# Photophysical properties of Zn-substituted cytochrome *c* investigated by single-molecule and ensemble-averaged spectroscopy†

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**The study of the structural reorganization and photophysical properties of Zn–Cyt*c* using the single-molecule and ensemble-averaged spectroscopy shows that the photoblinking behaviors of single-Zn–Cyt*c* depend on the folded and unfolded structures, whereas the fluorescence dynamics of Zn–Cyt*c* observed in the bulk phase are hardly affected by the conformational change of a protein.**

Since the protein structure is intimately related with the function of a protein, understanding the conformational change of a protein occurring in physiological condition is of critical importance. Numerous experimental and theoretical studies in the single-molecule and microscopic level have been performed to provide such information.[1] Studies of the protein dynamics in the single molecule level have given new information and insight on the reaction mechanism of various proteins occurring in physiological system, whereas most of the studies carried out in bulk phase have difficulty in revealing the important dynamic or mechanistic features hidden under ensemble-averaged results. To provide convincing and comprehensive assignments of protein kinetics, a combination of the single-molecule and the ensemble-averaged spectroscopy is highly desirable.

Zn-substituted cytochrome *c* (Zn–Cyt*c*) has served as an excellent model system for the study of protein structural dynamics. The Zn(II) ion of Cyt*c* is axially bound to two side chains of the Met80 and His18 amino acid residues (Fig. 1a). Beck and coworkers used time-resolved fluorescence spectroscopy to study the structural reorganization of Zn–Cyt*c* in physiological conditions.[1d,2] Unlike the wild type cytochrome *c* (Fe–Cyt*c*), the native Zn–Cyt*c* exhibits a long-lived fluorescence spectrum with resolved vibronic structure. Based on their results, they proposed that the dissociation of Zn(II)'s axial ligands induced by the laser irradiation leads to the reorganization of the protein conformation from its folded state to its transient or unfolded state. Here we have investigated the correlation between the structural reorganization and photophysical properties of Zn–Cyt*c* using the single-molecule spectroscopy as well as ensemble-averaged spectroscopy.

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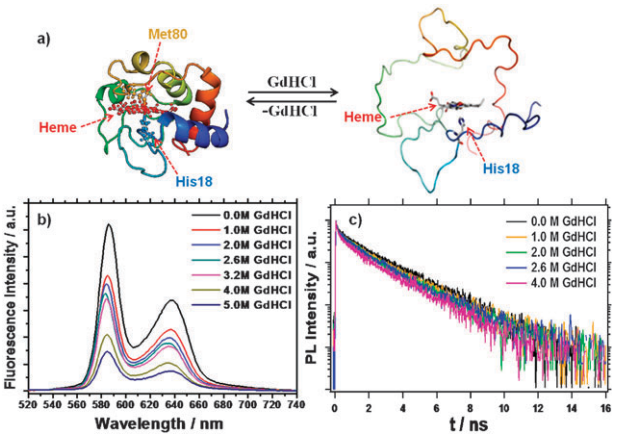


**Fig. 1** (a) Schematic illustration of the transition between folded and unfolded Zn–Cyt*c* induced by a denaturant such as GdHCl. (b) Fluorescence spectra of Zn–Cyt*c* as a function of the GdHCl concentration. (c) Fluorescence decay profiles of Zn–Cyt*c* as a function of the GdHCl concentration in 100 mM phosphate buffer (pH 7).

From the ensemble-averaged experiments (time-resolved fluorescence spectroscopy), we found that the observed photophysical properties of Zn–Cyt*c* are hardly affected by the protein conformational change induced by a denaturant, indicating that time-dependent fluorescence traces from a bulk sample are not sensitive to the structural reorganization of a protein followed by the ligand dissociation reaction. On the other hand, the quantitative analysis of fluorescence intensity trajectories (FITs) of the folded and unfolded single-proteins shows that the photoblinking behaviors of Zn–Cyt*c* are highly sensitive to the protein structure. In this communication, we will discuss the correlation between the structural reorganization and photophysical properties of Zn–Cyt*c*.

