Chemical Modification of the Structures and Functions of Proteins by the Cofactor Reconstitution Method

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A new strategy for the semi-synthesis of proteins and enzymes bearing non-natural functional groups is described. In line with our proposal that the replacement of native cofactors by modified cofactors may represent a useful and general methodology for introducing non-natural molecules into cofactor-dependent proteins and enzymes, we have shown that semi-synthetic cofactors bearing non-natural functional units can be incorporated in the proximity of the active sites of enzymes by cofactor reconstitution. A variety of non-natural building units such as a hydrophobic alkyl chain, a polyanion cluster, metal complexes, electron donors or acceptors, a photochromic moiety, peptides, and artificial receptors have successfully been introduced into hemoproteins (myoglobin, hemoglobin, and cytochrome b562) and their effects on the holoproteins have been examined. Incorporation of a long alkyl chain, for example, greatly facilitates conversion of a water-soluble protein into a membrane-bound protein. Attachment of a photosensitizer has enabled us to switch the enzyme activity by means of visible light. Artificial receptors introduced in the proximity of the active site can modulate the structures and activities of native proteins in response to specific guest molecules. This concept has been expanded to include flavoenzymes such as glucose oxidase. An immense variety of non-natural molecules are available that are potentially useful for protein engineering. By combination of the present cofactor reconstitution method with other techniques such as site-directed mutagenesis, chemical modification, peptide semi-synthesis, and non-natural amino acid incorporation using suppressor *t*-RNA, it should be possible to artificially manipulate native protein molecules with a similar degree of flexibility as that with which organic chemists manipulate small molecules.

Introduction

Significant advances in the field of protein and enzyme engineering have been made as a result of the development of recombinant DNA technology. However, this methodology is limited to the 20 natural amino acids that are genetically coded in DNA sequences. Considering natural enzymes, however, it is apparent that besides these 20 amino acids, many other components play key roles in determining their structural and functional properties. Cofactors, metal ions, heteroatoms, chromophores, saccharides, and fatty acids that are hybridized in post-translational manners efficiently give rise to a diverse range of functions that cannot be achieved solely by the simple peptide backbones of enzymes. These functionalities are thus of potential use in the chemical engineering of biomacromolecules. Derivatization of naturally occurring proteins and enzymes with artificial functional molecules represents one of the most promising approaches for the development of novel tailor-made bio-

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MICROREVIEWS: This feature introduces the readers to the authors' research through a concise overview of the selected topic. Reference to important work from others in the field is included.

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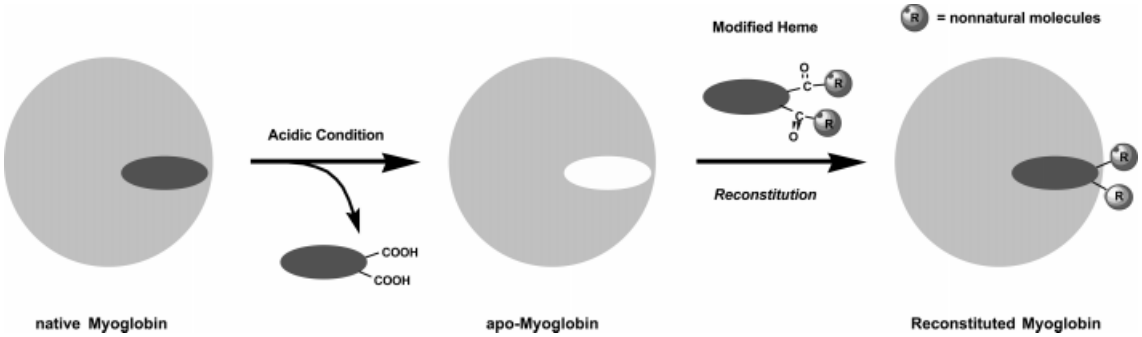


Scheme 1. General scheme of the cofactor reconstitution method for the incorporation of non-natural functional groups into hemoproteins

molecules, which should have potential applications in chemistry and chemical biology. It is gradually being demonstrated that non-natural molecules can confer sophisticated functions upon native proteins.

It is now desirable for chemists to screen and select useful non-natural molecules capable of regulating and/or of modifying enzyme functions on the basis of their precise biophysical and biochemical effects. Unfortunately, however, incorporation of such non-natural molecules into native proteins is generally rather difficult. Chemical reactions of proteins with organic reagents often provide mixtures of randomly modified proteins. In order to achieve specific modifications, Kaiser and co-workers proposed the concept of "chemical mutation", where a reactive amino acid (Cys or Ser, for example) is artificially modified with non-natural functional groups.[1−4] The great progress made in solid-phase peptide synthesis, as developed by Merrifield, has allowed the total synthesis[5,6] or the *de novo* design[7] of small proteins with no restrictions as to the non-natural amino acids that can be incorporated. The semi-synthesis of proteins represents a further alternative for the site-specific incorporation of non-natural molecules.[8−17] A biosynthetic method using aminoacylated suppressor *t*-RNA has recently been developed by Schultz and Chamberlin.[18−25] Although this method can be expected to be more general, the quantities of the mutant proteins produced and the types of non-natural amino acids amenable to it are somewhat restrictive. Thus, there remains a need for a convenient method by which the actions of a variety of non-natural molecules upon proteins can be monitored.

General Strategy for the Incorporation of Non-Natural Molecules into Native Proteins by Cofactor Reconstitution

It is generally established that under certain conditions the cofactor units of a number of cofactor-bearing enzymes and proteins, such as hemoproteins, flavoenzymes, NADH or PQQ-dependent enzymes, may be extracted from the active pocket.[26] The resultant apoenzymes or apoproteins can then combine with chemically modified cofactors. This technique has been widely used for the elucidation of cofactor−apoenzyme interactions at the molecular level for several decades.[27] It came to our attention that this methodology might be exploited for the introduction of non-natural molecules into proteins and enzymes, the reconstitution process allowing the incorporation of semi-synthetic cofactors bearing non-natural functional units in the proximity of the active sites of enzymes. For instance, the heme unit of myoglobin (Mb) can easily be replaced by porphyrin derivatives bearing various functional groups simply by mixing the artificial heme with apo-Mb in neutral aqueous solution. This is depicted in Scheme 1. These are suitable systems for examining how the non-natural moieties affect the structures and functions of native proteins.

Design and Synthesis of a Lipid-Anchored Hemoprotein

X-ray structural studies of native Mb have established that the two propionate ends of the protohemin are exposed to the protein surface[28] (Figure 1). Hence, various molecules bearing functional groups may be attached at a propionate site, with appropriate spacers so as to minimize the structural perturbation of the reconstituted Mb.

