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Invited Paper

Semisynthetic Hemoproteins Using Cofactor Engineering: Toward Supramolecular Protein-based Photosynthetic System

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INTRODUCTION

Proteins are the most fundamental functional molecules which play in every biological process, from energy production such as photosynthesis to life protection using immune response [1]. Recent significant advances in the field of protein engineering enable one to manipulate the structure of proteins precisely at the level of molecule or atom, [2–4] by tools such as site-directed mutagenesis, chemical modification, and semi- and/or total-synthesis of peptides and proteins. Site-specific manipulation of protein structure is considered to be a powerful approach, not only for understanding the relationship between the structure and the function of the protein macromolecules, but also for creating novel artificial proteins with enhanced properties including structural stability, binding specificity, and catalytic function. However, engineering of protein structure and/or function is rather limited as long as we simply use building blocks existing in nature. It is anticipated that methodologies to incorporate unnatural molecules into proteins can greatly expand our ability to rationally and systematically manipulate their structure and function [5–7].

Among innumerable biological systems, photosynthetic reaction center is one of the most elaborated natural systems [8,9]. The primary charge separation in photosynthesis is the key reaction for life on earth since it is the only reaction that converts light energy into chemical energy by a series of fast and efficient electron transfer (ET) steps. It has been established that, in photosynthesis, the protein matrix in which various pigments, e.g. bacteriochlorophylls, bacteriopheophytins, and quinones, are embedded, plays a major role in controlling efficient ET and charge separation. The electron and hole thus generated are transferred to opposite sides of the biomembrane each other, and are accumulated in a catalytic core to accomplish chemical reactions. In past several decades, numerous efforts have been directed to build artificial photosystems, consisting of organic/inorganic small building blocks, based on the concepts of natural photosynthesis [10–14]. The systematic investigations of photophysics of fully synthetic diad or triad molecules have provided us various fundamental insights into the mechanism of ET reactions [15,16]. On the other hand, for the purpose of understanding the chemical/physical principles of biological ET reaction mediated by protein molecules, Gray, Hoffman, McLendon and other researchers have intensively addressed to construct novel ET systems on proteins [17–22]. Unnatural photosensitizers or redox-active metal complexes were incorporated into protein frameworks, and then ET reactions proceeding in these systems were extensively examined. As a consequence, it is

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now generally accepted that biological ET events involving proteins, even though apparently very complicated, can be reasonably explained by using Marcus theory [15,16] or other extended ET theory.

In contrast to those developments of the mechanistic aspect on biological ET, general concepts and/or strategies to create protein-based ET systems remain to be less established. What sorts of synthetic molecules can be incorporated into the protein structure? What types of new functions can we produce from the semisynthetic proteins? Hybridization of proteins or enzymes with photoelectronic molecules is promising for the synthesis of sophisticated biomaterials such as photo-biocatalysts, bioelectronic or optobioelectronic devices, and biosensors [23–28]. It might also be useful as a tool to elucidate the mechanism of protein functions involving redox reactions. In this mini-review, we describe our recent efforts to construct novel protein-based ET systems and their properties, including topics of photo-control of the protein function, and photoinduced charge separation. Using cofactor reconstitution method, supramolecular ET systems were built in redox-active hemoprotein scaffold. Our research can be considered as a crossroad of protein engineering and supramolecular chemistry.

A COFACTOR RECONSTITUTION TECHNIQUE FOR THE INCORPORATION OF UNNATURAL MOLECULES INTO PROTEINS/ENZYMES

Cofactors, e.g. porphyrin derivatives, NAD(P)H, flavin, or PQQ, play crucial roles in expanding a chemical diversity of protein/enzyme functions, mainly providing the redox activity [29]. In most cases, the cofactor unit is noncovalently bound in a hydrophobic pocket provided by the folded polypeptide chain. Thus, in denatured form (usually under acidic pH condition), the cofactor can be easily removed from the protein, affording the corresponding apoprotein [30]. The resultant apoprotein can incorporate not only with the original cofactor (then recover its intact function), but also with a chemically modified cofactor under physiological condition, which is referred to cofactor reconstitution [31]. This technique has been widely applied to various cofactor-dependent proteins for the elucidation of cofactor-apoprotein interaction, and cofactor structure–holoprotein function relationship so far [32,33]. We re-lighted such a classical methodology and elaborately utilized it with the aim of introduction of unnatural molecules into proteins.

In order to demonstrate the validity of our idea, hemoproteins were employed as an initial model. X-ray crystal structure of horse heart myoglobin (Mb) reveals that both propionic acid groups of the protoheme are exposed to the periphery of protein

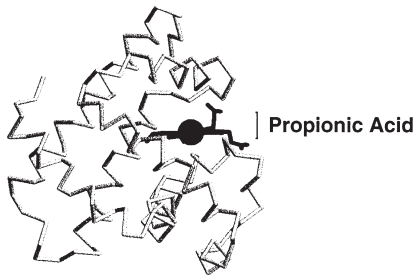


FIGURE 1 X-ray crystallographic structure of horse heart myoglobin.

surface (Fig. 1) [34]. Therefore, it is reasonably expected that reconstitution with synthetic heme cofactors modified chemically at the propionate may enable us to attach various types of molecules to the hemoprotein, particularly in the close proximity of its active sites. As mentioned below, we found that this strategy is very convenient and useful to construct semi-synthetic proteins which show unique functions and biophysical properties [35].

COVALENT ATTACHMENT OF PHOTOSENSITIZER TO PROTOHEME OF MYOGLOBIN: A PROTEIN-BASED DIAD SYSTEM WHICH CAN UTILIZE LIGHT ENERGY TO REGULATE ITS FUNCTION [36–40]

Photo-control of protein/enzyme activity is one of the most attractive targets in the field of protein engineering [23–28] Generally, the function of redox active proteins is strictly regulated by the electronic/redox state of their active site [41,42]. For instance, Mb or hemoglobin can bind molecular oxygen with heme only in the ferrous high spin state. Taken these aspects into consideration, we attempted to employ photoinduced ET that can inject and/or abstract an electron in response to external light stimuli. Site-specific attachment of a photosensitizer to Mb seems to be crucial to carry out this project.

