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Design of an Artificial Light-Harvesting Unit by Protein Engineering: Cytochrome b_{562}–Green Fluorescent Protein Chimera

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We have generated a novel model protein for an artificial light-harvesting complex composed of two proteins, cytochrome b_{562} (cytb_{562}) and enhanced green fluorescent protein (EGFP), in which two chromophores are fixed in each protein matrix. Cytb_{562} was appended to the N-terminus of EGFP via a Gly-Ser linker and the resultant fusion protein was successfully expressed in *Escherichia coli* as a mixture of the apo- and the holo-forms as to the cytb_{562} moiety. The fluorescence of EGFP was substantially quenched when the apo-form was reconstituted with hemin. Based on the fluorescence lifetime measurements, it appeared that light energy entrapped by EGFP is transferred to the heme of cytb_{562} by resonance energy transfer (energy transfer yield: 65%). Spatial organization of two chromophores using small and stable protein matrices will be promising toward the construction of an artificial light-harvesting complex by protein engineering.

Key Words: **cytochrome b_{562}; green fluorescent protein; fluorescence quenching; protein engineering; resonance energy transfer; light-harvesting complex.**

In the early step of photosynthesis, pigments absorb sunlight, and light energy is transferred from the light-harvesting complex (LHC) to the photosynthetic reaction center by resonance energy transfer. Within the LHC, several proteins maintain pigment molecules in the precise orientation and position that are optimal for light absorption and energy transfer (1, 2). Toward the construction of an artificial photosynthetic system, a number of attempts have been conducted so far by chemical (3, 4) and semisynthetic approaches (5, 6). Because natural biological systems are substantially more complex than synthetic models, chemical approaches have succeeded to mimic them to some extent and provided useful information on the photochemical event (1, 4). However, there have been no reports on the creation of an artificial photosynthetic reaction center (PRC) composing of several functional proteins possibly due to difficulties in reconstructing the unique structure of natural PRC. Hence, we have tried to create a simple model of protein-based artificial LHC because it might lead to the construction of an artificial PRC.

In LHC, there are subsets of s (sensitizing) and f (fluorescing) chromophores and efficient energy transfer is achieved from f- to s-chromophores (1). In this study, we have chosen two small proteins, enhanced green fluorescent protein (EGFP, 238 aa) and cytochrome b_{562} (cytb_{562}, 106 aa), having a chromophore per molecule, as a model of s- and f-proteins, respectively. EGFP is one of *Aequorea* GFP mutants (F64L, S65T) that show stable fluorescence (7), more than twofold molar absorption coefficient (ϵ_{488} = 55 mM^{-1} cm^{-1}) of wild-type GFP, and high quantum yield (0.60) (8). These characters are highly suitable for efficient collection of light energy. Cytb_{562} is a stable four-helix bundle protein and binds noncovalently a heme (iron-protoporphyrin IX) as the prosthetic group (9, 10). Although wild-type cytb_{562} does not exhibit fluorescence, the reconstitution of a zinc-porphyrin can yield cytb_{562} variant showing fluorescence (5). In addition, this protein can transfer an electron directly to electrodes (11) and the variants with different redox potentials were prepared (12), implying the availability for further investigation from an artificial LHC to PRC.

One of key requirements in the construction of an artificial LHC should be spatial arrangement of chromophores that act as an energy donor and an acceptor (1, 2). To fix the two chromophores of EGFP and cytb_{562}, the short peptide linker, Gly-Ser, was adopted to con-

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nect them. This article describes the preparation of the fusion protein (cytb_{562}–EGFP) as a model for an artificial LHC and the characterization of the fluorescence properties. The fluorescence lifetime measurements revealed that light energy entrapped by EGFP is transferred to the heme of cytb_{562}. This chimera, to our knowledge, is the first example showing successful organization of two chromophores using protein matrices by protein engineering.

MATERIALS AND METHODS

Gene construction. The gene encoding EGFP was obtained from pEGFP plasmid (Clontech, CA) by digesting with *Nco*I and *Not*I and cloned into pET32b(+) vector [a fusion expression system with *Escherichia coli* thioredoxin (TRX), Novagen, WI]. The cytb_{562} gene (13) subcloned into pUC118 (14) except periplasmic signal sequence was amplified by two PCR primers (forward, 5′-CGGAATTCCATGG-GATCCGCTGATCTTGAAGACAATATGC-3′; reverse, 5′-GGGGGTA-CCATGGAACCACGATACTTCTGGTGATAGGC-3′; both containing *Nco*I site, underlined). The amplified fragment was digested with *Nco*I and cloned into the pET32b(+)–EGFP vector above mentioned. DNA sequence was verified by conventional technique. The final gene construct encodes the fusion protein, TRX–cytb_{562}–EGFP, in which cytb_{562} and EGFP are connected by a Gly-Ser linker.