Upon increasing the concentration of the denaturant, guanidine-hydrochloride (GdHCl), the absorption maxima of the Soret band of Zn–porphyrin in Zn–Cyt*c* slightly blue-shifts from 421 nm whereas the Q band around 532 nm exhibits similar spectroscopic features (see Fig. S1 in ESI†). On the other hand, the fluorescence intensity decreases with the increasing GdHCl concentration, indicating that the environment of Zn–porphyrin is influenced by the GdHCl-induced conformational change. In order to further investigate the photophysical properties of Zn–porphyrin in the protein moiety, we have measured the fluorescence lifetime of Zn–porphyrin as a function of the GdHCl concentration (Fig. 1b). As depicted in Fig. 1c, Zn–Cyt*c* in the absence of the denaturant exhibits biexponential decay feature, with a major fast-decay component of ~200 ps along with a

slow-decay component ~2 ns. As shown in Fig. 1c and Table S1 (ESI†), upon increasing the concentration of GdHCl, Zn–Cyt*c* shows similar dynamic features with a fast decay followed by a slow decay regardless of the conformational change of a protein induced by a denaturant although the time constants slightly decrease with the increasing GdHCl concentration. This implies that the observed photophysical properties of Zn–porphyrin are not affected by the protein conformational change. These results are very consistent with those of Ensign *et al.*[1b] They reported that Zn–porphyrin in Zn–Cyt*c* mutant (E4C) is unaffected by the GdHCl-induced protein unfolding. However, these results are in contrast with the proposal by Beck and colleagues.[1d] They reported that the vibronic structure of the Q-band fluorescence spectrum of Zn–porphyrin in the folded Zn–Cyt*c* exhibits a biexponential response ($\tau_1$ = 120 ps, $\tau_2$ = 7 ns), with the 0–0 band decreasing and the 0–1 band increasing in intensity in a mirror-image fashion with respect to time. They interpreted these changes in terms of the sequential photodissociation of the Zn(II) ion's two axial ligands (Met80 and His18). Furthermore, they suggested that the breaking of the first axial-ligand bond is rate limited by the reorganization of the surrounding protein matrix, which in turn is damped by the surrounding solvent and the ligand dissociation process can induce the reorganization of the protein conformation from its folded state to unfolded state.[2] However, direct evidence for the ligand dissociation dynamics and conformational dynamics of Zn–Cyt*c* has not been provided. Generally, it is well known that Zn(II) ion of Zn–Cyt*c* in the native structure is bound to both His18 and Met80 in solution, implying that Zn(II) ion has a six-coordinate configuration.[3] On the other hand, Anni *et al.* suggested that Zn(II) ion in denatured state is penta-coordinated losing the contact with Met80,[4] based on the results obtained from the fluorescence line narrowing (FLN) studies of Zn–Cyt*c* by Logovinsky *et al.*[5] Thus, if the fast decay component was related to the fast ligand dissociation dynamics (Met80–Zn) as suggested by Beck and colleagues, the fast decay component observed in the fluorescence lifetime experiments would have disappeared in the denatured state because the Zn(II) ion in denatured protein is bound only to His18 as an axial ligand. However, our results show that Zn–Cyt*c* exhibits similar dynamic features of a fast decay followed by a slow decay regardless of the GdHCl concentration, indicating that the two relaxation dynamics of Zn–porphyrin in both folded and denatured Zn–Cyt*c* observed in this study are not the consequence of the sequential photodissociation of the Zn(II) ion's two axial ligands and the structural reorganization of a protein. Recently Luo *et al.* reported the ultrafast relaxation of Zn–porphyrin in myoglobin using femtosecond fluorescence up-conversion spectroscopy.[6] They analyzed three relaxation dynamics observed from Zn–porphyrin as follows: (i) the fast-decay component (several hundreds of femtoseconds) pertains to an efficient energy transfer from the hot $S_1$ Zn–porphyrin to Mb through a bond between the Zn ion and the proximal histidine; (ii) the slow one (several tens of picoseconds) arises from the water-induced vibrational relaxation of the hot $S_1$ Zn–porphyrin; and (iii) the very slow component is due to $S_1 \rightarrow T_1$ intersystem crossing of the surviving cold $S_1$ Zn–porphyrin. In this regard, the two relaxation dynamics observed in this study can be interpreted in terms of the vibration relaxation of the hot $S_1$ Zn–porphyrin and the lifetime of the singlet state of Zn–porphyrin rather than the structural reorganization and ligand dissociation. In other words, we suggest that the fluorescence dynamics of Zn–Cyt*c* in the bulk phase is not associated with the conformational change of a protein.