Tris(2,2′-bipyridine)ruthenium(II) complex ($Ru^{II}(bpy)_3$) was selected as a photosensitizer, because of its high chemical stability, high water-solubility, and the rich data on its electrochemical/photochemical properties [43]. Photoexcited state, $^{*}Ru^{II}(bpy)_3$, which has a relatively long lifetime (ca. 600 ns), is a powerful reducing species with a redox potential of -0.86 V vs NHE. In contrast, the oxidized state, $Ru^{III}(bpy)_3$, generated by oxidative quenching of the excited state, is a strong oxidant with a redox potential of $+1.26$ V vs NHE. These photophysical properties are perfectly suitable to control the redox state of myoglobin.

In order to introduce $Ru^{II}(bpy)_3$ into Mb by cofactor reconstitution method, a diad molecule,

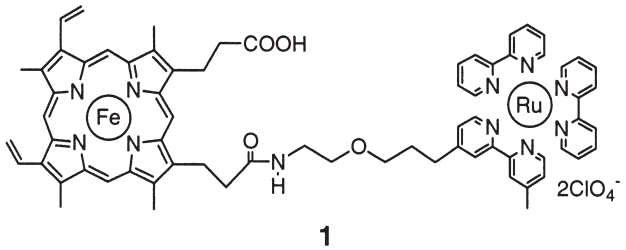


FIGURE 2 Molecular structure of $Ru^{II}(bpy)_3$–appended proporphyrin IX **1**.

$PP(Fe^{III}Cl)-Ru^{II}$ **1** (shown in Fig. 2) was designed. $Ru^{II}(bpy)_3$ was covalently attached to one of propionate groups of protoporphyrin IX via an appropriate spacer, an alkyl ether chain which has both high flexibility and moderate water-solubility, so as to minimize the structural perturbation of the reconstituted Mb. Reconstitution of **1** with apo-Mb was conducted according to the modified procedure reported previously (Fig. 3), [30,31] affording $Ru^{II}(bpy)_3$-appended Mb (Mb(**1**)). All biophysical data, including absorption spectra, electron paramagnetic resonance (EPR), circular dichroism (CD), and the stoichiometry of the heme over apo-Mb, confirmed that the artificial cofactor **1** is successfully inserted into the native heme crevice of apo-Mb without any significant structural changes.

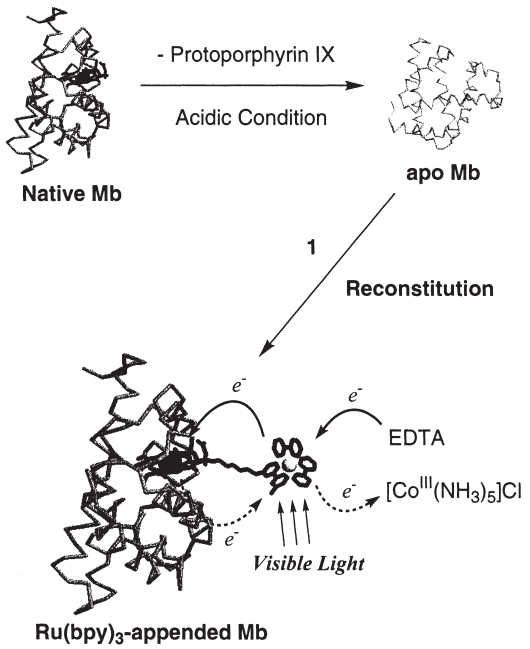
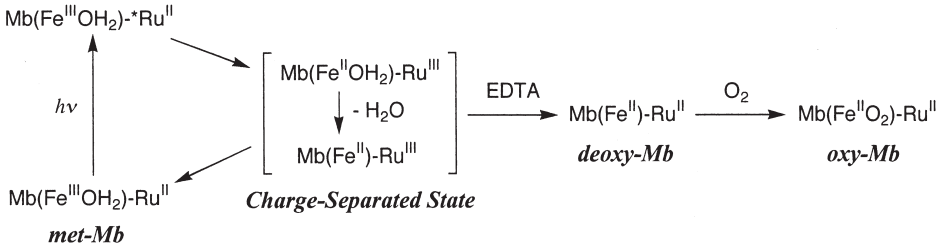


FIGURE 3 Schematic illustration of the reconstitution of **1** with apo-Mb and the photoactivation of the resultant Mb(**1**); solid line: electron injection system described in "Electron injection system: photo-control of oxygen-binding activity"; dashed line: electron abstraction system in "Electron abstraction system: photogeneration of oxy-ferryl species".

Electron Injection System: Photo-control of Oxygen-binding Activity [36–38]

To test the feasibility of our strategy, we initially conducted an electron injection system to achieve the reductive photoactivation of hemoproteins using the $Ru^{II}(bpy)_3$-appended Mb(**1**) (Fig. 3). Since $^{*}Ru^{II}(bpy)_3$ is a powerful reductant, [43] the photoinduced ET reaction from $^{*}Ru^{II}(bpy)_3$ to iron(III) center is reasonably expected to be thermodynamically favorable. The steady-state illumination of the visible light (>450 nm) to Mb(**1**) in the presence of EDTA, a sacrificial electron donor, under Ar, caused a remarkable absorption spectral change, in which the absorption maxima of the met-$Mb(Fe^{III}OH_2)$ shifted to those of deoxy-$Mb(Fe^{II})$. The resulting species was readily converted to oxy-$Mb(Fe^{II}O_2)$ upon exposure to air. More interestingly, photoirradiation of $Mb(Fe^{II})-Ru^{III}$ in the presence of EDTA under aerobic condition gave rise to the direct generation of oxy-$Mb(Fe^{II}O_2)$. It was clearly demonstrated that Mb(**1**) is capable of switching its oxygen-binding activity by responding to the external visible light. In contrast, photoreaction did not occur in an intermolecular system, that is, an equimolar mixture of native Mb and $Ru^{II}(bpy)_3$.

