

Award Accounts

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Development of New Methods to Introduce Unnatural Functional Molecules into Native Proteins for Protein Engineering

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Proteins are one of the most sophisticated biomolecules that play important roles in many biological processes and recently have attracted much attention as a key molecular player in new fields of nanobio-science and -technology. To use protein molecules, suitable molecular interfaces for maintaining, regulating, and/or expanding the structure and activity of natural proteins are highly desirable. In addition, development of flexible strategies and new concepts to manipulate protein structures at precise atomic levels is an important challenge from the viewpoint of organic chemistry. In this account, we highlight two unique chemistry-based strategies, developed by our group, for incorporating unnatural molecules into natural proteins, which include (i) reconstitution of chemically modified cofactors with apo-proteins or -enzymes, and (ii) post-(photo)affinity labeling modification of natural proteins. Using these methods, various unnatural molecules were incorporated into natural proteins without any genetic manipulation to produce semi-artificial proteins, of which the function can be regulated, modulated, and/or read-out with help of the incorporated unnatural functionalities.

1. Introduction

Naturally occurring proteins are one of the most sophisticated biomolecules, and they play key roles in many biological processes, such as photosynthetic energy production, selective molecular recognition/sensing, remarkable chemical conversion, and elegant regulation of biological systems, such as immune responses.[1] More importantly, the size of these biomacromolecules is usually in the nanometer range. Thus, proteins are key molecular players as well as DNA and RNA in the recently developing fields of nano-bioscience and nano-biotechnology.[2–5] When these biomacromolecules are employed in this field, however, proteins inevitably experience various abiological conditions that may suppress or completely abolish their elegant natural function. To accommodate such abiological conditions, suitable artificial interface molecules that enable to maintain, regulate, and/or expand the structure and activity of natural proteins are desirable.

Recent developments in new bioorganic methods, such as site-directed mutagenesis using expanded genetic codes, total- or semi-synthesis of enzymes and proteins, and protein splicing, are making it possible to introduce unnatural molecules into the framework of natural proteins.[6–9] In contrast, semi-artificial proteins, of which the function and activity can be positively modulated or extended by incorporating artificial molecules using these methods, are still limited. More flexible strategies and new concepts not only for a way to introduce an unnatural moiety into proteins, but also for a way to (re-)create new protein function should be established on the basis of chemical viewpoints.

As a novel chemistry-based method for protein engineering, we developed two unique strategies: (i) reconstitution of chemically modified cofactors with apo-proteins or -enzymes, and (ii) post-(photo)affinity labeling modification of natural proteins. Importantly, both of these methods are general and flexible for introducing unnatural functional molecules specifically close to the active site of natural proteins without the need for mutagenesis. As described below, we showed that various unnatural molecules, such as photo-sensitizers, redox-active molecules, lipid tails, artificial molecular recognition units, fluorescent chemosensors, etc., can be incorporated into natural proteins or enzymes by these methods to produce semi-artificial proteins, of which the functions are drastically expanded with help of the unnatural molecules.

2. Incorporation of Unnatural Molecules into Proteins by Cofactor Reconstitution Method[10]

Cofactor-dependent enzymes possess organic or inorganic cofactors, e.g., metal-centered porphyrin derivatives, nicotinamide ($NAD(P)^+$/NAD(P)H), flavin (FMN or FAD), or PQQ derivatives, in the active site and play crucial roles in many biological processes involving redox (oxidation and reduction) chemistry.[11] Since the cofactor unit is non-covalently bound to a folded protein matrix in most cases, it is readily removed from the holo-protein in the denatured state to yield an apo-protein with no catalytic activity. Interestingly, the