Protein preparation. The fusion protein with a thrombin cleavage site between TRX and cytb_{562} moieties was expressed in *E. coli* strain BL21(DE3)pLysS as described previously for the preparation of GFP fusion proteins (15). The fusion protein expressed (TRX–cytb_{562}–EGFP) was purified by affinity chromatography using $(His)_6$-tag between TRX and cytb_{562} moieties by Talon metal affinity resin (Clontech). After the purification, TRX and $(His)_6$-tag moieties were cleaved by thrombin (site-specific protease, Novagen) and TRX fragments and undigested fusion protein were removed by the affinity purification. The resultant fusion protein (cytb_{562}–EGFP) was applied to anion exchange chromatography (Poros PI, PE Biosystem; flow rate, 5.0 ml/min; mobile phase, 50 mM phosphate buffer, pH 9.0) to separate the apo- (apocytb_{562}–EGFP) and the holo- (holocytb_{562}–EGFP) forms. UV-visible spectroscopic analyses confirmed the separation of the two forms in the linear gradient of NaCl from 0 to 1 M. The apocytb_{562}–EGFP fraction was eluted prior to that of holocytb_{562}–EGFP and the protein content of the former fraction exceeded the latter fraction. Approximately 85% of fusion protein was collected as apocytb_{562}–EGFP. The fusion proteins are dialyzed against buffer used prior to spectral measurements. The reconstitution of apocytb_{562}–EGFP was performed with 1 mM dimethyl sulfoxide solution of hemin (Funakoshi Co., Japan). To obtain reconstituted holocytb_{562}–EGFP in high yield, all procedures were conducted at low temperature (4°C).

Spectroscopic measurements. UV-visible absorption and fluorescence spectra were recorded on V-550 UV-vis (JASCO, Japan) and F-2000 fluorescence (Hitachi, Japan) spectrophotometers, respectively. Time-resolved fluorescence measurements were carried out with a streak camera (Hamamatsu Photonics C4334, Japan) coupled with a spectrometer (JASCO CT-25CS, Japan). The 488 nm line obtained by converting the third harmonic of the pulsed Nd-YAG laser beam (EKSPLA PL2143B, Lithuania, wavelength 355 nm for excitation and PG-401, Lithuania) optical parametric generator for the wavelength conversion) was used for excitation. Photon was collected perpendicularly to the laser beam. The laser power was 6 mJ per pulse and the best time resolution of this system was 15 ps. All experiments were performed at room temperature.

RESULTS AND DISCUSSION

Design of the Fusion Protein and Separation of the Two Forms

Since s- and f-chromophores are properly fixed in natural LHC, much of efforts are directed to coordinate organic compounds in the construction of artificial antennae. As a result, molecular architecture tends to have a complex structure and often have multiple donors for a single acceptor for efficient collection of light energy. On the contrary, we focused our attention to make a simple energy transfer system consisting of an energy donor (EGFP) and an acceptor (cytb_{562}). The important point in the preparation of a bifunctional fusion protein is the design of linkers (15). In this study, the length of linker could also be critical determinant for fixing two chromophores and for efficient energy transfer. It seemed that a long linker could not control the orientation and the position of chromophores precisely, while a rigid short linker might prohibit the proper folding of the fusion protein. Hence, we decided to use a flexible short linker, Gly-Ser, for creating cytb_{562}–EGFP.