ET kinetics of Mb(**1**) was examined by laser flash photolysis experiment, in the absence of EDTA under anaerobic condition, which led to the photoactivation mechanism as shown in Scheme 1. In the initial step, very rapid ET from $^{*}Ru^{II}(bpy)_3$ to iron(III)-center of heme produces the charge-separated (CS) state (with a first-order rate constants of $4.4 \times 10^7\ s^{-1}$), $Mb(Fe^{II})-Ru^{III}$. The CS state thus generated readily reverts to an original ground state by charge recombination (with a first-order rate constant of $(2.0–3.7) \times 10^7\ s^{-1}$) in the absence of EDTA. On the other hand, in the presence of EDTA,



SCHEME 1 Photoinduced ET scheme of Mb(**1**) in the electron injection system.

the oxidized $Ru^{III}(bpy)_3$ in the CS state can retrieve an electron from EDTA (with a second-order rate constant of $1.0 \times 10^{10}\,M^{-1}\,s^{-1}$) in competition with the recombination process, yielding the active deoxy-Mb(Fe^{II}), which can bind molecular oxygen. No quenching of $^*Ru^{II}(bpy)_3$ was observed in an intermolecular system, which agrees well with the result of the steady-state photolysis described above.

As a result of covalent attachment of photosensitizer onto the cofactor, Mb was successfully converted to a light-responsive protein which can achieve photo-switching of the intact function by efficient photoinduced ET reactions. This strategy has been successfully applied to cytochrome b_{562} (Cyt-b_{562}), an electron transporting protein [44].

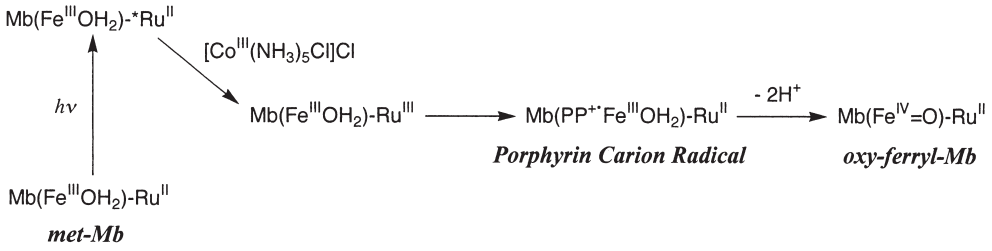
Electron Abstraction System: Photogeneration of Oxy-ferryl Species [39,40]

In contrast to the extensive researches on ET driven by high reductive potential, in which the reducing ability of the excited state of photosensitizer is exploited, there have been only a few examples demonstrating that a protein is oxidatively activated by light [45–47] We considered that semi-synthetic Mb(**1**) might be a suitable system for the oxidative photoactivation (Fig. 3).

To carry out the oxidative activation of Mb(**1**), we focused on the strong oxidizing ability of $Ru^{III}(bpy)_3$. $Ru^{III}(bpy)_3$ generated by oxidative flash quenching [48,49] might abstract an electron from the resting-state Mb($Fe^{III}OH_2$), since the ET reaction is expected to be thermodynamically favorable. In the presence of chloropentamminecobalt(III) complex ($[Co^{III}(NH_3)Cl]Cl_2$), a sacrificial oxidative quencher of $^*Ru^{II}(bpy)_3$, the steady-state photoirradiation of Mb(**1**) showed a remarkable absorption spectral change where the absorption maxima of the met-Mb($Fe^{III}OH_2$) shifted to those of oxy-ferryl-Mb(Fe^{IV}=O). The photoproduct was satisfactorily identified to oxy-ferryl-Mb(Fe^{IV}=O), a high-valent iron(IV) porphyrin, by absorption spectra, EPR, and reactivity test. In sharp contrast, only photodegradation of the polypeptide framework (dimerization and/or cleavage) was observed in an intermolecular system. These results clearly indicated that precise spatial arrangement of photosensitizer is one of the essential factors to abstract an electron from the active site deeply buried in the protein matrix.

Laser flash photolysis experiment gave us an interesting ET mechanism that the oxy-ferryl-Mb(Fe^{IV}=O) generated via the porphyrin cation radical as a key intermediate, not via the direct 1e-oxidation of metal-center (Scheme 2). In the initial step, $^*Ru^{II}(bpy)_3$ is oxidatively quenched by $[Co^{III}(NH_3)Cl]Cl_2$ to yield the strong oxidant, $Ru^{III}(bpy)_3$. $Ru^{III}(bpy)_3$ thus generated efficiently retrieves an electron from the macrocyclic porphyrin ligand (with a first-order rate constants of $7.1 \times 10^5\,s^{-1}$), which is followed by iron(III) oxidation by the porphyrin cation radical with concurrent deprotonation of water coordinated to the 6th position of iron(III) heme to yield the oxy-ferryl species. Consistent with this mechanism, it is demonstrated that the rate of the first step (i.e. the porphyrin radical generation) is not affected by pH and H/D exchange of solvent, whereas the second slower step (i.e. oxy-ferryl-Mb(Fe^{IV}=O) formation) shows both significant pH dependence and isotope effect. This can be reasonably explained by the involvement of proton-coupled process in the second ET step. Since the more basic condition facilitated the deprotonation of coordinated-water, formation of the oxy-ferryl-Mb(Fe^{IV}=O) is greatly accelerated (with a first-order rate constants of $4.0 \times 10^4\,s^{-1}$ at pH 7.5, and $2.0 \times 10^5\,s^{-1}$ at pH 9.0). Careful simulation of the pH profile of the kinetics indicated that the protonation/deprotonation equilibrium of the protein matrix plays an important role to regulate the oxy-ferryl-Mb(Fe^{IV}=O) formation in a heme pocket of Mb. It is most likely that the deprotonation of a specific amino acid residue (one of the most promising candidate is Lys 45, see Fig. 4) facilitates the formation of oxy-ferryl-Mb(Fe^{IV}=O) through the conformational change of distal His of Mb.

