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Energy migration within hexameric hemoprotein reconstituted with Zn porphyrinoid molecules†

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Photosensitizers, Zn protoporphyrin IX and Zn chlorin e_6, are completely inserted into each heme pocket of a hexameric apohemoprotein. The fluorescence quenching efficiencies upon addition of methyl viologen are 2.3 and 2.6 fold-higher than those of the partially photosensitizer-inserted proteins, respectively, indicating that the energy migration occurs within the proteins.

An array of natural pigments achieves efficient capture of sunlight in natural photosynthetic systems.[1] For example, LH2, a simple light harvesting complex from purple bacterium containing a precise array of eighteen Mg-bacteriochlorin molecules, demonstrates energy migration *via* successive and rapid energy transfer within protein matrices.[2] To mimic such a structure and function, a number of efforts to reproduce an array of photosensitizers (especially porphyrin derivatives) have been undertaken using synthetic,[3] supramolecular,[4] and coordination-bonding[5] approaches. Several proteins have also been found to provide appropriate scaffolds for accumulating photosensitizers by supramolecular interactions[6] or covalent modifications.[7] In this context, we have focused on the use of the native oligomer of hexameric tyrosine-coordinated heme protein (HTHP) from the marine bacterium *Silicibacter pomeroyi*[8] as a scaffold for construction of a new array of photosensitizers. Dobbek and coworkers reported that HTHP is a C_6-symmetric toroidal hexamer with each domain including a 75-residue peptide chain consisting of three α-helices and a non-covalently bound iron protoporphyrin IX, heme *b* cofactor. According to the crystal structure, the heme–heme distance is 18 Å (Fig. 1b). Although the native function of HTHP remains unknown, the unique structure and its peroxidase and catalase activities have been investigated.[8] Here, we report that reconstitution of the hemoprotein with zinc porphyrinoid complexes[9] provides an array of photosensitizers with well-defined orientations in the HTHP matrices (Fig. 1). The modified HTHP is found to provide a model of the light harvesting complex which demonstrates energy migration within an array of chromophores. The present system will serve as the versatile strategy to create a light harvesting complex based on a series of porphyrinoid photosensitizers.

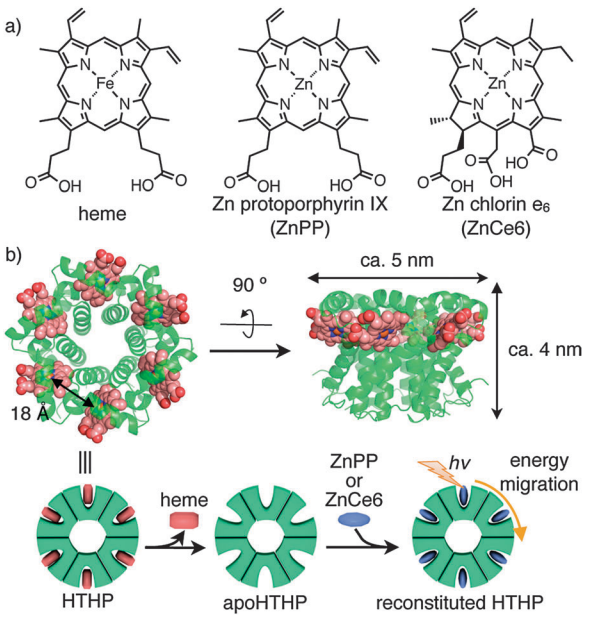

Fig. 1 (a) Molecular structures of heme and Zn porphyrinoids, ZnPP and $ZnCe_6$. (b) Crystal structure of HTHP (PDB ID; 2OYY) and schematic representation of the preparation of reconstituted HTHP.

HTHP is expressed in a recombinant *E. coli* system and purified by anion exchange and size exclusion chromatography (SEC). Analytical SEC (Fig. 2a) and DLS (dynamic light scattering, Table S1, ESI†) reveal a monodisperse species with a hydrodynamic diameter of 5.4 nm, which is consistent with the value expected from the hexameric structure observed in X-ray crystallography,[8]

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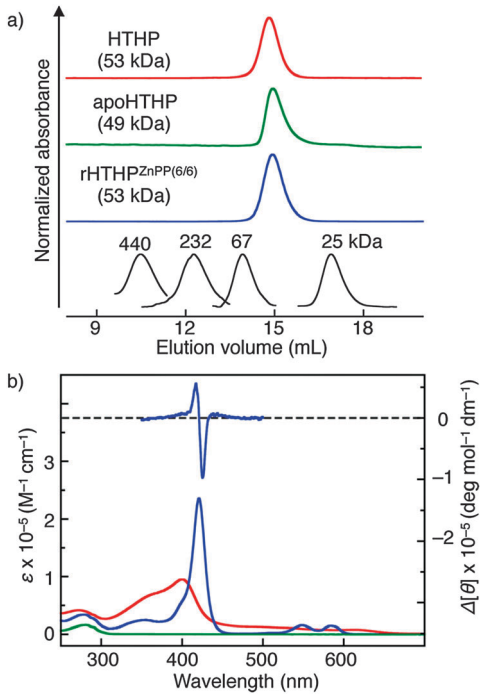