If the fusion protein expressed in *E. coli* folds properly, the absorption spectrum should be identical to the sum of the spectrum of cytb_{562} and EGFP. Since cytb_{562} binds heme in the periplasm (13), the chimera expressed in cytoplasm was a mixture of the apo- and the holo-forms as to the cytb_{562} moiety (data not shown). To investigate the effect of cytb_{562} moiety on the fluorescence property of EGFP, separation of the two forms was indispensable. An ordinary heme extraction technique from holocytb_{562} with 2-butanone under acidic condition (16) cannot be applied to obtain apocytb_{562}–EGFP because EGFP lost its fluorescence in such harsh condition. Finally, we found that the two forms can be successfully separated by anion exchange chromatography. One of the fractions showed clear Soret band around 420 nm, while the other fraction exhibited the absorption spectrum that is basically identical to that of EGFP (Fig. 1, inset). Furthermore, apocytb_{562}–EGFP appeared to be readily reconstituted with exogenous hemin. Hence, we could successfully compare the difference of spectroscopic characteristics between the two forms. The UV-visible absorption spectrum of holocytb_{562}–EGFP prepared by the reconstitution of the apo-form with hemin is shown in Fig. 1. It shows a sharp Soret band at 418 nm and two visible bands of wild-type cytb_{562} with the additional characteristic band of EGFP at 488 nm. Judging from these spectroscopic features, it can be concluded that the fusion protein was successfully constructed.

Fluorescence Properties

Figure 2 shows the fluorescence spectra of the apo- and holo-forms of the fusion proteins and the equimo-

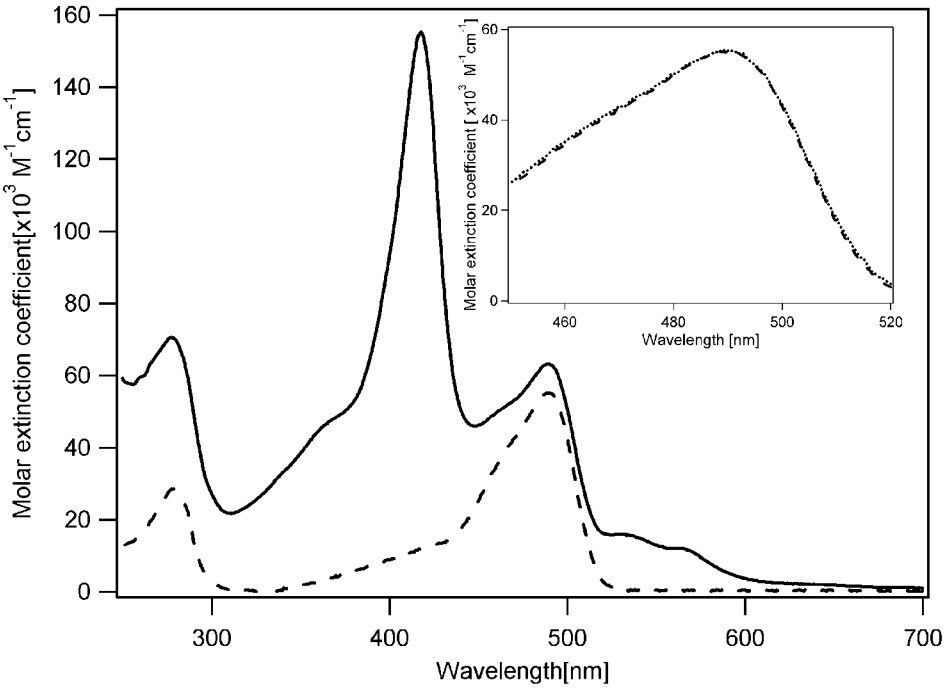

FIG. 1. UV-visible absorption spectra of holo- (solid line) and apo-cytb_{562}–EGFP (broken line) in 10 mM Tris–HCl buffer containing 100 mM NaCl at pH 7.5 and 15°C, respectively. (Inset) Dotted and broken lines are the spectra of EGFP and apocytb_{562}–EGFP, respectively.

lar mixture of holocytb_{562} and EGFP. It revealed that the apocytb_{562}–EGFP showed a strong fluorescence (peak maximum at 508 nm) upon the excitation at 488 nm, being consistent with the spectrum of EGFP. This implies that cytb_{562} appended to the N-terminus of EGFP does not affect the inherent fluorescence. On the