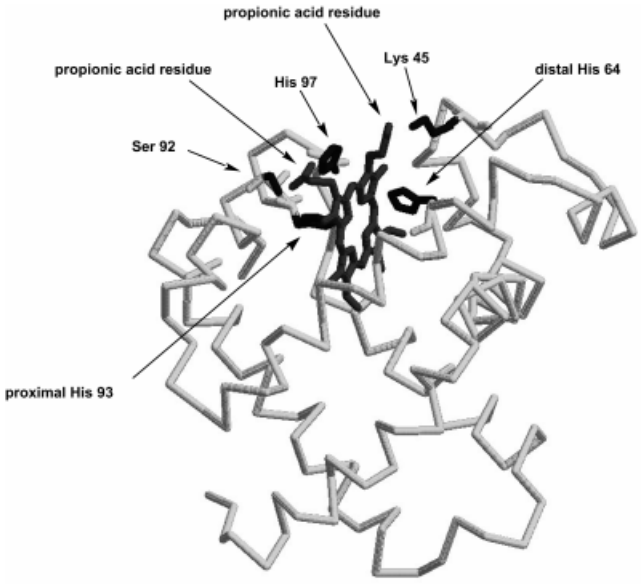
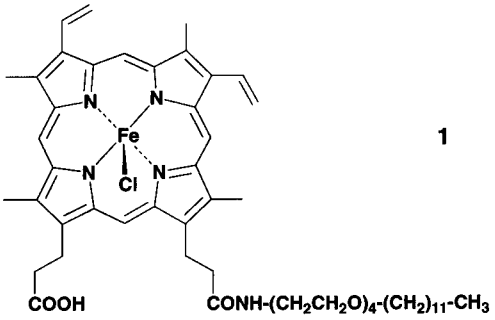


Figure 1. 3D structure of horse heart myoglobin from X-ray crystal analysis

As an initial example, we attempted to incorporate a hydrophobic long alkyl chain into myoglobin, a water-soluble globular hemoprotein.[29] We functionalized protohemin, the naturally occurring prosthetic group of most hemoproteins, with a hydrophobic dodecyl group (**1**) or a short ethyl disulfide (**2**) and according to a slight modification of a literature procedure incorporated the resulting molecules into the heme crevice of apomyoglobin (apo-Mb).



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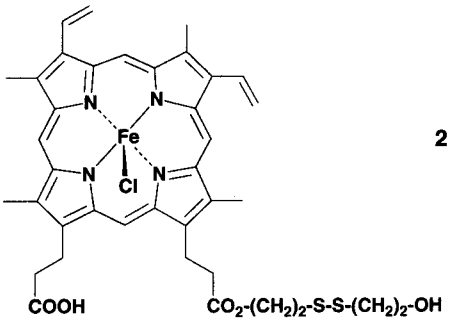

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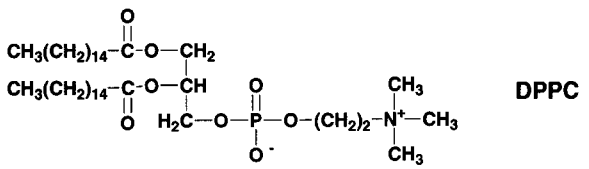

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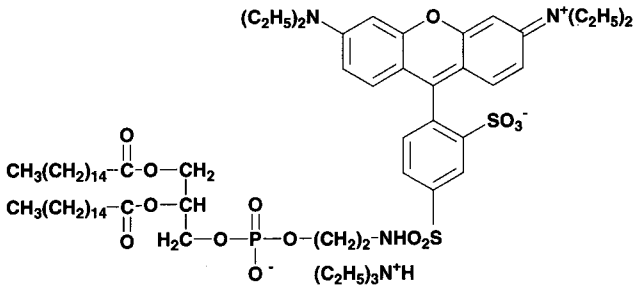


DPPE-Rhodamine B

Characterization of the reconstituted Mb was conducted as follows. Spectrophotometric titration of the modified hemins with apo-Mb supported the 1:1 stoichiometry of the complex formation. The absorption spectrum of the met-form [oxidized form; Fe(III) state] of the semi-synthetic Mb bearing an alkyl chain showed a sharp Soret band at 409 nm and a Q-band at 630 nm. Reduction of the met-form with $Na_2S_2O_4$ and subsequent aerial oxidation gave absorption spectra corresponding to those of the deoxy [reduced state; Fe(II)] and oxy (oxygen-bound state) forms, respectively. These spectra were almost identical to those of native Mb. The absorption spectra of the Mbs obtained by replacement of the axial (sixth) ligand with fluoride, cyanide, or azide were essentially identical to those of the corresponding forms of native Mb. The electron paramagnetic resonance (EPR) spectra were consistent with typical high-spin iron(III) species (g = 5.9 and 2.0). Their circular dichroism (CD) spectra were also similar to that of the native Mb [CD peaks at 195 nm (positive), 208 and 222 nm (negative)]. These data confirmed that the artificial cofactors had been successfully inserted into the active site pocket of apo-Mb.

The effect of the introduced anchor chain on the Mb binding and orientation to the lipid membrane was evaluated by means of various physicochemical measurements. Gel filtration experiments using Sepharose 4B and an ultrafiltration assay (cut-off molecular weight 100000) indicated that the lipid-anchored Mb(**1**), but not native Mb or Mb(**2**), was bound to the lipid bilayer membrane composed of dipalmitoylphosphatidylcholine (DPPC) in aqueous dispersion. Quenching of the fluorescence of the membrane-bound probe with the anchored Mb strongly supported the above results.[30]

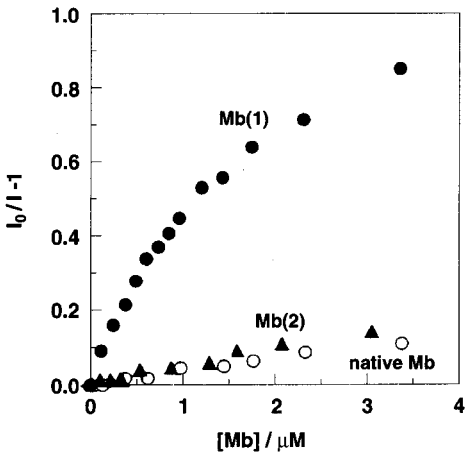


Figure 2. Stern-Volmer plot for the emission quenching of the membrane-bound DPPE-rhodamine B by myoglobin derivatives; I_0: the emission intensity in the absence of Mb, I: the emission intensity in the presence of the appropriate amount of Mb

The fluorescence intensity of DPPE-rhodamine B embedded in the DPPC membrane matrix was efficiently lessened by the addition of Mb(**1**), but not by native Mb or Mb(**2**) (Figure 2). An immobilized cast film of DPPC containing the lipid-anchored Mb(**1**) displayed anisotropic EPR signals, these being dependent on the angle of the cast film plane in relation to the applied magnetic field, implying that the Mb molecules are attached to the lipid bilayer surface in a fixed orientation by inserting the anchor chain into

the hydrophobic region of the lipid membrane. Such EPR anisotropy was not observed in the absence of an anchor [i.e. with native Mb or Mb(**2**)]. These results clearly demonstrate that the attachment of a single anchor chain to a water-soluble Mb not only enhances the membrane affinity, but also regulates the molecular orientation in relation to the membrane, as in the case of naturally occurring membrane-bound proteins (see Figure 3).