In this section, we described that $Ru^{II}(bpy)_3$-appended Mb can utilize the light energy to control its function in reductive or oxidative manner. Very rapid ET between the photosenistizer and heme in semi-synthetic Mb through the covalent bond is essential for these photoactivation processes. However, in this system, high concentration of sacrificial redox reagents is inevitably required to carry out the vectorial electron injection or abstraction into or from



SCHEME 2 Photoinduced ET scheme of Mb(**1**) in the electron abstraction system.

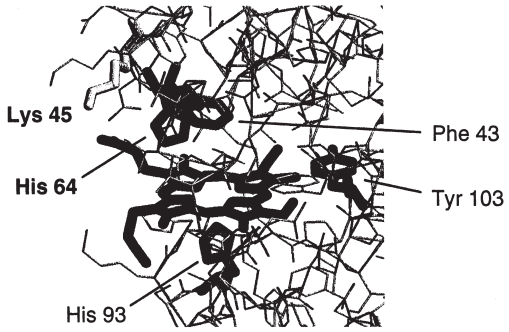


FIGURE 4 The detailed structure of the heme pocket of horse heart Mb.

the active site. Semi-synthetic proteins in which multi-step intramolecular photoinduced ET proceeds to generate an active species will be expected to be more elaborate protein-based ET system which mimic the important function of natural photosynthesis.

A PROTEIN-BASED TRIAD SYSTEM MIMICKING SEVERAL ESSENTIAL FACTORS OF NATURAL PHOTOSYNTHESIS [50–52]

Along the line described above, we next challenged to attach an additional electron acceptor to $Ru^{II}(bpy)_3$ of Mb(**1**) diad molecule, which might be photo-activated via stepwise intramolecular ET within a single-molecule.

Triad compounds, $PP(Fe^{III}Cl)–Ru^{II}–BXV^{4+}$ **2**, $PP(Zn)–Ru^{II}–BXV^{4+}$ **3**, shown in Fig. 5 were synthesized using the stepwise coordination chemistry to the Ru center. Iron(III) and Zn(II) complexes were used in order to examine the effect of the metal center of porphyrin for ET properties. A cyclic viologen (BXV^{4+}) was selected as an electron acceptor, and a catenane-type mechanical linkage was adopted as the connector between $Ru^{II}(bpy)_3$ and BXV^{4+}, according to the elegant reports by Dürr and co-workers that the diad molecule consisting of $Ru^{II}(bpy)_3$ and BXV^{4+} in the [2]catenane effectively stabilizes the CS state [53,54]. Reconstitution of **2** and **3** were readily carried out in spite of these bulkiness.

Vectorial, Multistep, and Proton-coupled Electron Transfer [51]

ET behavior of the triad systems was investigated by laser flash photolysis under anaerobic condition. The transient absorption obviously clarified the ET mechanism as shown in Schemes 3 and 4. Photo-excitaion of the $Ru^{II}(bpy)_3$ moiety of Mb(**2**) gives rise to an initial CS state (with $> 2.0 \times 10^8\ s^{-1}$ of a first-order rate constant), $Mb(Fe^{III}OH_2)–Ru^{III}–BXV^{3+\cdot}$, which is followed by thermal ET to generate a second intermediate, $Mb(PP^{+\cdot}Fe^{III}OH_2)–Ru^{II}–BXV^{3+\cdot}$. $Mb(PP^{+\cdot}Fe^{III}OH_2)–Ru^{II}–BXV^{3+\cdot}$ thus generated is subsequently converted into the final CS state (with $6.6 \times 10^3\ s^{-1}$ at pH 7.0), $Mb(Fe^{IV}{=}O)–Ru^{II}–BXV^{3+\cdot}$, with concomitant deprotonation as observed in the diad system. Thus, the generation of the final CS shows strong pH dependence. It is remarkable that the lifetime of the final CS state is found to be longer than 2 ms, which is practically comparable to the value of the natural photosynthesis (Scheme 3).

On the other hand, in the case of the zinc porphyrin center (Mb(**3**)), photoexcitation of $Ru^{II}(bpy)_3$ moiety at 460 nm also yields a vectorial two-step ET relay leading to the final CS state, $Mb(Zn^{+})–Ru^{II}–BXV^{3+\cdot}$, via the intermediate CS state, $Mb(Zn)–Ru^{III}–BXV^{3+\cdot}$ (Scheme 4). However, the lifetime of the final CS state (biexponential analysis estimated it to be 1–18 μs) is more than 1000-fold shorter than that in the iron porphyrin center (Mb(**2**)).