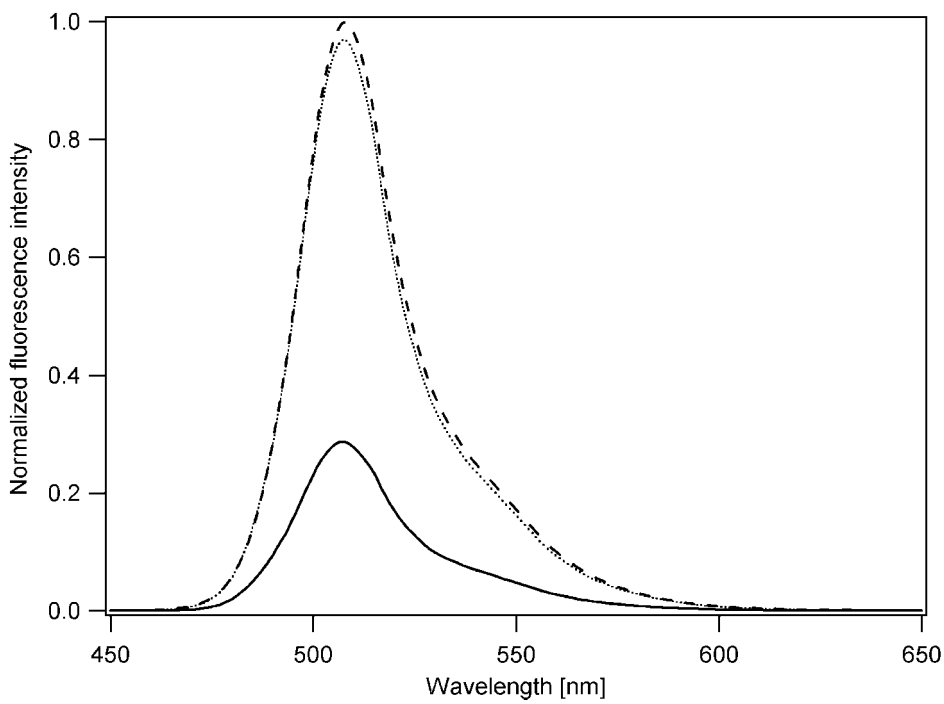

FIG. 2. Fluorescence spectra of holocytb_{562}–EGFP in 10 mM Tris–HCl buffer containing 100 mM NaCl at pH 7.5 and 20°C (solid line). Broken line corresponds to that of apocytb_{562}–EGFP before the reconstitution with hemin. Dotted line is that of equimolar mixture of holocytb_{562} and EGFP.

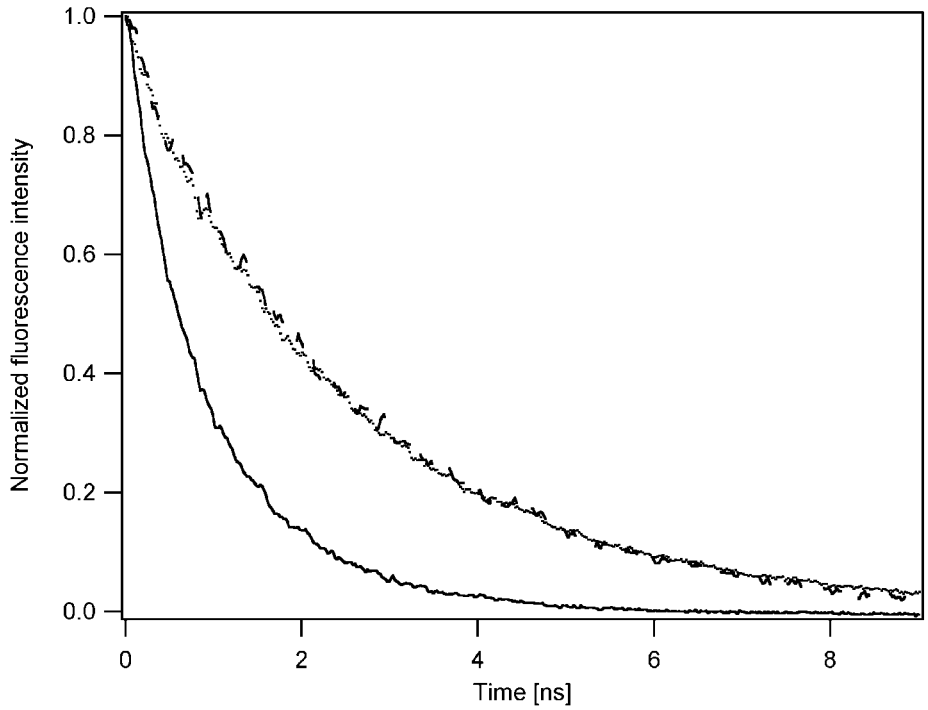

FIG. 3. Fluorescence decay of holocytb_{562}–EGFP in 10 mM Tris–HCl buffer containing 100 mM NaCl at pH 7.5 and 20°C (solid line). Broken and dotted lines correspond to those of apocytb_{562}–EGFP and EGFP, respectively. Data for the fluorescent emission changes were obtained at 500–520 nm.