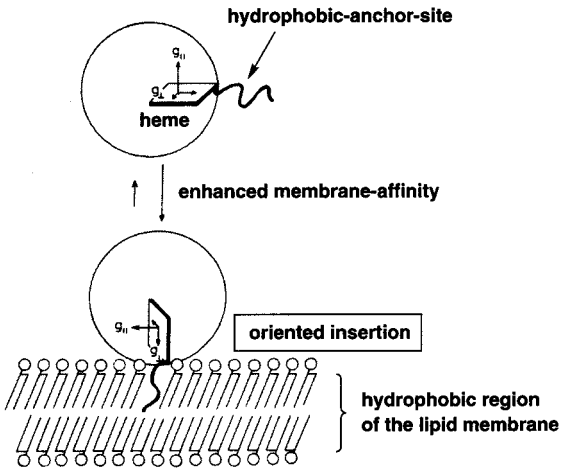
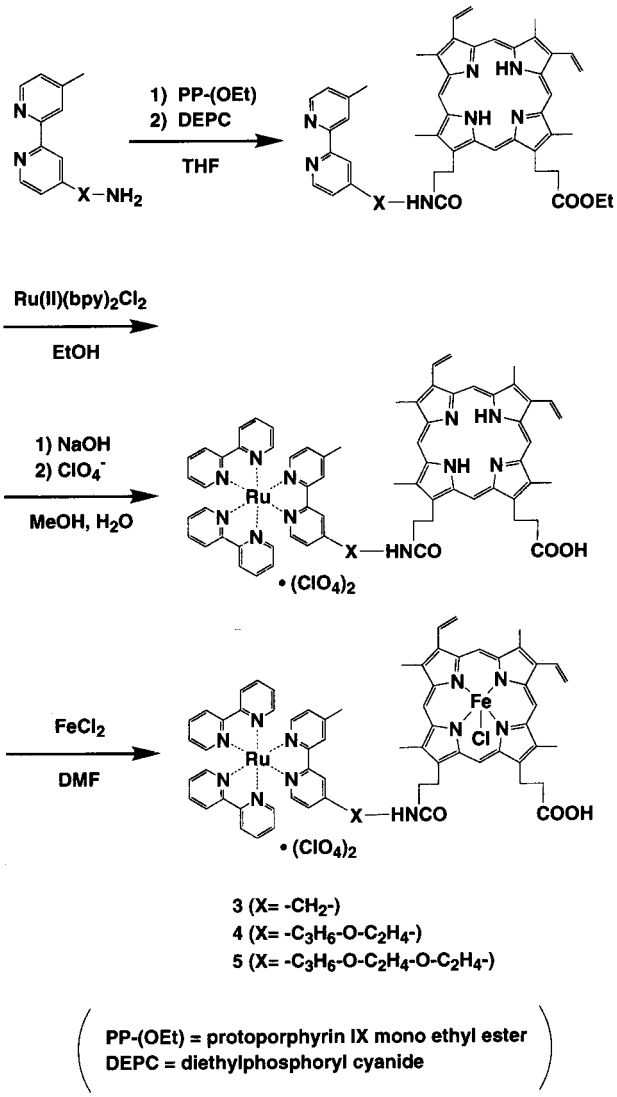


Figure 3. Schematic representation of the directional anchoring of lipid-anchored myoglobin

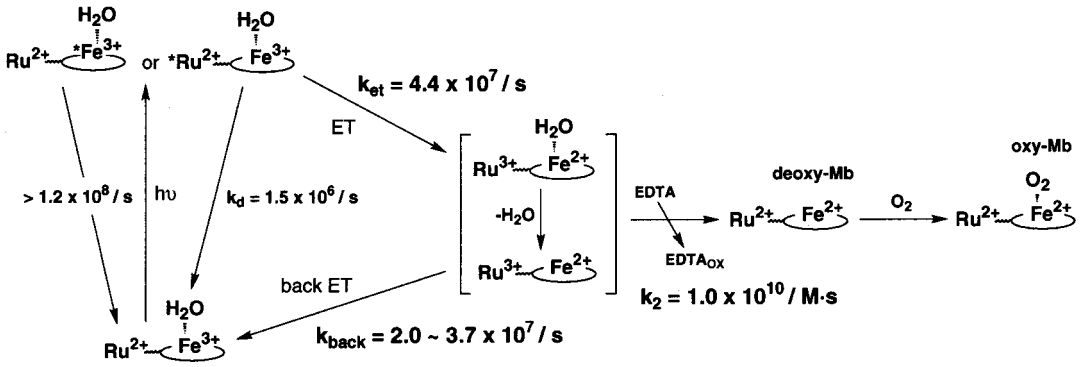
Photo-Activatable Hemoproteins

The redox state of iron in the active site of hemoproteins is crucial with regard to their enzymatic activity. Mb and hemoglobin, for example, can bind oxygen with the heme in the ferrous state, but not when it is in the ferric state. The catalytic cycle of P-450 monooxygenase is initiated by reduction of the ferric heme (resting state) with P-450 reductase. We have proposed an approach which allows switching of the redox state of such systems by photoinduced electron transfer (ET), following the covalent attachment of a photosensitizer [$Ru(bpy)_3$] to the hemoprotein (Mb or cytochrome) using cofactor reconstitution.[31]

Protohemin-bound $Ru(bpy)_3$ [$Ru(bpy)_3$-hemins, **3**–**5**] were designed and synthesized as shown. Protoporphyrin IX monoethyl ester was condensed with amino derivatives of 2,2′-bipyridine (bpy) in the presence of diethyl cyanophosphate, then complexed with bis(bpy)dichlororuthenium [$Ru(bpy)_2Cl_2$]. Ester hydrolysis was followed by iron insertion into the resultant ruthenium tris(bpy)-protoporphyrin IX, to yield the target $Ru(bpy)_3$-hemins.[32] The reconstituted Mb was characterized by methods similar to those described in the case of the lipid-anchored Mb. Furthermore, the redox potential ($E_{1/2}$) of the iron site [Fe(II)/Fe(III)] was determined to be 70 ± 2 for Mb(**3**), 71 ± 10 for Mb(**4**), and 80 ± 6 mV for Mb(**5**) *vs.* NHE (normal hydrogen electrode), values which are of the same order as that for native Mb (50 ± 10 mV). All these data indicate that the $Ru(bpy)_3$-hemins were successfully reconstituted with apo-Mb.