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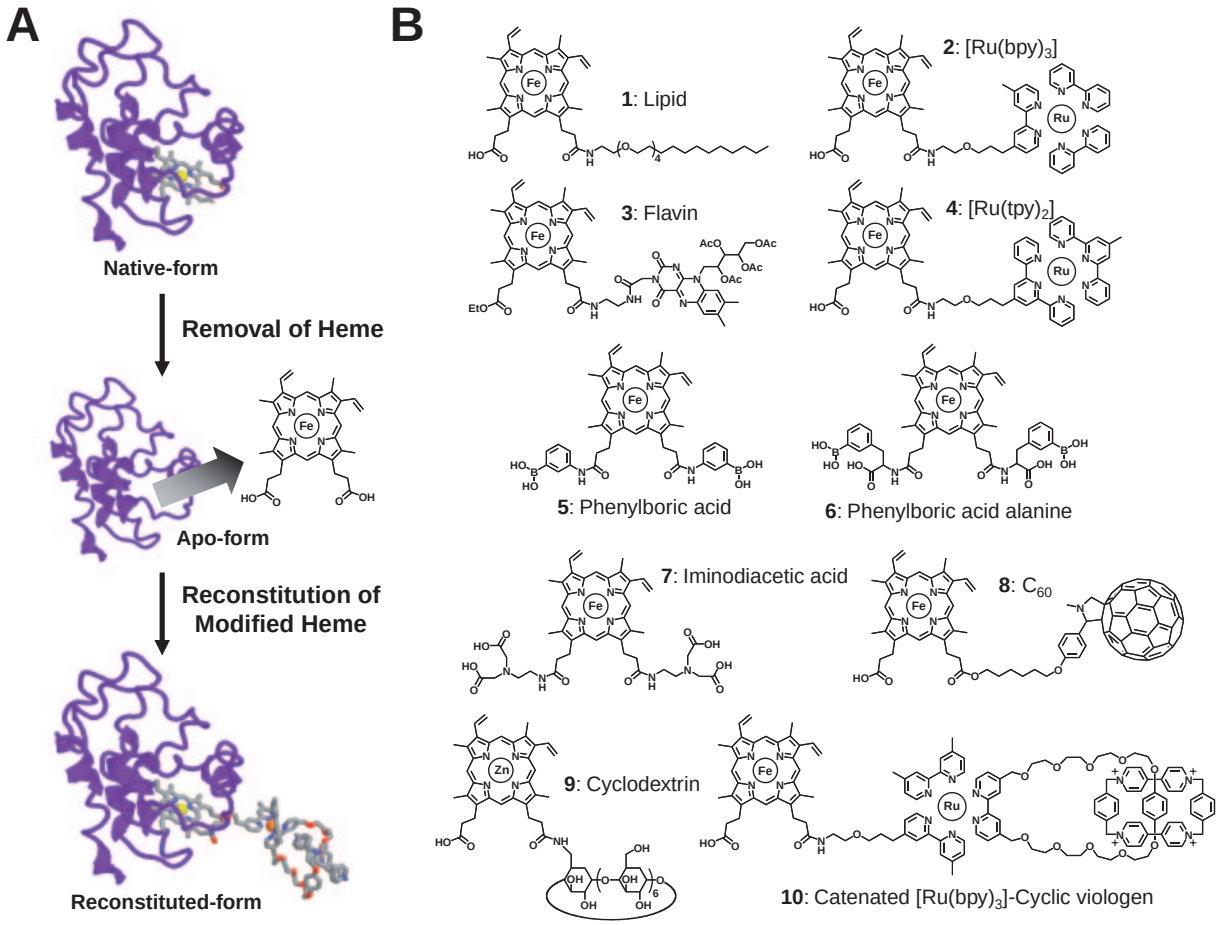

Scheme 1. A: Cofactor reconstitution of hemoprotein: in this figure, heme **10** in Scheme 1B is reconstituted to Mb. B: Synthetic heme analogues previously reported by us: **1**, lipid-anchored heme;[14] **2**, [Ru(bpy)$_3$]-anchored heme;[15] **3**, flavin-anchored heme;[17] **4**, [Ru(tpy)$_2$]-anchored heme;[18] **5**, phenylboric acid-anchored heme;[19] **6**, phenylboric acid alanine-anchored heme;[19] **7**, iminodiacetic acid-anchored heme;[20] **8**, C_{60}-anchored heme;[24] **9**, β-cyclodextrin-anchored heme;[25] **10**, Catenated [Ru(bpy)$_3$]–cyclic viologen-anchored heme.[16]

resulting apo-protein can re-bind the original cofactor under appropriate conditions and regain its full activity.[12] This is called cofactor reconstitution. Cofactor reconstitution has been used mainly among enzymologists and structural biologists to investigate the protein–cofactor interactions and the structure–activity relationships of the cofactor-dependent enzymes. For such purposes, several attempts to use cofactors bearing a slight structural modification for the reconstitution with apo-proteins have been reported.