Fig. 2 (a) Analytical SEC traces and (b) UV-vis absorption and CD spectra of HTHP (red), apoHTHP (green) and rHTHP$^{ZnPP(6/6)}$ (blue). In (a), black traces show protein standards, and red, green, blue and black traces were detected by absorptions at 402, 280, 421, and 280 nm, respectively. In (b), the upper blue line is a differential CD spectrum generated by the subtraction of the CD spectrum of rHTHP$^{ZnPP(1/6)}$ from that of rHTHP$^{ZnPP(6/6)}$.

and ESI-TOF MS (Fig. S1, ESI†) shows the desired mass numbers of the multiply ionized holo-hexameric species: found m/z = 3318.2 and 3539.4; calcd m/z = 3318.5 (z = 16+) and 3539.7 (z = 15+). The apo-form of HTHP (apoHTHP) was prepared by a conventional method using acid and 2-butanone,[10] and the resulting protein has no absorption in the visible region (Fig. 2b). The hexameric structure in the apo-form was confirmed by analytical SEC and DLS measurements. In addition, the CD spectrum of apoHTHP in the far-UV region is consistent with that of HTHP, showing that the α-helices are maintained in the absence of the heme cofactors (Fig. S2, ESI†). Addition of excess amounts of Zn protoporphyrin IX (ZnPP) into an apoHTHP solution under pH-neutral conditions yields reconstituted HTHP (rHTHP$^{ZnPP(6/6)}$), where 6/6 represents the complete incorporation of the zinc complex into the six heme pockets in apoHTHP.[11,12] The UV-vis absorption spectrum of rHTHP$^{ZnPP(6/6)}$ has maxima at 421, 548, and 584 nm (Fig. 2b). This pattern is similar to that of tyrosine-coordinated ZnPP in human serum albumin.[13] Analytical SEC measurements for rHTHP$^{ZnPP(6/6)}$ indicate that the protein has the same elution volume as HTHP. DLS measurements indicate a hydrodynamic diameter of 5.6 nm and confirm that the thermodynamically stable hexameric structure is maintained.

Addition of apoHTHP into a rHTHP$^{ZnPP(6/6)}$ aqueous solution increases the intensity of fluorescence derived from ZnPP moieties (Fig. S4, ESI†), indicating re-equilibration toward reconstituted HTHP with less than six ZnPP molecules, rHTHP$^{ZnPP(n/6)}$, where n represents the apparent number of the photosensitizer molecules in the six heme pockets.[14] The fluorescence lifetime (τ) of rHTHP$^{ZnPP(6/6)}$ was determined to be 1.43 ± 0.01 ns, which is slightly shorter than that of rHTHP$^{ZnPP(1/6)}$ (τ = 1.56 ± 0.01 ns). Taken together with lower fluorescence intensity in rHTHP$^{ZnPP(6/6)}$, it appears that singlet–singlet annihilation occurs in the protein hexamer.[3a–c,4c,15] The visible absorption spectrum of rHTHP$^{ZnPP(n/6)}$ is similar to that of rHTHP$^{ZnPP(6/6)}$, indicating that the coordination environments of the two proteins are similar (Fig. S5, ESI†). This re-equilibration is also confirmed by a differential CD spectrum obtained by subtracting the spectrum of rHTHP$^{ZnPP(1/6)}$ from that of rHTHP$^{ZnPP(6/6)}$. The observed split type Cotton effect (Fig. 2b) induced by ZnPP–ZnPP exciton coupling strongly suggests the formation of conformationally-defined Zn porphyrin arrays.[16–18] These findings also indicate that the ZnPP molecules can be incorporated into each subunit of apoHTHP while maintaining the intrinsic hexameric structure, whereas re-equilibration upon addition of apoHTHP provides a mixture of incompletely-reconstituted photosensitizer-containing proteins.

