flexible structure compared to the folded protein, the probability of the van der Waals interaction between Zn-porphyrin and amino acids will be significantly increased with the protein unfolding.

### References

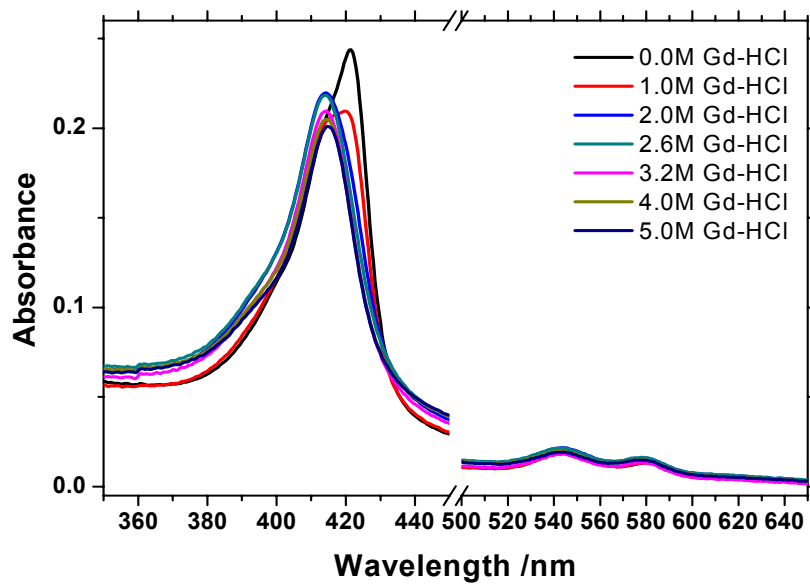
1. a) J. M. Vanderkooi, F. Adar and M. Erecinsk, *Eur. J. Biochem.* 1976, **64**, 381-387.; b) S. Ye, C. Sben, T. M. Cotton and N. M. Kostić, *J. Inorg. Biochem.* **1997**, 65, 219-226.
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**Table S1.** The fluorescence decay times of Zn-Cytc as a function of the concentration of GdHCl in 100 mM phosphate buffer (pH 7.0).

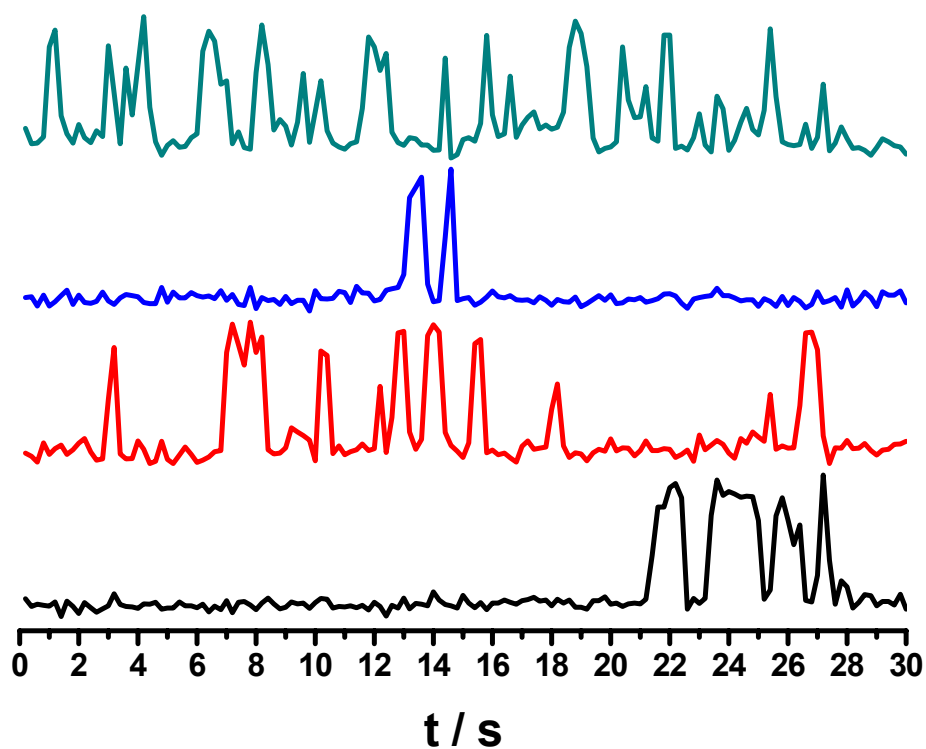
[GdHCl] / M	Zn-Cytc			
	$\tau_1$ / ns	$a_1$	$\tau_2$ / ns	$a_2$
0.0	$0.19 \pm 0.01$	0.43	$1.95 \pm 0.02$	0.57
1.0	$0.19 \pm 0.01$	0.43	$1.82 \pm 0.02$	0.57
2.0	$0.17 \pm 0.01$	0.45	$1.65 \pm 0.01$	0.55
2.6	$0.19 \pm 0.01$	0.46	$1.69 \pm 0.02$	0.54
4.0	$0.16 \pm 0.01$	0.52	$1.56 \pm 0.02$	0.48