Since photoexcited $Ru(bpy)_3$ [$*Ru(bpy)_3$] is a powerful reductant (redox potential of −0.80 V *vs.* NHE), the photoinduced ET from $*Ru(bpy)_3$ to iron(III) of met-Mb should be a spontaneous reaction with a high driving force (0.87−0.88 V). When a solution of $Ru(bpy)_3$-Mbs was irradiated with visible light (cut-off wavelength below 450 nm) in the presence of EDTA (ethylenediaminetetraacetic acid, a sacrificial donor) under Ar, the absorption maxima of the met-Mb apparently shifted to match those of deoxy-Mb. The resulting species was rapidly converted to oxy-Mb upon exposure to air, confirming that deoxy-Mb was indeed the photoproduct. Irradiation with visible light did not affect the spectra of $Ru(bpy)_3$-hemins (which do not have an apo-Mb skeleton). No reaction occurred in an intermolecular control system [an equimolar mixture of $Ru(bpy)_3$ and native met-Mb]. Mb(**5**) was photoreduced almost as efficiently as Mb(**4**). The reaction of Mb(**3**), on the other hand, was an order of magnitude less efficient than the other two processes, showing that the spacer structure has a marked effect on the photoreduction efficiency.



Scheme 2. Photoactivation (photoinduced ET) scheme of $Ru(bpy)_3$-Mbs

The kinetic mechanism and the net photoactivation properties of semi-synthetic Mbs were examined by laser flash photolysis and steady-state photoirradiation under anaerobic conditions, which led to the mechanistic picture shown in Scheme 2.[33] Photoelectron transfer from the excited state of $Ru(bpy)_3$ to ferric heme (resting state) produces Mb with ferrous heme (active state), which is able to bind oxygen. In the absence of the sacrificial donor, EDTA, the charge-separated state rapidly reverted to the original state through back electron transfer.

Light-induced activation of $Ru(bpy)_3$-Mb was subsequently examined under aerobic conditions. Steady-state photoirradiation quantitatively converted met-Mb into oxy-Mb. It is notable that the photogeneration of oxy species is not significantly affected by O_2, even though O_2 is a typical quencher of photoexcited $Ru(bpy)_3$. This is mainly due to the accelerated intramolecular electron transfer from $*Ru(bpy)_3$ to heme rather than to O_2 in $Ru(bpy)_3$-Mbs. In fact, the excited state of $*Ru(bpy)_3$ in $Ru(bpy)_3$-Mb was not influenced by the O_2 concentration, whereas it was significantly quenched in the intermolecular system. Thus, by switching the light on and off under air, we were able to fully regulate the oxy form, an active state of Mb, by means of visible light (Figure 4).

Furthermore, using $Ru(bpy)_3$-Mbs in the presence of a sacrificial acceptor, $[Co^{3+}(NH_3)_5Cl]$, an oxidized (Fe^{4+}-heme)-Mb was photoproduced, the key step of this process being an intramolecular electron abstraction reaction.[34] UV/visible spectra, electron paramagnetic resonance measurements, and reactivity tests identified the photooxidized Mb as a ferryl species (i.e. an Fe^{4+}-heme), without damage of the protein structure (Figure 5).

The transient absorption of the laser flash photolysis provides direct evidence that the ferryl-Mb is photogenerated via the porphyrin radical cation as an intermediate and consequently the rate constants for each step can clearly be determined.[35] In the initial step, the photoexcited $Ru^{2+}(bpy)_3$ is oxidatively quenched by $[Co(NH_3)_5Cl]^{2+}$, a sacrificial acceptor, to yield $Ru^{3+}(bpy)_3$. The triply-charged product $Ru^{3+}(bpy)_3$ (a powerful oxidant with a redox potential of +1.25 V *vs.* NHE) efficiently abstracts an electron from the porphyrin ring (with a first-order rate constant of $8.5 \times 10^5\ s^{-1}$), which is followed by iron(III) oxidation by the porphyrin radical (with a first-order rate constant of $4.0 \times 10^4\ s^{-1}$ at pH 7.5). Consistently, the rate of the fast step of the porphyrin radical generation is found to be independent of the pH, whereas the slower step of ferryl heme formation shows a pH dependence. This represents the first direct evidence that shows that the photooxidation of ferric heme to ferryl heme proceeds via a porphyrin radical intermediate.

These examples demonstrate that both the introduction and the abstraction of an electron are artificially photoregulatable by the site-specific introduction of a non-natural

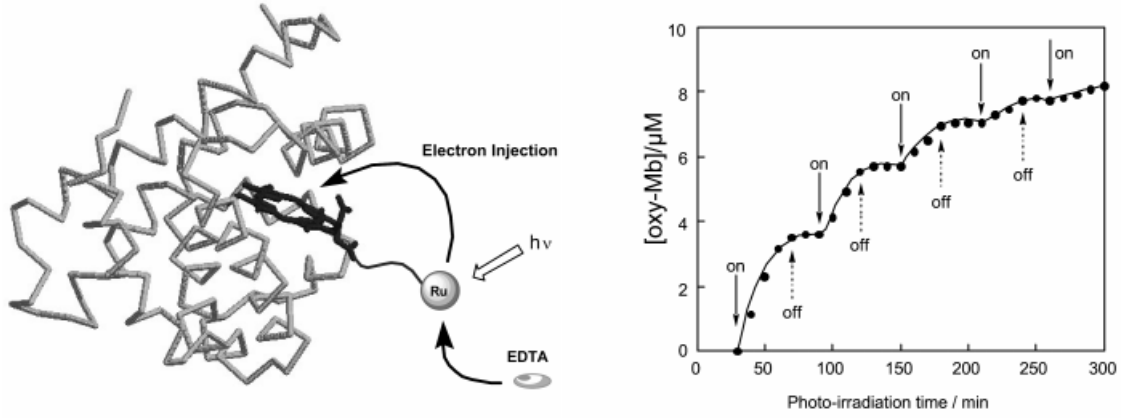


Figure 4. Photoelectron-transfer triggered activation and ON-OFF switching of $Ru(bpy)_3$-Mbs; visible light (> 450 nm) led to switching according to the appropriately labelled arrows; the concentration of oxygen-bound Mb (oxy-Mb) was monitored spectrophotometrically