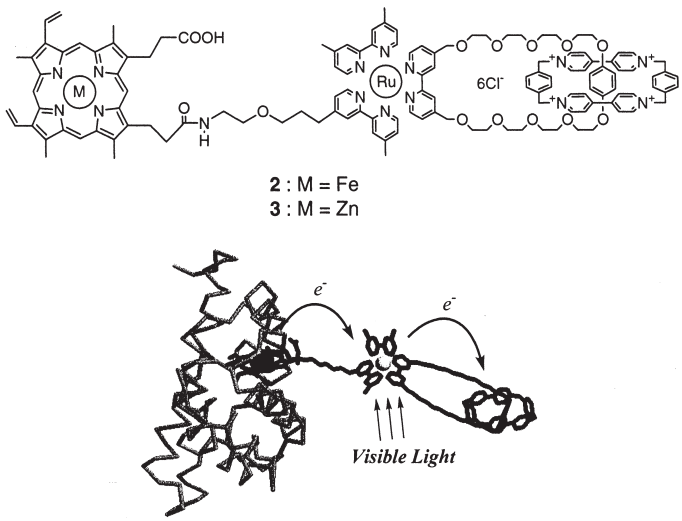
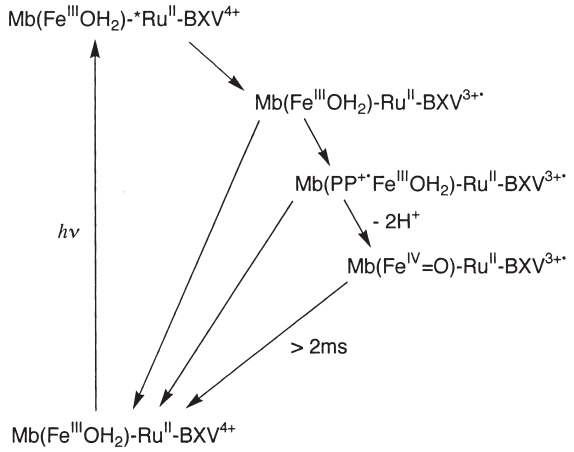


FIGURE 5 Molecular structure of the triad compounds **2** and **3** and photoinduced ET flow of their reconstituted Mb.



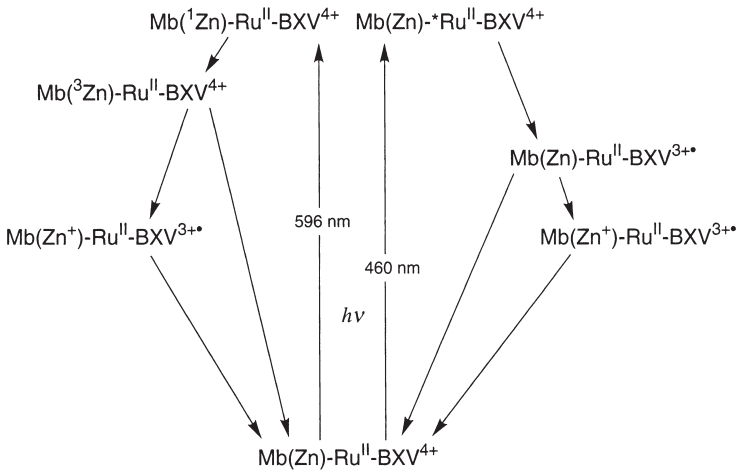
SCHEME 3 Electron transfer pathways for Mb(**2**).

Why does such difference in the recombination rate between the iron system and the zinc system arise, although the driving force for the charge recombination of Mb(Fe^{IV}=O)–Ru^{II}–BXV$^{3+\cdot}$ and Mb(Zn$^+$)–Ru^{II}–BXV$^{3+\cdot}$ are very similar ($-\Delta G =$ 1.30 eV)? It can be explained by the involvement of a proton coupling process into the iron system. According to a related system previously reported by English and co-workers, [55,56]one-electron reduction of the oxy-ferryl species of Mb(Fe^{IV}=O) which is a reverse reaction of Mb(Fe^{IV}=O) formation is a proton-coupled process. This is regulated by the protonation or deprotonation of the distal His 64 as a rate-limiting step (see Fig. 4). The deprotonation state facilitated the formation of the CS state and suppressed the charge recombination, rendering the CS state especially long-lived. A control experiment of the triad molecule **2** without apo-Mb matrix produced neither the long-lived CS, nor the stepwise ET. This clearly demonstrated that partial wrapping of the triad molecule by the protein matrix plays a crucial role in controlling the ET pathway and stabilizing the CS state. It should be noted that, despite the significant differences in its size and structure, the semi-synthetic Mb(**2**) does mimic several essential factors of natural photosynthesis, including multistep, vectorial, and protein-assisted and proton-coupling ET, for the long-lived charge separation.

Protein Matrix Effect [52]

Are there any protein matrix effects for ET in this system, besides the protein-assisted proton-coupling step? The systematic comparison of ET properties of semi-synthetic systems in the presence and the absence of protein matrix should help to elucidate the role of proteins in the biological ET events. In order to investigate how protein matrix affects to the ET processes, two semi-synthetic system, in which the triad molecule **3** is incorporated into myoglobin or cytochrome b_{562}, were prepared by the cofactor reconstitution. Photophysics of these two semi-synthetic proteins was examined in detail (Scheme 4), and the data were compared with the triad **3** itself in a homogeneous solvent.

Photoexcitation of the ZnPP moiety at 596 nm of Mb(**3**) or Cyt-b_{562}(**3**) yields a direct long-distance ET from the triplet state of ZnPP (^{3}ZnPP) to BXV^{4+} to generate final CS state, Mb(Zn$^+$)–Ru^{II}–BXV$^{3+\cdot}$ or Cyt-b_{562}(Zn$^+$)–Ru^{II}–BXV$^{3+\cdot}$. On the other hand, direct ET from the excited singlet state, not the triplet state, of ZnPP (^{1}ZnPP) to the BXV^{4+} moiety occur in **3** in the absence of the protein matrix. When the Ru^{II}(bpy)$_3$ moiety of Mb(**3**) or Cyt-b_{562}(**3**) is excited at 460 nm, a stepwise ET relay leads to the same final CS state as that generated in the direct ET pathway, with an intermediate, Mb(Zn)–Ru^{III}–BXV$^{3+\cdot}$ or Cyt-b_{562}(Zn)–Ru^{III}–BXV$^{3+\cdot}$. The lifetime of the corresponding final CS state were determined to be 300 ns for **3** in the absence of the protein matrix, 1–18 μs for Mb(**3**) and 600–900 ns for Cyt-b_{562}(**3**), the



SCHEME 4 Electron transfer pathways for Mb(**3**) after excitation at the Ru^{II}(bpy)$_3$ moiety and ZnPP moiety. Cyt-b_{562}(**3**) shows essentially same ET behavior as Mb(**3**).