In order to further study the changes in the photophysical properties induced by the conformational changes of Zn–Cyt*c*, we have measured the single-molecule photoblinking properties of Zn–Cyt*c*. The fluorescence lifetime of the native single-Zn–Cyt*c* embedded in 1% polyvinyl alcohol (PVA) film is determined to be 0.2 and 2.5 ns (see Fig. 2a), which are slightly longer than that measured in the bulk phase (0.2 and 2 ns). On the other hand, the FITs of the folded and denatured single-protein encapsulated in lipid vesicles exhibit one-step photo-bleaching and on–off blinking behaviors as shown in Fig. 2b, which are the characteristic features of the single-molecule. The quantitative analysis of the FITs of the folded and denatured Zn–Cyt*c* shows that the determined duration time at off-state of Zn–porphyrin ($t_{Off}$) is significantly longer than the lifetime of the triplet state of Zn–porphyrin in the folded and denatured Zn–Cyt*c* (10 and 1 ms, respectively).[1c,7] This result suggests that the dark state (off-state) is not the triplet state. However, the on-state duration time of the folded Zn–Cyt*c* is sensitive to oxygen whereas that of the denatured Zn–Cyt*c* is not (Table 1), implying that nonradiative decay process from on- to off-state of the folded form is affected by oxygen, a triplet quencher. These results indicate that although the dark state is not the triplet state, the transition to the dark state can take place through the triplet state. In this respect, the increase of $t_{On}$ and the decrease of $t_{Off}$ observed for the folded Zn–Cyt*c* upon oxygen exposure is probably due to the decrease of the transition probability from the triplet state to the dark state, because ground-state oxygen efficiently

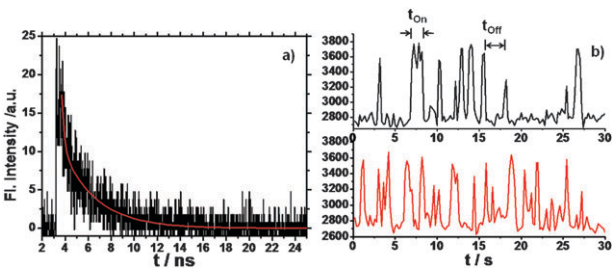


**Fig. 2** (a) Fluorescence decay profile of single Zn–Cyt*c* embedded in 1% polyvinyl alcohol (PVA) film ($\lambda_{Ex}$ = 405 nm). The theoretical fits obtained from the fitting analysis are shown in red. (b) Typical FITs of single-molecule Zn–Cyt*c* encapsulated in lipid vesicles (black: without GdHCl under Ar; red: with GdHCl under Ar).