Based on this knowledge, we envisaged that a synthetic cofactor tethering an unnatural functional moiety could be incorporated into an apo-enzyme during the cofactor reconstitution process, producing new holo-enzyme bearing the unnatural unit proximal to the active site. As a proof-of-principle experiment, we used myoglobin (Mb) as a first model protein among various cofactor-containing proteins. Mb is abundant in muscle cells of mammals and is involved in oxygen-storage and oxygen transport to mitochondria for oxidative phosphorylation.[13] As well as other hemoproteins including hemoglobin, peroxidases, or cytochrome families, Mb contains an iron-centered protoporphyrin IX (Fe–PPIX) as the natural cofactor. As shown in Scheme 1A, the propionate ends of Fe–PPIX are exposed out to the surface of Mb. Therefore, we decided to attach an unnatural unit at the propionate end(s) of Fe–PPIX and synthesized various Fe–PPIX derivatives as shown in Scheme 1B, all of which were accepted as the cofactor for apo-Mb, yielding semi-artificial holo-Mb without any significant loss of the activity.

Our first successful demonstration of the cofactor reconstitution-based protein modification was the lipid-anchored Mb (**1**–Mb).[14] Despite the presence of an unusual hydrophobic lipid tail, the reconstituted **1**–Mb showed ligand-binding properties almost identical with that of native Mb. Furthermore, it was demonstrated that, by virtue of the lipid tail, **1**–Mb could be incorporated into a phospholipid bilayer membrane in an anisotropic orientaion. Controlling the orientation of protein molecules within a two-dimentional matrix is of importance to synthesize sophisticated bio-materials or -catalytic systems.

Later, we extended the reconstitution method to create semi-artificial hemoproteins with new properties. One example includes a photo-functional Mb bearing ruthenium tris(bipyridine) complex, [Ru(bpy)$_3$], as a photo-sensitizer at the Mb surface (**2**–Mb).[15] In biological systems, the activity of hemoproteins is determined by the redox state of the active site, i.e. Fe–PPIX,