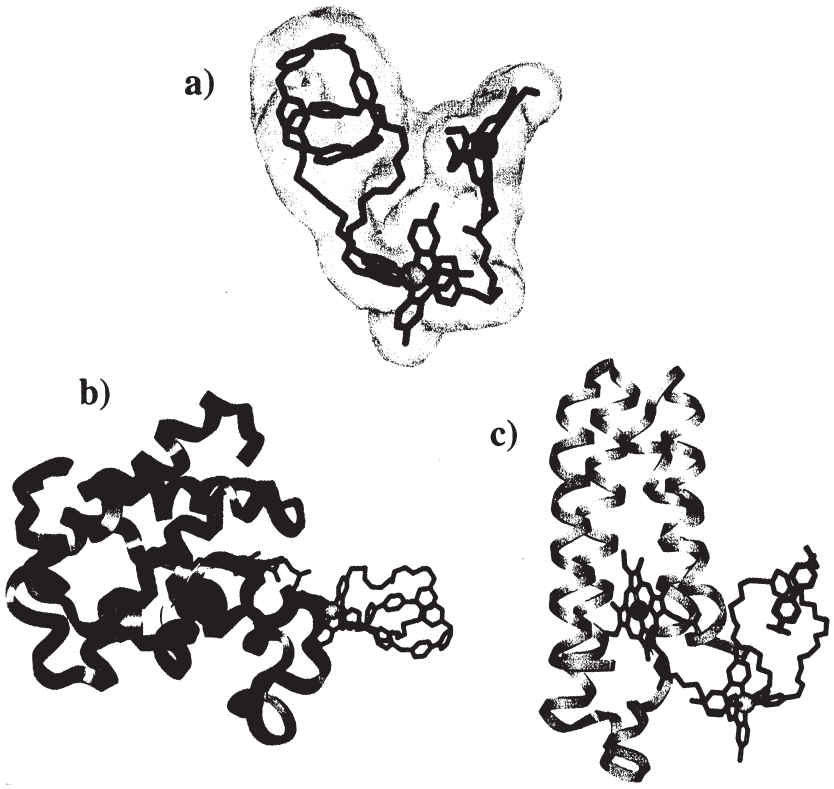


FIGURE 6 Energetically minimized three-dimensional structures generated by molecular modeling of (a) **3**, (b) Mb(**3**), (c) Cyt-b_{562}(**3**) in water.

values of which are greatly affected by the types of protein matrix.

Molecular modeling study of the three systems showed that, in the absence of protein matrix, **3** tend to fold into a U-shaped conformation with ZnPP and BXV^{4+} moieties roughly in face-to-face arrangement (with a donor–acceptor distance of ca. 9 Å), whereas in the presence of protein, a U-shaped conformation of **3** changes to more extended ones in Mb(**3**) and Cyt-b_{562}(**3**) (Fig. 6). Moreover, electrostatic interaction between each protein surface and positively charged BXV^{4+} determines the donor–acceptor distance to be ca. 23 Å in Mb (positively charged surface) and ca. 16 Å in Cyt-b_{562}(negatively charged surface). These results are in good agreement with the differences of their photophysical behaviors, which may suggest that a designed protein surface can rationally regulate ET processes. Similar finding was recently reported by Willner and co-workers [57].

SUPRAMOLECULAR APPROACH FOR THE CONSTRUCTION OF TRIAD SYSTEM ON A PROTEIN SURFACE [58]

As we described above, the cofactor reconstitution method made it possible to construct the diad and triad ET systems on protein surfaces. However, chemical synthesis of protoheme-photosensitizer hybrids is still rather difficult and annoying. A novel strategy utilizing self-assembly processes may be a more flexible alternative.

As a strong association force in aqueous solution, hydrophobic interaction was employed to our triad system. β-cyclodextrin-appended zinc-protoporphyrin IX (PP(Zn)–CD) **4**, its reconstituted Mb (Mb(**4**)), and adamantane-modified $Ru^{II}(bpy)_3$–BXV^{4+} (AD–Ru^{II}–BXV^{4+}) **5** in Fig. 7 were designed as parts of such a self-assembled ET system. We confirmed that the adamantane moiety of **5** is spontaneously bound with a CD interior of Mb(**4**), to form a noncovalently-linked donor-sensitizer-acceptor triad system on myoglobin surface (the binding constant is ca. $5 \times 10^5\ M^{-1}$). Photoexcitation of the $Ru^{II}(bpy)_3$ moiety of the Mb(**4**)·**5** complex leads to a stepwise ET to generate the final CS state, Mb(Zn^+)–CD:AD–Ru^{II}–$BXV^{3+\cdot}$ with the lifetime of ca. 640 ns (Scheme 5). It is clear that stepwise and noncovalent ET reactions (coupling of the mechanical linkage and the hydrophobic linkage in this case) take place in our supramolecular protein-based triad. In sharp contrast, the CS state does not generate by the photoexcitation of ZnPP moiety of Mb(**4**)·**5** complex. Furthermore, neither ZnPP radical nor viologen radical was detected in a mixture of native Mb(Zn) and **5** under the same experimental condition. These results indicated that direct long-distance ET pathway is not effective for the final CS state in these control systems.