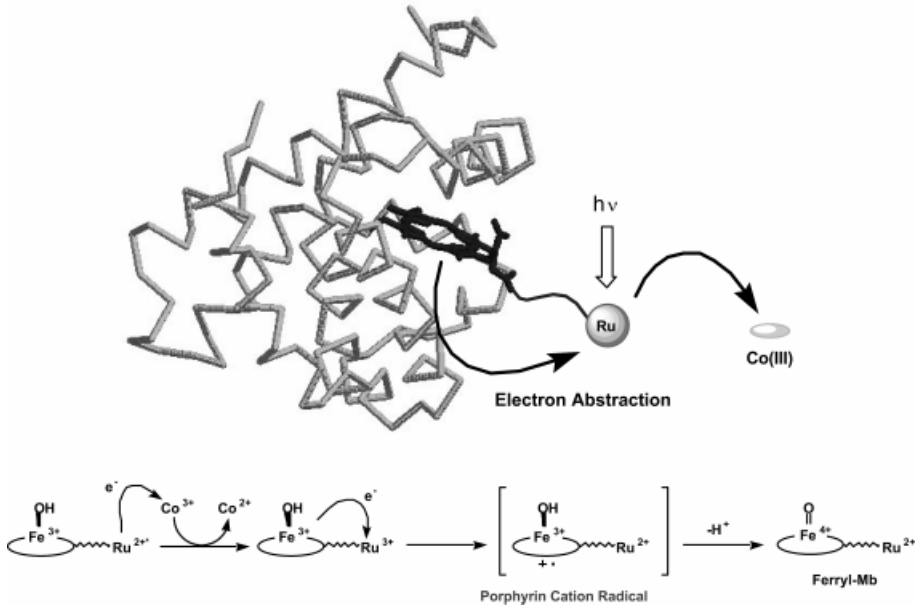


Figure 5. Photooxidation reaction scheme of $Ru(bpy)_3$-Mbs in the presence of a sacrificial Co(III) complex; a transient absorption clearly demonstrated that oxidation proceeds via the porphyrin radical cation

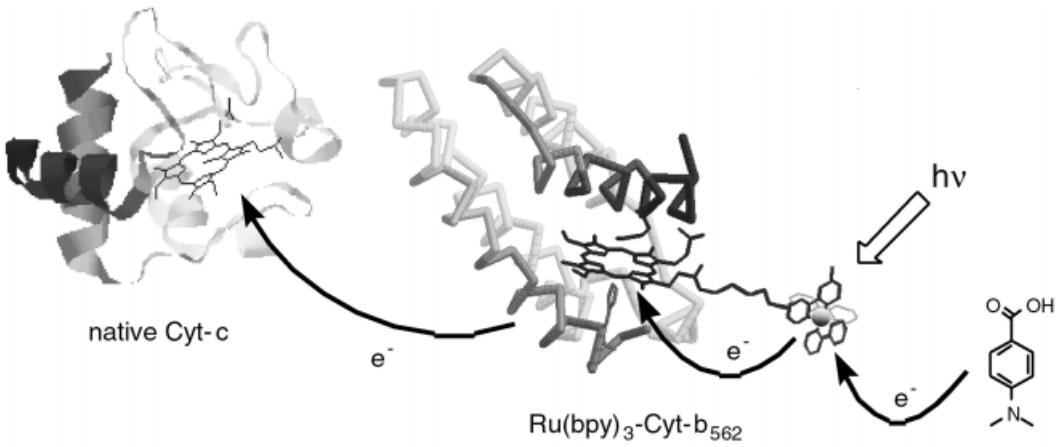


Figure 6. Tandem photoreduction of Cyt-c mediated by $Ru(bpy)_3$-Cyt-b_{562}; dimethylaminobenzoate was used as a sacrificial donor

photosensitizer. The same strategy has recently been applied to cytochrome b_{562} (Cyt-b_{562}), an electron transporting enzyme.[36] Semi-synthetic $Ru(bpy)_3$-Cyt-b_{562} is not only activated by visible light, but also transfers an electron to Cyt-c, another counterpart. The tandem electron transfer system is photo-facilitated using the derivatized cytochrome (Figure 6). In summary, we have demonstrated that it is possible to achieve photo-switching of the activities of these reconstituted hemoproteins.

Willner and co-workers have elegantly combined the cofactor reconstitution method with chemical modification in order to prepare a novel photoenzyme.[37] They changed the iron center of Mb to cobalt by reconstitution with cobalt-protoporphyrin, and then modified the peptide backbone with eosin, an organic photosensitizer [Co-Mb(Eosin)] (Figure 7).

The redox state of the cobalt center was regulated by visible light irradiation. Interestingly, a highly reduced Co(I) hydride was generated, which was subsequently used for the catalytic hydrogenation of acetylenedicarboxylic acid to maleic acid. The reconstituted Co-Mb(Eosin) was coupled to the redox enzyme lactate dehydrogenase, LDH, using ferrocene as a reversible electron mediator.[38] The coupled multi-enzyme system was able to photoproduce ethylene from acetylene at the Co-Mb(Eosin) and simultaneously oxidize lactic acid to pyruvic acid at the LDH, in the presence of *N*-(methylferrocene)caproic acid as an electron shuttle. Ogoshi and co-workers used the reconstituted Mb

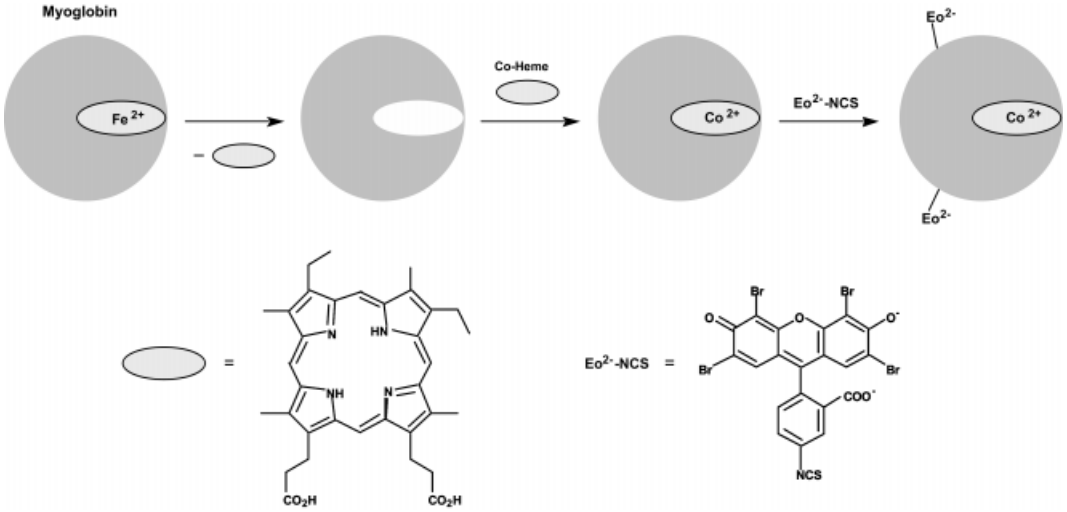


Figure 7. Preparation of a photoenzyme by combination of the reconstitution method with chemical modification; after reconstitution with cobalt heme and apo-Mb, the polypeptide backbone was chemically modified with an eosin derivative