other hand, holocytb_{562}–EGFP exhibited much weaker fluorescence compared to the apo-form, indicating the positive effect of the heme in the fluorescence quenching. It should be noticed that the equimolar mixture of holocytb_{562} and EGFP did not show any quenching although the concentration of chromophores was adjusted to the same as the holocytb_{562}–EGFP solution. These results clearly indicate that there is an intramolecular interaction between the two chromophores in holocytb_{562}–EGFP.

Time-Resolved Fluorescence Analyses

Typical experimental results in the fluorescence lifetime measurements were shown in Fig. 3. The fluorescence decay profiles of EGFP and apocytb_{562}–EGFP were almost overlapped and were fitted to monoexponential curve with a lifetime of 2.4 ns. On the other hand, the holocytb_{562}–EGFP exhibited biphasic decay profile expressed by two lifetimes and two amplitudes (Fig. 3 and Table 1). Because EGFP and apocytb_{562}–EGFP showed the same lifetime, the environment around EGFP chromophore seemed not to be altered by either appending cytb_{562} or the reconstitution of that part. Hence, the component with long lifetime in the fluorescent decay of holocytb_{562}–EGFP may be deduced to the presence of partially denatured apocytb_{562}–EGFP that has fluorescence but not capable of binding heme. In fact, apocytb_{562} itself is easily denatured compared to the holo form. We therefore fix one of the lifetimes to that of apocytb_{562}–EGFP (2.4 ns) and fitted the fluorescence decay profile with biexponential equation. The analysis yields the predominant component (more than 90%) with short lifetime of 0.84 ns. It is evident that this component is owing to the neighboring cytb_{562} moiety. The rate constant for the fluorescence quenching (k_q) caused by the heme in holocytb_{562}–EGFP can be estimated by the equation, $k_q = 1/\tau_2 - 1/\tau_1$, where τ_2 and τ_1 are the fluorescent lifetimes of holo- and apo-cytb_{562}–EGFP, respectively

TABLE 1

Parameters Obtained in Time-Resolved Fluorescence Measurements

	A	τ_1/ns	B	τ_2/ns	r
EGFP[a]	1.0	2.4	—	—	0.999
apob_{562}–EGFP[a]	1.0	2.4	—	—	0.999
holob_{562}–EGFP[b]	0.08	2.4	0.92	0.84	0.999

[a] Fluorescence decay profile was fitted by the equation $I(t) = A_{exp}(-t/\tau_1)$ [A, amplitude; τ_1, fluorescence lifetime].

[b] Fluorescence decay profile was fitted by the equation $I(t) = A_{exp}(-t/\tau_1) + B_{exp}(-t/\tau_2)$ [A and B, amplitudes; τ_1 and τ_2, fluorescence lifetimes].