·  $\tau_1$  and  $\tau_2$  denote the shorter and longer lifetimes of the fluorescence, respectively.

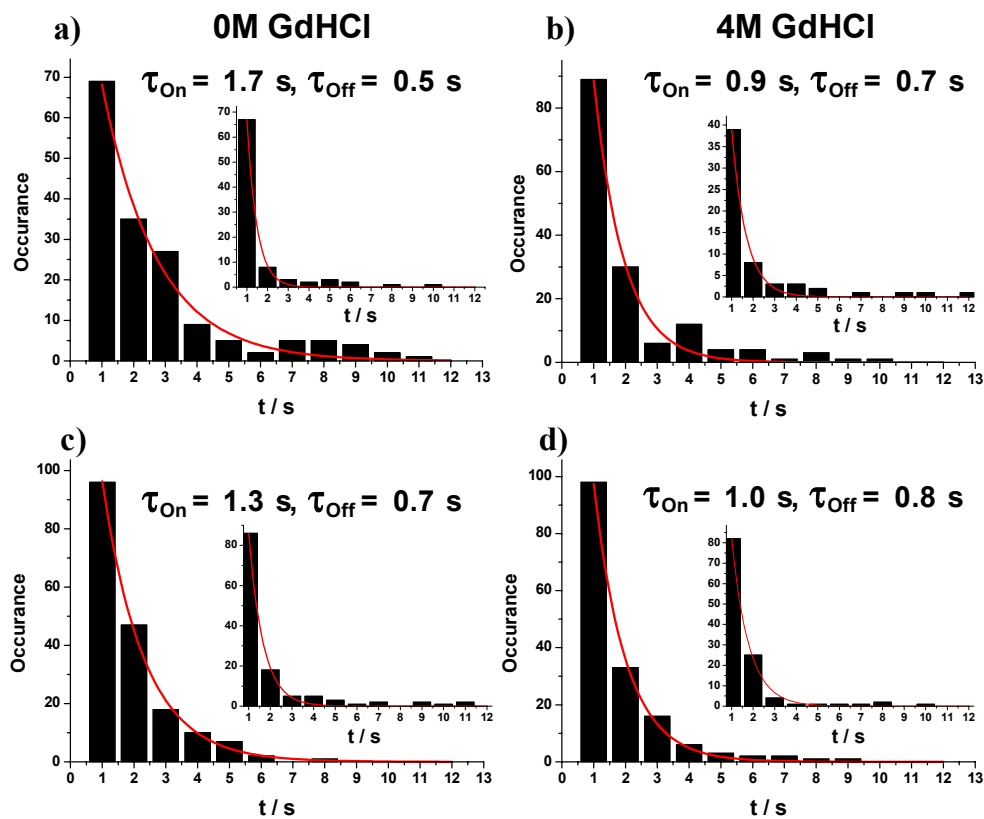
·  $a_1$  and  $a_2$  are the preexponential factors.



**Fig. S1.** Absorption spectra of Zn-Cytc as a function of a denaturant (GdHCl) in 100 mM phosphate buffer (pH 7.0).



**Fig. S2.** Representative FITs of single-molecule Zn-Cytc encapsulated in vesicles. (Black: without GdHCl under air; Red: without GdHCl under Ar; Blue: with GdHCl under air; Green: with GdHCl under Ar).



**Fig. S3.** (a and c) Histograms of the duration times at on-state of folded and unfolded Zn-Cytc under air atmosphere, respectively. (b and d) Histograms of the duration times at on- and off-state of folded and unfolded Zn-Cytc under argon atmosphere, respectively. The red solid lines denote the single exponential fitting results. The insert shows the histograms of the duration times at off-state determined in each experimental conditions.