as a simple model system for the elucidation of biological electron transfer. Zinc porphyrin was covalently linked to benzoquinone via an oligoglycine spacer (heme **6**) and was subsequently inserted into the heme pocket of apo-Mb.[39]

These authors recently synthesized an artificial heme bearing polycarboxylic acid groups (heme **7**) and then reconstituted it with apo-Mb. An anionic cluster domain was formed on the exterior of the heme crevice, similar to the hydrophobic domain of the lipid-anchored Mb, which acted as a binding site for cationic molecules.[40] The emission of the zinc porphyrin was effectively quenched upon binding of a cationic quencher such as methylviologen or cytochrome-c (Figure 8).[41]

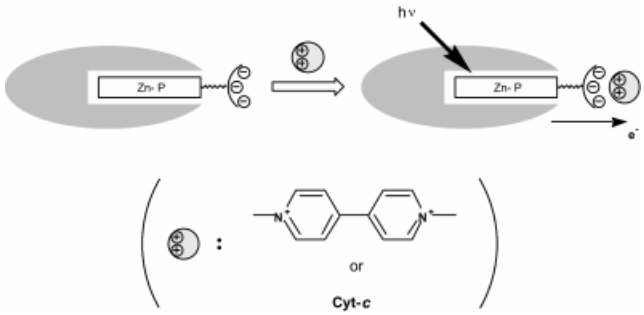
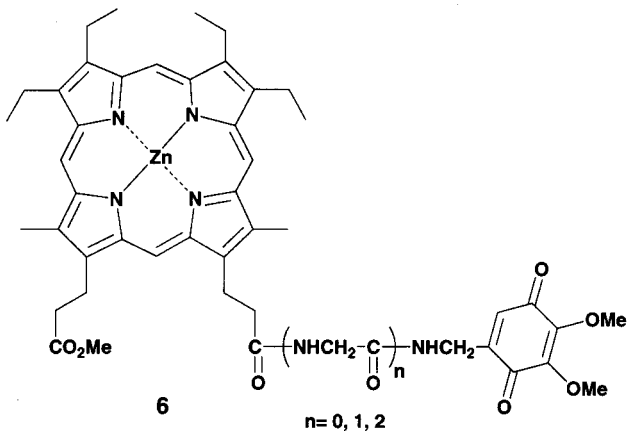


Figure 8. The introduction of an anionic cluster on the surface of myoglobin using the reconstitution method made it possible to bind a cationic viologen or cytochrome-c



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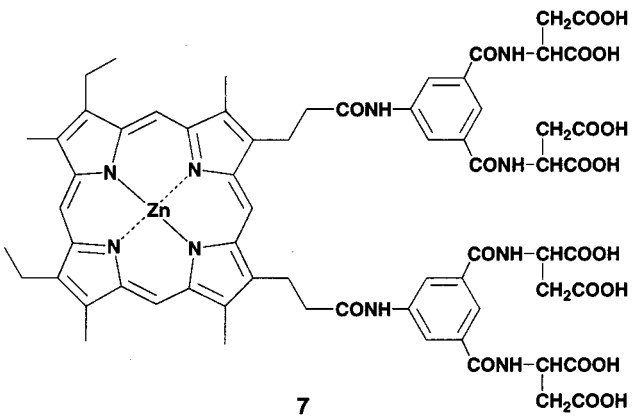

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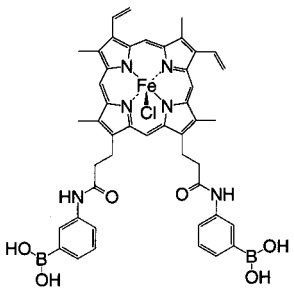
Incorporation of Artificial Receptors into Hemoproteins

Artificial receptors are anticipated to be promising molecules for the regulation of protein structure and activity. A



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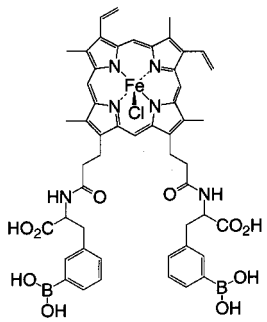

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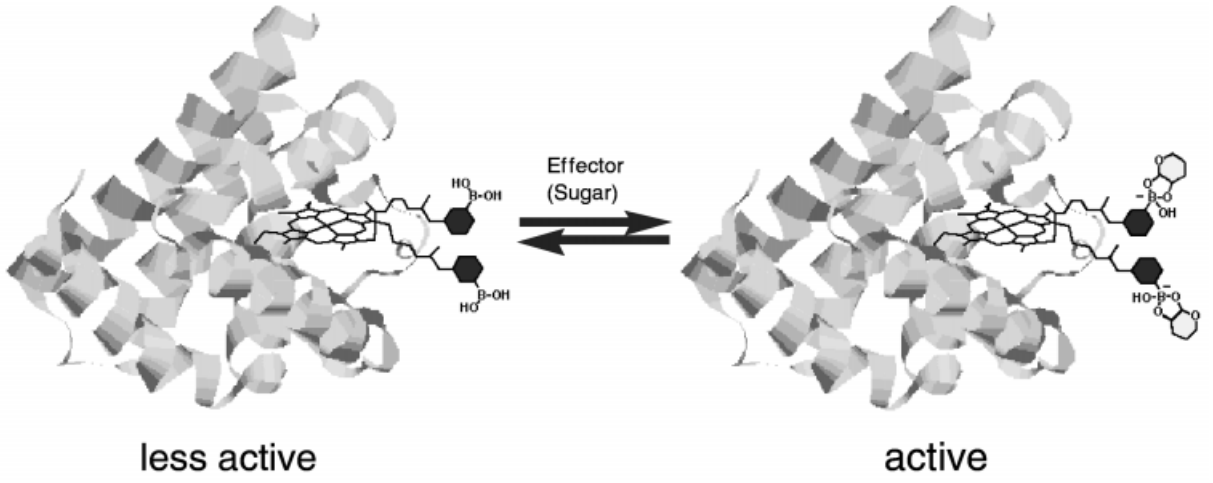
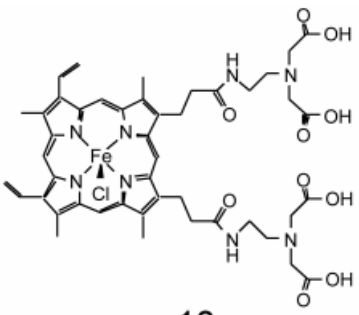