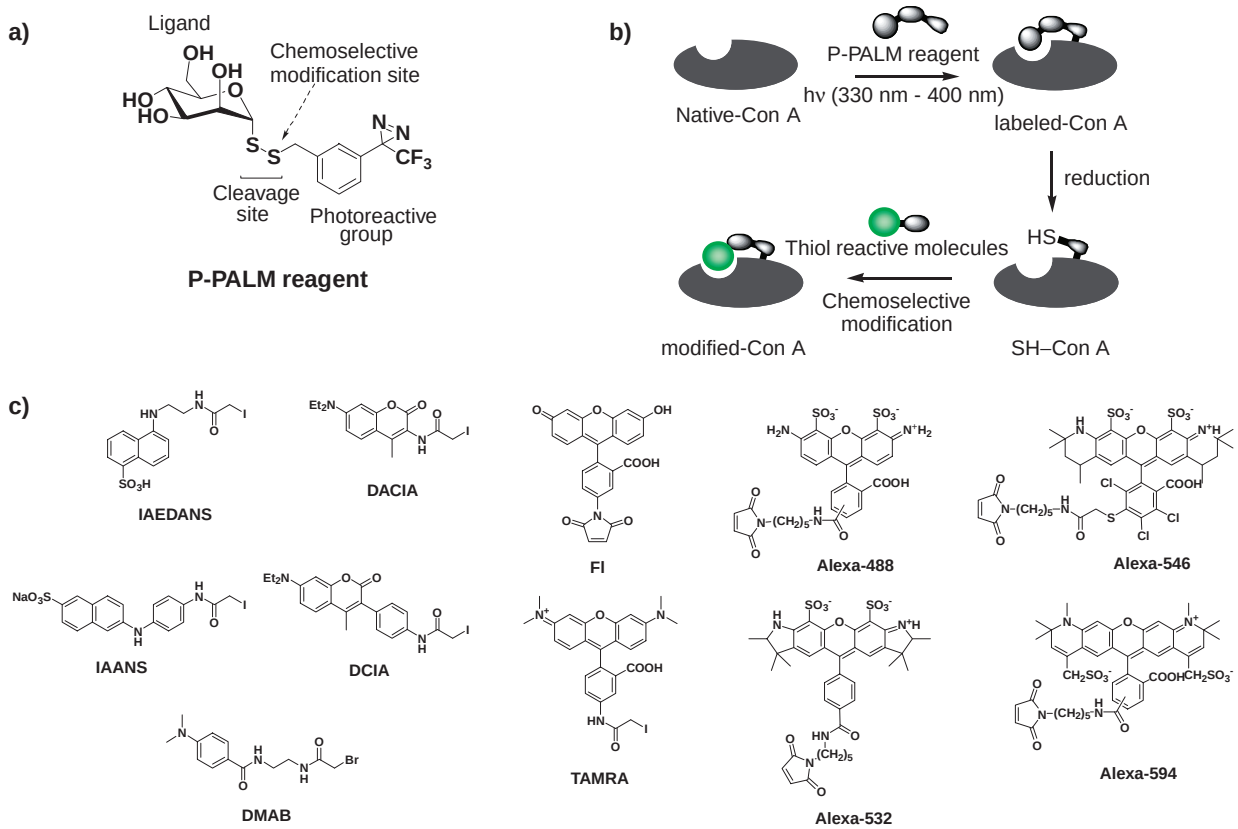

Fig. 1. a) Molecular structure of a P-PALM reagent. b) P-PALM scheme for the semisynthesis of fluorophore labeled-Con A. c) Molecular structure of various thiol reactive fluorophore introduced on Con A by us.

which is dynamically regulated by electron relay systems involving multiple redox-active proteins. Therefore, we reasoned that the activity of **2**–Mb could be photo-regulated by injecting or abstracting an electron to or from the Fe–PPIX by using unique photo-redox properties of $[Ru(bpy)_3]$. Indeed, we demonstrated that **2**–Mb in an inactive resting-state could be photoconverted to oxygen-storage state or substrate-oxidizable state via photo-induced electron transfer. The more complicated supramolecular electron relay system based on donor/sensitizer/acceptor triad was successfully attached to the Mb surface (**10**–Mb).[16] In **10**–Mb, photo-initiated vectorial, multistep, and proton-coupled electron transfer was carried out, achieving a long-lived charge-separated state with the lifetime of over 2 ms, which approaches that of the natural photosynthetic reaction center. These and other studies, including flavin- (**3**–Mb)[17] and ruthenium bis(terpyridine) complex-appended myoglobin (**4**–Mb),[18] clearly indicate that the direct attachment of photo-/redox-active molecules at the cofactor can facilitate electron or hole transfer to the active site which is, in most cases, deeply buried in the protein matrix. The findings described above will provide a new strategy and concept for creating protein-based nano-electronic devices.

As represented by several signal transduction processes, such as Ca^{2+}-, cAMP/GMP-, or IP_3-signaling, the allosteric regulation of protein function by small molecules is fundamental to organize complex protein networks. We demonstrated that such allosteric enzymes could be created by tethering an artificial molecular recognition site to the cofactor by using cofactor reconstitution method. For examples, Mb modified with boronic acid for sugar-binding (**5**– and **6**–Mb) can modulate the aniline hydroxylase activity in response to a sugar, such as D-fructose.[19] A chelator-attached Mb (**7**–Mb) can respond to transition-metal cations and alter its ability to accept an electron via an allosterically induced conformational change.[20] Given the current difficulty of (re-)designing an allosteric protein by rational approach, incorporation of artificial receptor molecules into proteins should be a valid chemical strategy.

Following our reports, other researchers have also reported that the reconstitution of synthetic heme analogues can confer unique catalytic activity to Mb.[21] It should be pointed out that this strategy should be generally applicable to other hemoproteins as well as cofactor-dependent enzymes containing flavin or PQQ. Indeed, we reconstituted synthetic heme with cytochrome b_{562} and hemoglobin to produce semi-artificial hemoproteins with new properties.[22] Willner and co-workers have prepared various semi-synthetic enzymes by using cofactor reconstitution toward constructing efficient biocatalysis systems.[23] These results indicate that a set of chemical modification of natural cofactors and the reconstitution with the corresponding apo-proteins represents a powerful methodology to confer new functions on native proteins.