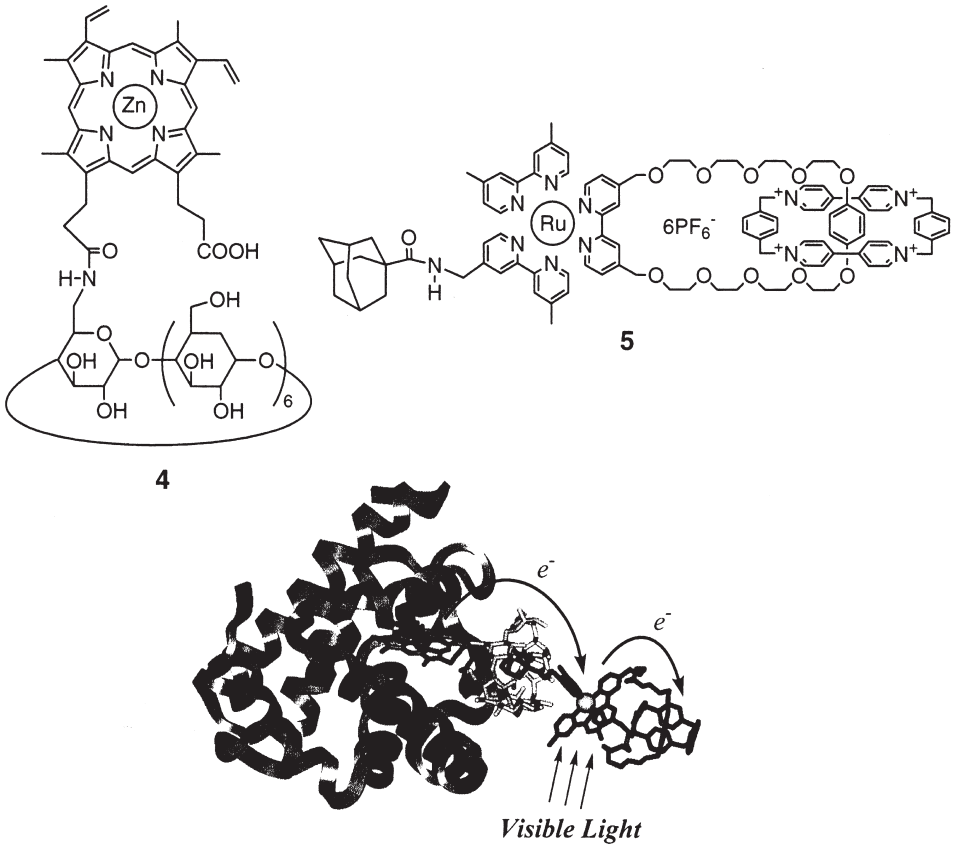
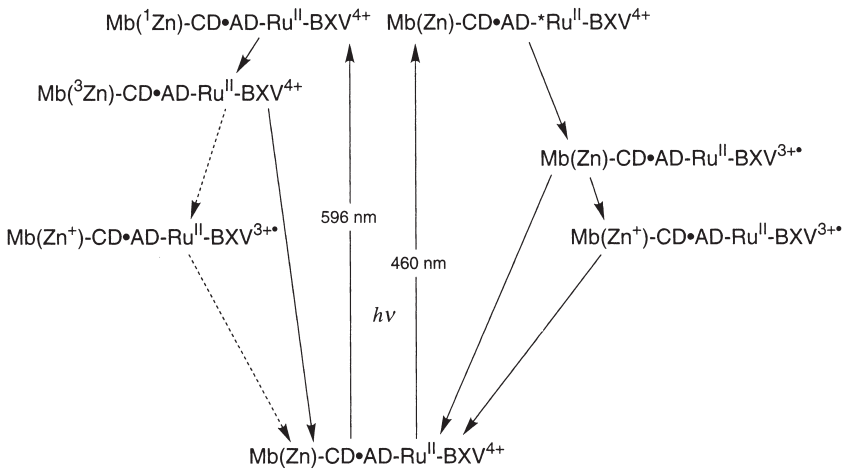


FIGURE 7 Molecular structures of β-CD–appended zinc proporphyrin IX **4** and adamantane-modified $Ru^{II}(bpy)_3$–BXV^{4+} **5** and the self-assembled Mb(**4**)·**5** complex.

It is well-known that cyclodextrin can act as a versatile host for various nonpolar small molecules, including photo-/redox-active molecules such as quinones, ferrocenes, and tetraphenylporphyrin derivatives [59–61]. Therefore, the present CD-appended Mb(**4**) might be useful as a protein scaffold not only for the construction of novel supramolecular photosystems, but also for the systematic evaluation of driving-force dependence of ET.

HEMOPROTEIN-PHOTOSENSITIZER-SEMICONDOCTOR HYBRID FOR THE CONSTRUCTION OF NOVEL PROTEIN-BASED ELECTRODES [62]

Very recently, we made an attempt to hybridize the $Ru^{II}(bpy)_3$-appended Mb with semicondoctor TiO_2 to create a novel protein-based photoelectrode. It has been already established by Grätzel and co-workers that nanocrystalline TiO_2 electrode coated with



SCHEME 5 Electron transfer pathways for the self-assembled Mb(**4**)·**5** complex after excitation at the $Ru^{II}(bpy)_3$ moiety and the ZnPP moiety.