Figure 9. Schematic representation of the sugar-responsive enzymes bearing phenylboronic acid moieties; the newly generated negative charges at the boronate ester groups upon sugar binding are crucial with regard to the activity switching of the engineered Mb

water-soluble phenylboronic acid as a saccharide binder and iminodiacetic acid as a transition metal chelator were selected for initial trials. These two receptors can bind appropriate guest molecules even in aqueous solution.

Phenylboronic acid groups were successfully introduced in the proximity of the heme crevice by reconstitution using the modified hemes **8** (Bph-Mb) and **9** [*m*-(Bphe)$_2$-Mb].[42−44] Both phenylboronic acid-appended Mbs were stabilized by approximately 2 kcal/mol upon complexation with D-fructose. Spectrophotometric pH titrations demonstrated that the coordinated water molecules on the iron centers of the active site become increasingly basic upon sugar binding. The CD (intensified ICD) and paramagnetic NMR spectra proved that sugars bound to the phenylboronic acid sites cause the rearrangement of the heme crevice in such a way as to reinforce the heme−apoprotein interactions. Such sugar-induced changes in the structure may be due to electrostatic interactions between the generated boronate anion and the positively-charged moiety of the heme crevice. The oxygen storage activity of the phenylboronic acid-appended Mbs (**8** and **9**) was consequently enhanced by the addition of sugars (Figure 9). As an interesting con-



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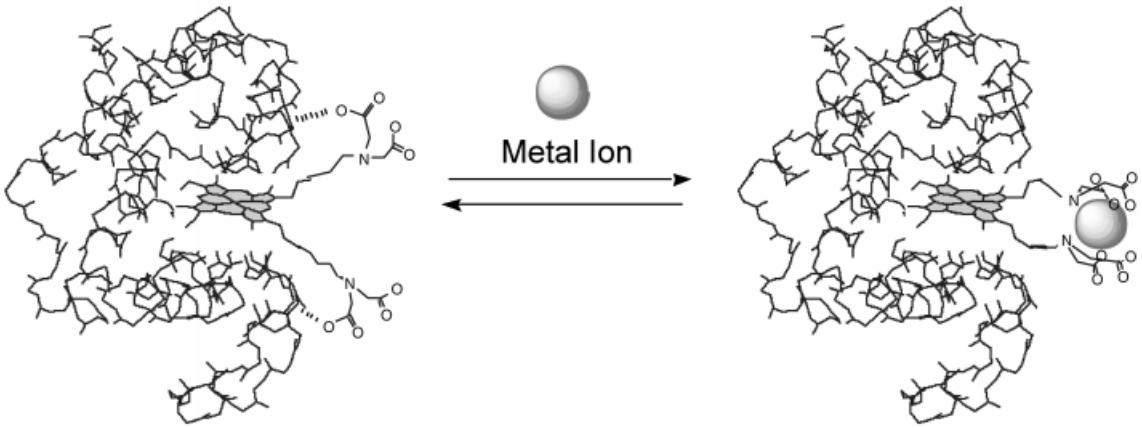


Scheme 3. Representation of transition metal binding by IDA2-Mb (a reconstituted Mb with heme **10**); two iminodiacetic acid moieties cooperatively bind transition metal cations in the proximity of the heme crevice

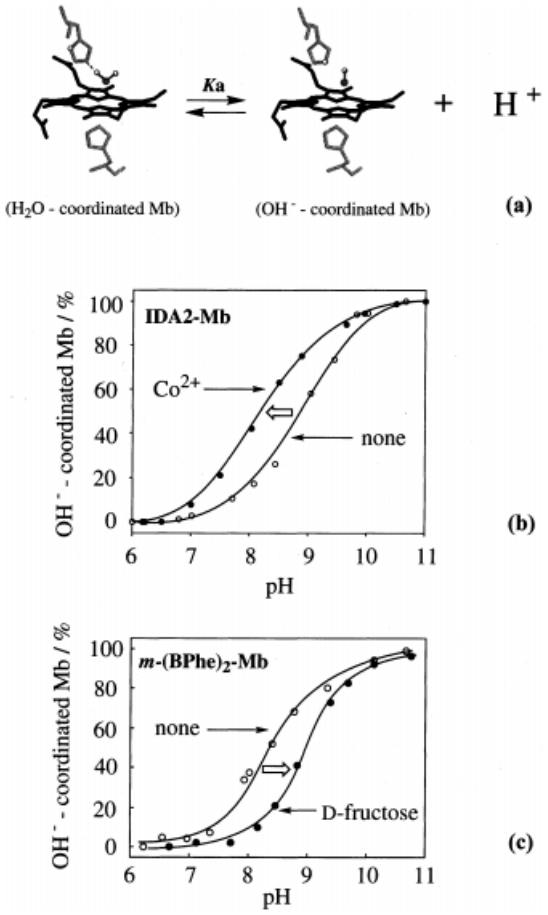
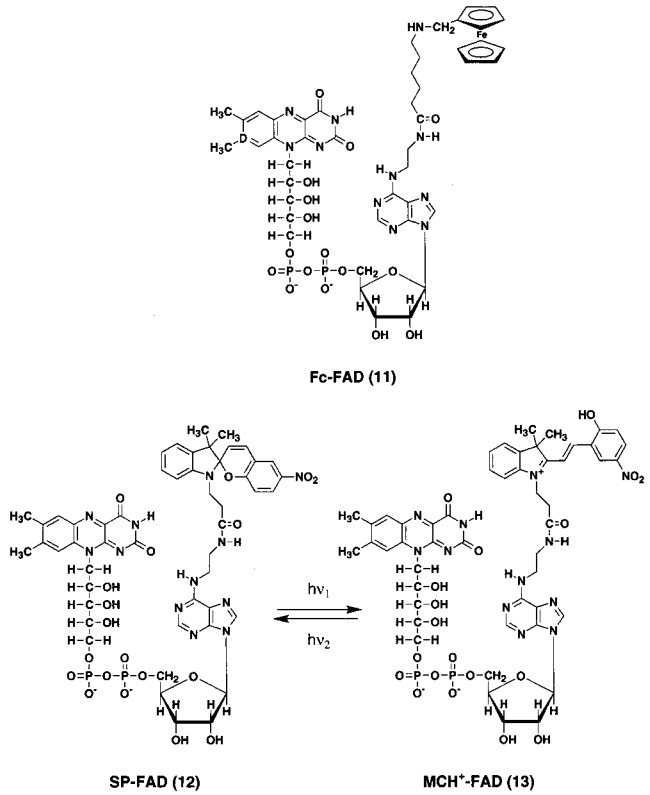