3. Development of Post-Photoaffinitiy Labeling Modification for Protein Engineering

3.1 Post-Photoaffinity Labeling Modification (P-PALM).[26] Here, we propose another new semisynthetic method, called post-photoaffinity labeling modification (P-PALM), to introduce a reactive tag for artificial molecules (a signal transducer,

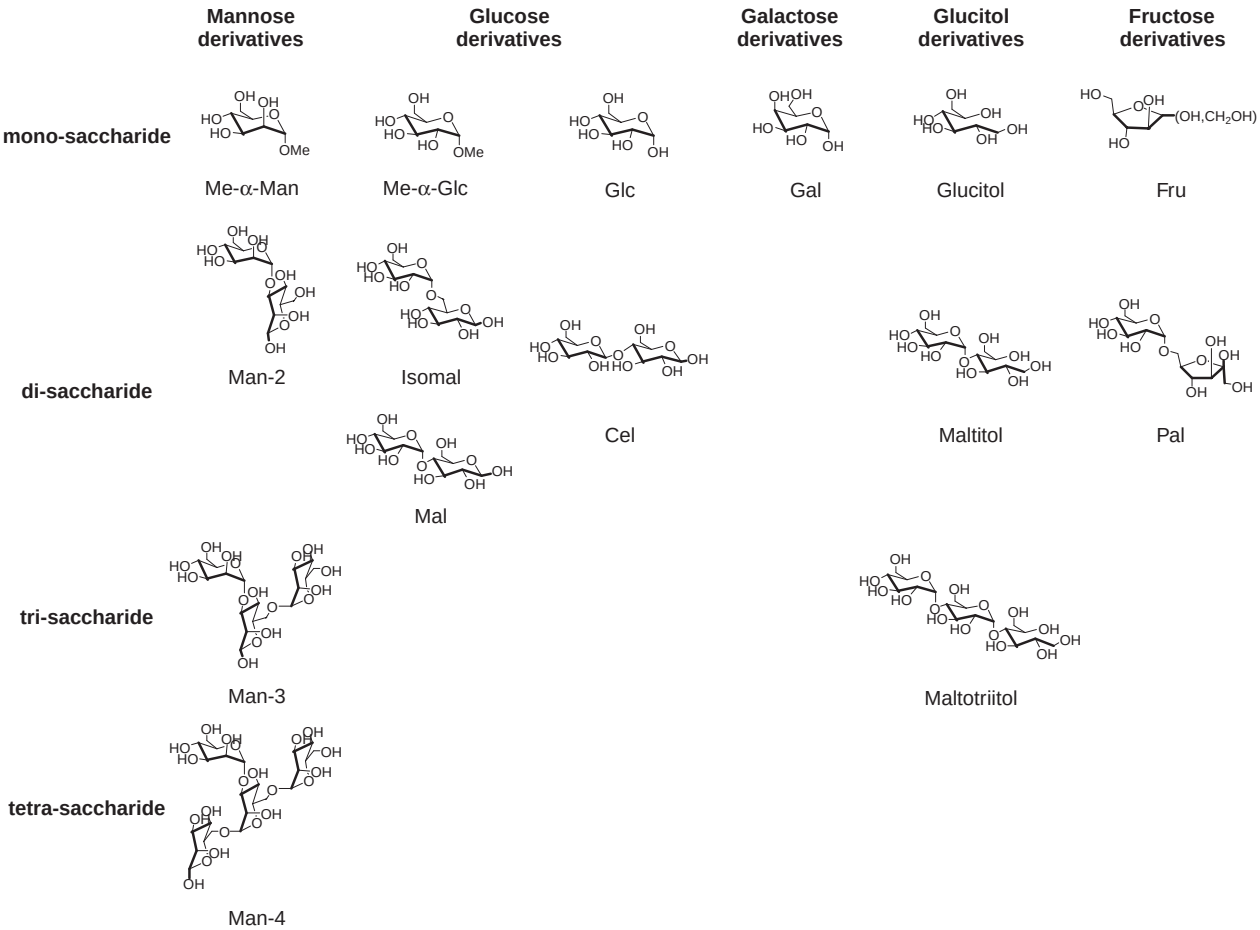

Fig. 2. Saccharide structures used in this account.

an artificial receptor, etc.) proximal to the active site of a target protein. In a proof-of-principle experiment, concanavalin A (Con A),[27] an extensively studied lectin (saccharide-binding protein), was used as a model protein.