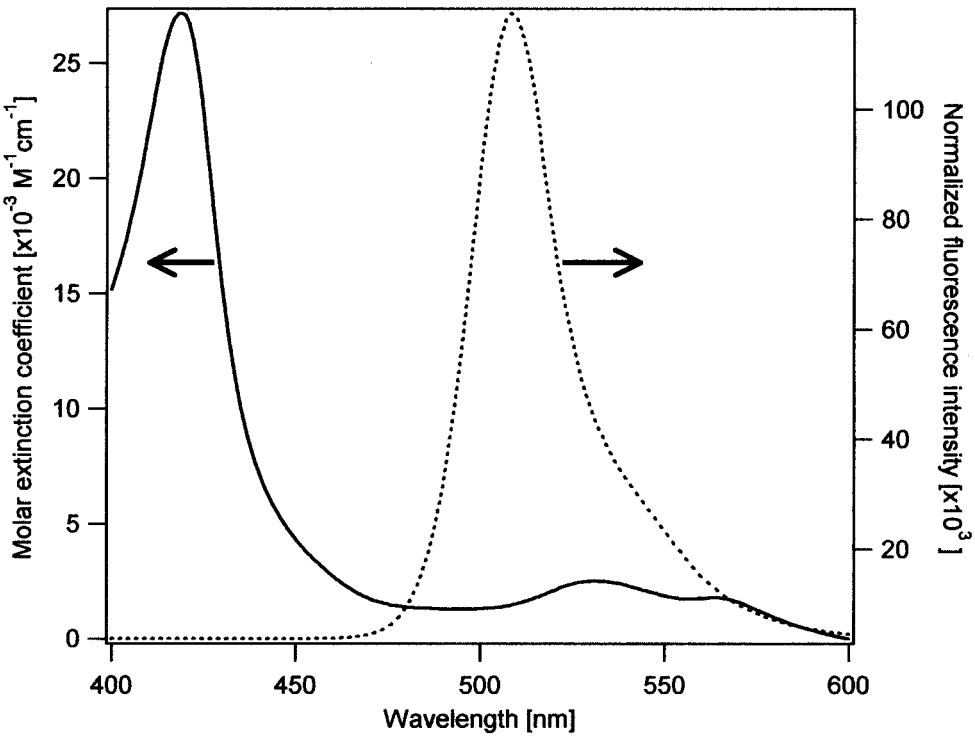

FIG. 4. Spectral overlap between the normalized fluorescence of EGFP (dotted line) and the electronic absorption of cytb_{562} (solid line).

(Table 1). Based on the estimated value of k_q (7.7×10^8 s^{-1}), it is likely that the fluorescence quenching is associated with energy transfer rather than electron transfer because the presumable distance between two chromophores (at least 35 Å based on the tertiary structures of cytb_{562} and EGFP) is too far to occur electron transfer.

Supposing the singlet-singlet energy transfer, the interchromophore distance between cytb_{562} and EGFP (R) can be estimated by the equation $k_q = (1/\tau_1)\,(R_0/R)^6$, where R_0 is the characteristic transfer distance (16). R_0 is defined by the equation $R_0 = 9.7 \times 10^3 (J\kappa^2 n^{-4} \phi_D)^{1/6}$, where J, κ are the spectral and geometrical factors, respectively; n is the refractive index of medium and ϕ_D is the quantum yield of EGFP (17). The J value is calculated based on Fig. 4 showing the spectral overlap between EGFP emission (donor) and cytb_{562} absorption (acceptor). Spatial allocation of donor and acceptor is important factor for determination of energy transfer efficiency because it determines not only distance but also geometrical factor. Assuming that the geometrical factor, κ^2, was a limiting value (2/3, which is available when the orientation of mutual acceptor and donor assumes to be random), R_0 can be calculated to be 46 Å. Given the parameters obtained, R can be calculated to be 41 Å, being within a reasonable interchromophore distance roughly estimated by molecular modeling (around 35~40 Å, data not shown). Even though the distance between chromophores was calculated using the limited value, R was comparable to the value of a model, implying that the estimation error could be derived from geometrical factor. Hence, the fluorescence quenching observed with holocytb_{562}–EGFP may be deduced to the result of energy transfer. In addition, energy transfer yield, $E = 1 - \tau_2/\tau_1$ (18), can be calculated to be 65%.

In conclusion, we have succeeded in designing a novel model protein for an artificial LHC by protein engineering. In our research, we chose a short peptide linker and it seemed to work favorably without disturbing correct folding of the chimera protein. Moreover, energy transfer efficiency of holocytb_{562}–EGFP is considerably high without any optimization. However, there may be a room for designing other chimera proteins with higher energy transfer efficiency by varying the linker properties or the order in combining two kinds of protein. In comparison with chromophores in the natural LHC II, R_0 of the chimera protein (46 Å) is comparable to that of chlorophyll *b* and chlorophyll *a* (42 Å, 18), however, the distance between pigments in natural LHC II is much closer (13 ± 1 Å, 2) than that of the chimera protein (approximately 40 Å). In consequence, energy transfer efficiency that depends on the sixth power of the distance between pigments is quite different between the natural system (~100%) and ours (65%). Hence, the distance between chromophores will be a key issue for improving our artificial LHC system. It has been demonstrated that the prosthetic group of cytb_{562} can be replaced by synthetic metalloporphyrins. Therefore, semisynthetic approaches (5,

19, 20) showed one way to make the chimera for the construction of an artificial PRC. In addition, a wide variety of the spectral variants of GFP can be promising for the further extension to collect wide range of light energy. Further study on characteristics of different pairs of cytb_{562} and GFP variants is now underway.

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