Stern–Volmer plots of steady-state and time-resolved emission against the concentration of methyl viologen dichloride (MV^{2+}) are shown in Fig. 3a and Fig. S8, ESI,† respectively. Quenching of steady state fluorescence by MV^{2+} (Fig. S9, ESI†) was observed at relatively high concentrations ([MV^{2+}] >1 mM), whereas no changes in lifetimes upon the addition of MV^{2+} were observed (Fig. S10, ESI†).[19] This indicates static quenching of fluorescence of rHTHP$^{ZnPP(n/6)}$ by MV^{2+}. The slopes of the Stern–Volmer plots for steady state fluorescence of rHTHP$^{ZnPP(n/6)}$ were determined to be 21 M^{-1} (n = 6) and 9.2 M^{-1} (n = 1) as apparent binding constants. The actual binding constant of MV^{2+} for rHTHP$^{ZnPP(6/6)}$ evaluated by UV-vis spectral changes to form the charge-transfer complex

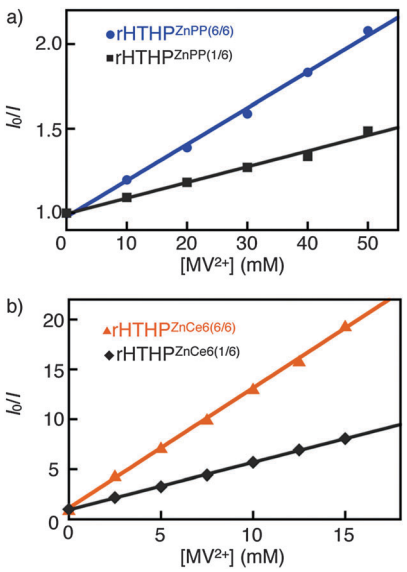

Fig. 3 Stern–Volmer plots of steady-state fluorescence against the concentration of methyl viologen as a quencher molecule for (a) rHTHP$^{ZnPP(n/6)}$ and (b) rHTHP$^{ZnCe_6(n/6)}$. Solid lines show corresponding least square fitting. Conditions: [ZnPP] = 4.0 μM or [$ZnCe_6$] = 2.0 μM in 100 mM potassium phosphate buffer, pH 7.0, at 25 °C under N_2 atmosphere, λ_{ex} = 421 nm (rHTHP$^{ZnPP(n/6)}$) and 418 nm (rHTHP$^{ZnCe_6(n/6)}$), λ_{em} = 592 nm (rHTHP$^{ZnPP(n/6)}$) and 645 nm (rHTHP$^{ZnCe_6(n/6)}$).

(Fig. S11, ESI†) is consistent with that of $rHTHP^{ZnPP(1/6)}$ (*ca.* 9 M^{-1}). Taken together, these results indicate that the higher apparent value of the protein hexamer fully occupied by photosensitizers is a result of efficient quenching which occurs due to the energy migration within the ZnPP molecule array.[20]

Similar results were obtained using Zn chlorin e_6 ($ZnCe_6$) instead of ZnPP (Fig. 1a). The reconstituted protein, $rHTHP^{ZnCe_6(6/6)}$, was also characterized by analytical SEC, DLS, UV-vis and CD spectroscopic measurements (Fig. S13 and Table S1, ESI†). The fluorescence intensity of $rHTHP^{ZnCe_6(n/6)}$ is found to depend on the *n* value, the ratio of the bound photosensitizer for apoHTHP. The apparent binding constants of MV^{2+} derived from the Stern–Volmer plots for steady state fluorescence of $rHTHP^{ZnCe_6(6/6)}$ and $rHTHP^{ZnCe_6(1/6)}$ are 1.2×10^3 M^{-1} and 4.7×10^2 M^{-1}, respectively (Fig. 3b). In contrast, the actual binding constants of MV^{2+} for $rHTHP^{ZnCe_6(n/6)}$ determined by UV-vis spectral changes (Fig. S15, ESI†) are 5×10^2 M^{-1} ($n = 6$) and 4×10^2 M^{-1} ($n = 1$).[21] Therefore, the 2.6-fold greater apparent binding constant of $rHTHP^{ZnCe_6(6/6)}$ relative to $rHTHP^{ZnCe_6(1/6)}$ suggests that the energy migration occurs within the $ZnCe_6$ array as well as $rHTHP^{ZnPP(6/6)}$.[20]

In conclusion, the present study demonstrates that the oligomeric hemoprotein is a versatile and useful model for detecting energy migration within assembled porphyrinoid photosensitizers with well-defined orientations in the heme binding sites. The present system is expected to contribute to generation of new efficient photo-catalysts and devices which harness the biological light harvesting function.

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16 The CD spectrum of $rHTHP^{ZnPP(1/6)}$ shows only a positive Cotton effect in the region from 360 nm to 480 nm and is generally consistent with that of $rHTHP^{ZnPP(0.7/6)}$, indicating a lack of cooperative binding of the cofactor molecules for adjacent monomers (Fig. S7, ESI†).
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21 The difference between the binding constants of MV^{2+} for $rHTHP^{ZnCe_6(n/6)}$ and $rHTHP^{ZnPP(n/6)}$ may be derived from the number of carboxylate groups of the cofactors.