Figure 10. Opposite pK_a shifts induced by the same guest molecule in IDA2-Mb and *m*-(BPhe)$_2$-Mb; (a) illustration of the acid-base equilibrium of the coordinated water at the iron center of heme; (b) spectrophotometric pH titration curves of IDA2-Mb with or without the Co(II) cation; (c) pH titration curves of *m*-(Bphe)$_2$-Mb in the absence or presence of D-fructose

trol, we prepared a Mb randomly modified with phenylboronic acid units (with an average degree of modification of 8 units per Mb). This randomly modified Mb did not show any pK_a shift of the coordinated water or any change in function in response to sugars. This control experiment clearly demonstrates that the *active-site directed incorporation* of a non-natural molecule, which is one of the characteristics of the cofactor reconstitution method, is far more effective than a random modification.

Interestingly, opposite responses were induced upon complexation of transition metals by the iminodiacetic acid-appended Mbs (heme **10** in Scheme 3, IDA2-Mb).[45] In the case of IDA2-Mb, the negative charges of the free IDA2 site were neutralized upon binding transition metal cations such as Co(II) or Zn(II). IDA2-Mb exhibited the acidic pK_a shift and the less intense ICD spectrum associated with transition metal binding, suggesting that the heme-apoMb interactions are weakened, in contrast to the behavior of Bph-Mbs (Figure 10). The slight opening of the heme crevice facilitated reduction of the met-form of Mb with ascorbate, depending on the transition metal concentration. It is conceivable that the fine modulation of the surface charges

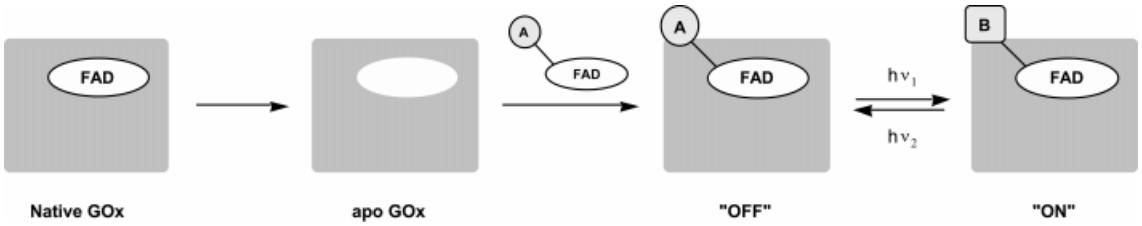


of a protein with a specific guest molecule can greatly affect its structure and activity.

These results imply that the concepts of host-guest chemistry, which have been established over the last few decades, represent powerful tools for the artificial control of the function of native enzymes. Similar ideas are now being applied to hemoglobin, an oxygen-transporting tetrameric hemoprotein, with the aim of regulating the oxygen-transporting activity using artificial receptors located on the protein surface.[46]

Extension of the Reconstitution Method to Flavoenzymes

Application of the cofactor reconstitution method for the incorporation of non-natural molecules is not limited to the aforementioned hemoproteins. Willner and co-workers recently extended this technique to flavoenzymes such as glucose oxidase and amino acid oxidase. They synthesized a ferrocene-appended flavin adenine-dinucleotide (Fc-FAD, **11**) and reconstituted it with apo-glucose oxidase (Gox).[47] The electrical communication between the reconstituted Gox and the electrode was greatly improved owing to the presence of the pendant ferrocene as an intramolecular electron mediator. Consequently, the amperometric sensing of glucose could be achieved using a modified electrode with the ferrocene-appended Gox. As a further example, these authors covalently attached a photochromic spiropyrane to FAD at its adenine ring (SP-FAD, **12**) and reconstituted it with apo-Gox.[48] Non-charged spiropyrane (SP) was pho-



Scheme 4. Schematic representation of reconstitution and photo-switching of an SP-appended glucose oxidase [bearing flavin adenine dinucleotide (FAD) as a cofactor]

toisomerized to protonated merocyanine (MC, **13**) at the Gox surface. The photoisomerizable enzyme was assembled as a monolayer on an Au electrode. The bioelectrocatalyzed oxidation of glucose in the presence of ferrocenecarboxylic acid or ferrocene-1,1′-dicarboxylic acid (acting as electron mediators) occurred with the enzyme in the MC-FAD-Gox state, whereas its function in the photoisomerized state, SP-FAD-Gox, was blocked. When 1-[1-(dimethylamino)ethyl]-ferrocene was used as a mediator, the enzyme exhibited bioelectrocatalytic activity in the oxidation of glucose when it was in the SP-FAD-Gox state, whereas its function was blocked in the MC state (Scheme 4). The authors claimed that the directionality of this enzyme photoswitch stemmed from the electrostatic affinities of the oxidized mediators to diffusively penetrate the protein and reach appropriate electron-transfer distances so as to oxidize the reduced flavin center. They succeeded in photoswitching the activity of a reconstituted flavoenzyme by tuning the surface charge distribution with visible light.

Conclusion

We have demonstrated that the reconstitution of chemically-modified cofactors with apoenzymes represents one of the most powerful methodologies for the design and semi-synthesis of artificially functionalized proteins and enzymes. Active-site-directed modification is a unique feature of this method. A wide variety of non-natural molecules, such as a hydrophobic alkyl chain, a polyanion cluster, a metal complex, electron donors or acceptors, a photochromic moiety, peptides, and artificial receptors have been successfully introduced and their effects on the holoenzymes have subsequently been assessed. This concept has recently been expanded to include not only hemoproteins but also flavoenzymes. Chemists have already produced a number of organic and/or inorganic artificial molecules with intriguing functions. It is envisaged that the controlled derivatization of native enzymes with such a broad range of non-natural molecules will be valuable for the creation of supramolecular proteins with novel biological functions. By combination of the cofactor-reconstitution technique described here with other techniques such as site-directed mutagenesis, chemical modification, peptide semi-synthesis, and non-natural amino acid incorporation using suppressor *t*-RNA, we should one day be able to artificially manipulate protein molecules with the same versatility as that with which organic chemists manipulate small molecules.

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