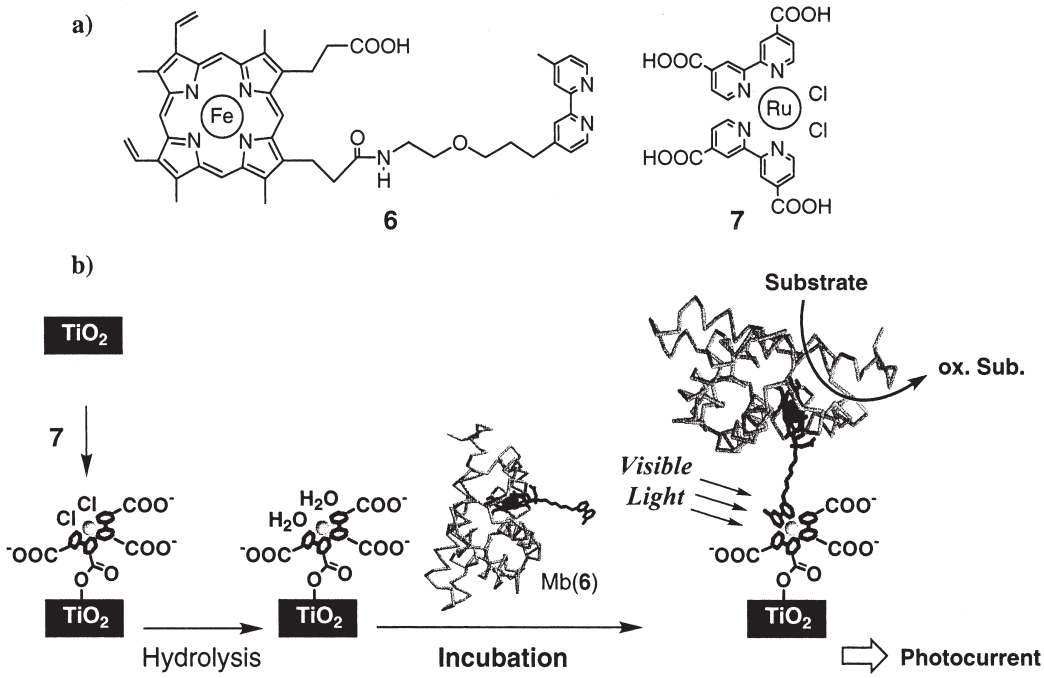


FIGURE 8 (a) Molecular structure of bpy-appended proporphyrin IX **6** and bis-$Ru^{II}(bca)_2Cl_2$ **7** (b) Immobilization scheme of Mb(**6**) on TiO_2/ITO electrode via coordination chemistry.

tris(2,2′-bipyridyl, 4,4′-carboxylate)ruthenium(II) complex ($Ru^{II}(bca)_3$) can perform as a efficient solar cell, in which electron injection from $^*Ru^{II}(bpy)_3$ to the conduction band of TiO_2 is a key process [63]. Thus, it is reasonably expected that, in Mb–$Ru^{II}(bpy)_3$–TiO_2 system, the formation of high-valent oxo-ferryl-species and generation of photocurrent may take place concomitantly by vectorial, stepwise photoinduced ET. To construct this triad system consisting of protein/inorganic hybrid materials in the orientation of Mb–$Ru^{II}(bpy)_3$–TiO_2, coordination chemistry was employed as schematically shown in Fig. 8.

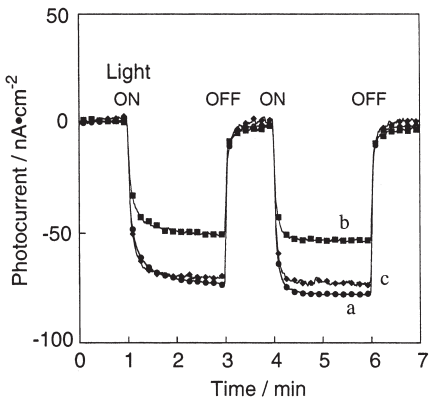


FIGURE 9 Photocurrent generation of Mb–$Ru^{II}(bpy)_3$–TiO_2/ITO electrode in the presence of guaicol as a oxidative substrate. (a) intact Mb–$Ru^{II}(bpy)_3$–TiO_2/ITO electrode after incubation, (b) after treatment with 3M Gdn·HCl, (c) after re-incubation of apo-Mb with electrode of (b)

2,2′-bipyridine-appended Mb was prepared by reconstitution (Mb(**6**)) and the subsequently hybridized with a TiO_2/ITO electrode coated with bis-$Ru^{II}(bca)_2Cl_2$ **7** as shown in Fig. 8. Photocurrent measurement in the presence of guaicol, a typical substrate for oxo-ferryl-Mb, clearly indicated that, the anode photocurrent increases by the incubation of Mb(**6**) in 1.9-fold, whereas no change was observed in the case of native Mb. This suggests that the bpy-appended Mb(**6**) was successfully immobilized on the surface of TiO_2/ITO electrode via a formation of tris-$Ru^{II}(bca)_2(bpy)$ complex. In addition, removal of apo-Mb matirx by washing of the Mb(**6**)-treated electrode with 3 M Gdn·HCl resulted in a decrease of the photocurrent, then it was recovered by the re-incubation with apo-Mb, indicating an important effect of protein matrix to the efficient photocurrent generation (Fig. 9).

This is the first example that a protein-based triad system is constructed on the semicondoctor support. This type of bio/inorganic hybrid may lead to a light-driven chiral oxidation catalyst with the concurrent generation of photocurrent or hydrogen gas in the future.

CONCLUSION

This mini-review has addressed our recent advances in the construction of protein-based ET systems. It has been demonstrated that a set of chemical modification of cofactors and their reconstitution

represents one of the most powerful methodology to confer the photofunction on naturally occurring enzymes/proteins. Efficient electrical communication between the cofactor buried in the interior of protein matrix and the photosensitizer exposed to the exterior solvent is achieved by this active-site directed modification. Following our reports, Willner and co-workers extended this concept to flavin or PQQ-dependent enzymes such as glucose oxidase and amino acid oxidase [64,65]. They also succeeded in the reconstituion of these enzymes on electrode interfaces to produce novel biosensors [26–28]. Combination of other techniques, such as site-directed mutagenesis, chemical modification of protein surface, and supramolecular approach may greatly expand our ability to create the semi-synthetic proteins/enzymes with novel chemical/physical properties.

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