We designed several P-PALM reagents to use with Con A. One of which is shown in Fig. 1a. The reagents had three functionalities in the molecular structure, that is, (i) a high affinity ligand (α-D-mannoside) to bind to Con A (a target protein) selectively, (ii) a photo-reactive group (trifluoromethylphenyldiazirine) to label the protein by photoirradiation, and (iii) a suitable cleavage site (disulfide bond) to remove the ligand after photolabeling and to generate a chemoselective modification site (thiol group, in this case). Using this P-PALM reagent, the protein modification was conducted as shown in Fig. 1b. When the P-PALM reagent was bound to the binding pocket of Con A, photo-labeling was carried out by UV-light irradiation ($330\,\text{nm} < \lambda < 400\,\text{nm}$ for trifluoromethylphenyldiazirine). Subsequently, the labeled Con A was purified by gel chromatography, followed by affinity column chromatography. Then, treatment of the labeled Con A with dithiothreitol (DTT) or tris(2-carboxyethyl)phosphine (TCEP) gave a unique α-toluenethiol site (SH–Con A), which was chemoselectively modified with a thiol reactive molecules, such as haloacetyl or maleimide group. The conventional peptide digestion, HPLC separation, and tandem mass spectroscopy of the labeled Con A indicates that the photolabeling site can be assigned to Tyr100, a proximal amino acid in the saccharide-binding pocket. It is clear that the photoaffinity-labeling process of the present P-PALM method is remarkably site-selective.

Figure 1c displays several type of thiol reactive fluorophores introduced on Con A by us. In the next section, we describe that various artifical molecules can be used to construct sophisticated biosensors based on Con A.

3.2 Fluorescent Saccharide Biosensor.[26] Various fluorophore-tethered Con As could be easily prepared from SH–Con A by simply mixing with the haloalkylated species. One of the representatives is EDANS–Con A bearing 5-((2-aminoethyl)amino)naphthalene-1-sulfonic acid (EDANS) fluorophore (an environmentally sensitive probe) as a signal transducer. It is interesting that a spectral change in the emission of EDANS–Con A took place by addition of methyl-α-D-mannoside (Me-α-Man, Fig. 2), one of the strongest ligands for native Con A (Fig. 3a). Upon increasing the concentration of Me-α-Man, the emission intensity decreased, and the emission peak maximum slightly red-shifted. This implies that the EDANS unit moves into a more polar microenvironment than the original position upon complexation of EDANS–Con A with Me-α-Man (Fig. 3b). It is noteworthy that the binding process of EDANS–Con A with Me-α-Man can be directly monitored by the fluorescence signal change. Fluorescence titration gave a typical saturation curve, from which a binding constant ($\log K = 3.89$) for Me-α-Man was determined. This