**Table 1** On- and off-state duration times of Zn–Cyt*c* in lipid vesicles

| | Under air conditions | | Under Ar atmosphere | |
|---|---|---|---|---|
| [GdHCl]/M | $t_{On}$/s | $t_{Off}$/s | $t_{On}$/s | $t_{Off}$/s |
| 0.0 | 1.7 ± 0.1 | 0.5 ± 0.1 | 1.3 ± 0.1 | 0.7 ± 0.1 |
| 4.0 | 1.0 ± 0.1 | 0.7 ± 0.1 | 1.0 ± 0.1 | 0.8 ± 0.1 |

 

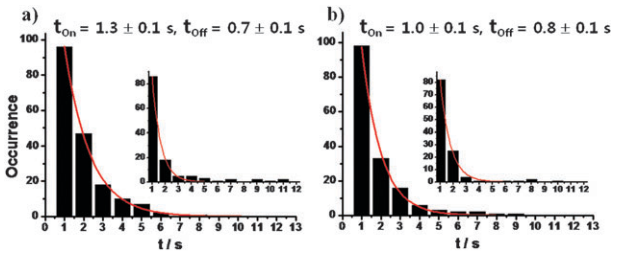


**Fig. 3** Histograms of the duration times at on- and off-state of folded (a) and unfolded (b) Zn–Cyt*c* under argon atmosphere. The red solid lines denote the single exponential fitting results. The insets show the histograms of the duration times at off-state. The error is the standard deviation determined by the fitting.

quenches the Zn–porphyrin in the triplet state to ground state *via* energy transfer.

It is an interesting observation that the on-state duration times of Zn–Cyt*c* under air and Ar atmospheres are significantly decreased with the protein unfolding, whereas the off-state duration times are slightly increased by the protein unfolding reaction, indicating that the $t_{On}$ and $t_{Off}$ observed for Zn–Cyt*c* significantly depend on the protein structure (Fig. 3 and Table 1). Here we now discuss the origin of the off-state (or dark state) observed for the folded and denatured Zn–Cyt*c*. From many studies for the off-state in a variety of molecular systems composed of metal–porphyrins and polymers, it is generally accepted that the blinking behavior stems from the intermolecular charge-transfer between the excited state of molecule and the surrounding polymer matrix.[8] Thus, the simplest scenario is that the observed off-state in our study is a charge-transfer state formed by the intermolecular or intramolecular charge-transfer. Generally, the formation of a long-lived Zn–porphyrin cation-radical can induce the transition to the dark state of single molecules.[9] Morishima *et al*. reported that ZnTPP cation-radicals can survive in some confined molecular environments for minutes.[9a] Hochstrasser *et al*. suggested that the involvement of cation radicals of chlorophylls may be responsible for the occurrence of dark states of single molecules of light-harvesting complexes (LH2), and dark states also occur when the oxygen pressure is very low, so it is quite likely that the triplet states react with the matrix in electron-transfer (ET) processes to produce cation-radicals.[10] Furthermore, Durrant *et al*. observed direct ET from immobilized Zn–Cyt*c* into a nanocrystalline $TiO_2$ electrode on the nanosecond timescale and the resultant photogenerated charge-separated state is remarkably stable, with a $t_{1/2}$ of 0.2 s.[9b,11] Furthermore, theoretical and experimental studies for the intramolecular ET in the photoexcited Zn–Cyt*c* show that the long distance ET in photoexcited Zn–Cyt*c* is originated by a hole-transfer through the occupied molecular orbitals.[12] Therefore, according to the previous studies and our results, we suggest that the off-state observed for Zn–Cyt*c* is a charge-separated state formed by the intramolecular ET in the protein matrix. In this respect, the shorter on-state duration time observed for the denatured Zn–Cyt*c* 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 (see ESI†).

In conclusion, we have observed the correlation between the structural reorganization and photophysical properties of Zn–Cyt*c* using the single-molecule and the ensemble-averaged spectroscopy. Results presented here show that the photobleaching behaviors of single-Zn–Cyt*c* significantly depend on the structure of a protein, whereas the fluorescence dynamics of Zn–Cyt*c* observed in the bulk phase are hardly affected by the conformational change of a protein induced by a denaturant. This implies that a combination of the single-molecule and the ensemble-averaged spectroscopy is necessary to provide comprehensive assignments of protein dynamics. In future work, we intend to verify the intramolecular electron transfer of a protein and the correlation between the dark state and the oxygen.

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