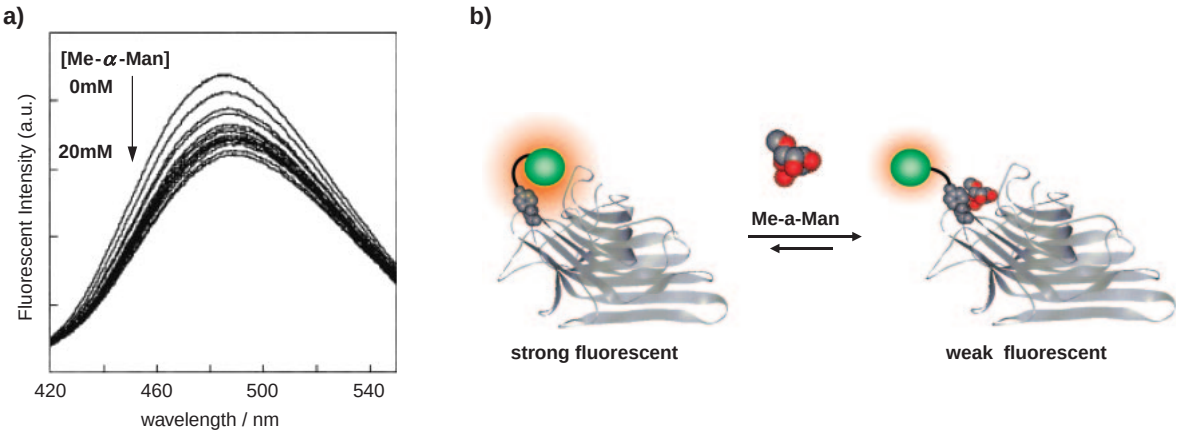

Fig. 3. a) Spectral changes in the fluorescence of EDANS–Con A upon addition of Me-α-Man (0–20 mM). [EDANS–Con A] = 5 μM, in 50 mM HEPES buffer (pH 7.0) containing 1 mM $CaCl_2$, 1 mM $MnCl_2$, and 0.1 M NaCl. Temperature: 15 ± 1 °C, $\lambda_{ex} = 340$ nm. b) Schematic illustration of the sensing mode of EDANS–Con A.

value is almost comparable to that of native Con A ($\log K = 4.04$) determined by isothermal titration calorimetry (ITC). More importantly, the sugar selectivity and binding affinity of the EDANS–Con A, evaluated by the fluorescence titration, are almost identical to those of native Con A (Table 1). This indicated that EDANS–Con A is a fluorescent saccharide biosensor retaining the natural selectivity and affinity of Con A.[27]

Molecular mechanism of the fluorescence response of EDANS–Con A to saccharide binding was strongly supported by fluorescence depolarization measurements. The fluorescence anisotropy ratio decreased upon addition of Me-α-Man. A decrease in the anisotropy is usually due to an increase in the freedom of the fluorophore in motion. Thus, the results clearly imply that the EDANS moiety is restricted in the binding pocket of Con A without sugars in the original state, and the environment changes into one of the rather higher mobility upon complexation with Me-α-Man. Both the slight red-shift in the fluorescence and decrease in the fluorescence anisotropy upon saccharide complexation reasonably supported such a mechanism.

As a control experiment, the EDANS group was randomly modified in Con A and fluorescence titration with Me-α-Man was conducted. No significant changes were observed in this control, indicating that the site-selective attachment of a EDANS group is crucial to the above-mentioned fluorescent response of EDANS–Con A.

3.3 Fluorescent Ratiometric Biosensor.[28] It is naturally anticipated that the introduction of two or more unnatural molecules is capable of conferring more sophisticated functions upon a native protein. In order to carry out the site-selective incorporation of two distinct fluorophores into Con A, we employed a controlled acylation reaction in addition to P-PALM. It is reported that Con A bears a loose binding site for hydrophobic substances distinct from the sugar-binding pocket.[29] Thus, it is expected that an appropriately hydrophobic fluorophore is bound there so that it reacts at the vicinal site of the hydrophobic domain. To test the hypothesis, a coumarin-appended active ester (AMCA-SE, Fig. 4a) was reacted with the native Con A. The site selectivity was examined by using a conventional peptide-mapping technique via trypsin digestion, and the labeling site was assigned to Lys114, indicating that the reaction selectively occurred at pH 5.0 to tether AMCA at Lys114 of Con A. In contrast, multiple peptide peaks were observed for the reaction under basic conditions (pH 8.5), suggesting that the reaction has a rather low selectivity at pH 8.5.

On the basis of the above acylation result, the scheme for the double modification of Con A was established as shown in Fig. 4b. Labeled Con A, prepared by photoaffinity labeling, was selectively acylated with AMCA-SE under acidic conditions to afford the AMCA-labeled Con A. Using the P-PALM method, the labeled ligand unit was cleaved off, and fluorescein was attached to the produced thiol site via the maleimide/SH reaction to yield doubly modified AMCA–Fl–Con A. Each step was followed by matrix-assisted laser desorption/ionization time-of-flight mass (MALDI-TOF MS)

Table 1. Comparison of the Assosiation Constants of Semi-synthetic Con A with Those of Native Con A and PBA

Saccharide	log *K*				
	native-ConA[c)]	EDANS–ConA	APET–Con A	APET-ape–ConA	PBA
Me-α-Man	4.04	3.89	—[a)]	—[a)]	—[a)]
Me-α-Glc	3.48	3.20	—[a)]	—[a)]	—[a)]
Fru	—[a)]	—[a)]	2.85	2.99	2.28
Glucitol	—[b)]	—[a)]	3.52	3.63	3.68
Man-2	4.48	3.76	—[a)]	—[a)]	—[a)]
Isomal	3.23	3.11	4.08	—[a)]	—[a)]
Mal	3.20	2.76	—[a)]	—[a)]	—[a)]
Cel	—[a)]	—[a)]	—[a)]	—[a)]	—[a)]
Pal	—[b)]	3.20	5.18	3.20	2.76
Maltitol	—[b)]	3.00	6.84	5.75	4.51
Man-3	5.40	5.40	—[a)]	—[a)]	—[a)]
Maltotriitol	—[b)]	3.00	5.26	5.84	4.56
Man-4	5.30	5.45	5.81	—[a)]	—[a)]

a) Precise values cannot be determined because of low affinity ($\log K < 2$). b) No data were reported in previous literatures.[27] c) Reported values determined by ITC.

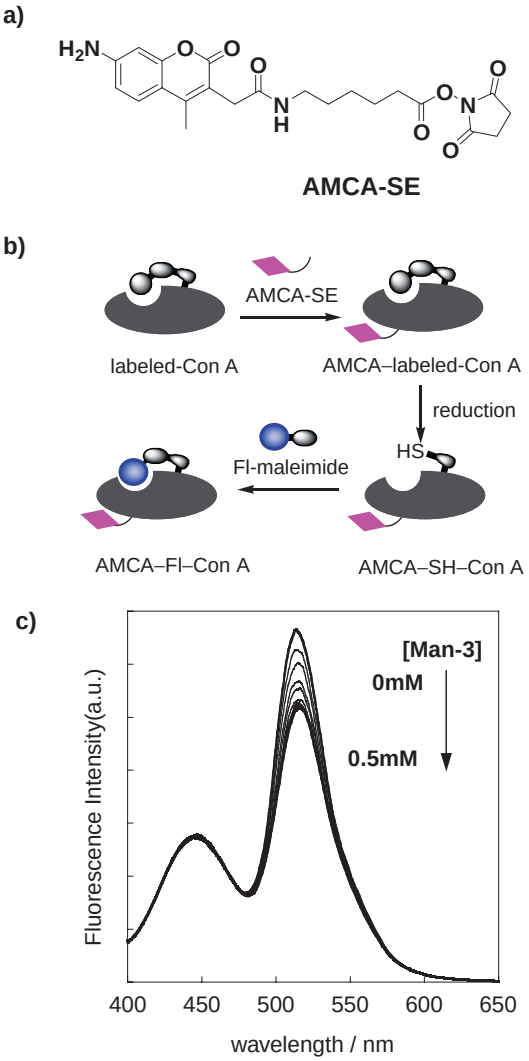

Fig. 4. a) Molecular structure of AMCA-SE. b) Scheme for double modification of Con A by coupling P-PALM with the controlled acylation to afford semisynthetic AMCA–Fl–Con A. c) Fluorescence spectral change of AMCA–Fl–Con A upon addition of Man-3 (0–0.5 mM). [AMCA–Fl–Con A] = 0.1 μM, in 50 mM HEPES buffer (pH 7.5) containing 1 mM $CaCl_2$, 1 mM $MnCl_2$, and 0.1 M NaCl. Temperature: 20 ± 1 °C, $\lambda_{ex} = 350$ nm.

spectroscopy, and AMCA–Fl–Con A was characterized by UV–visible, fluorescence, and mass spectroscopies.

These two distinct fluorescence peaks of AMCA–Fl–Con A may be potentially useful for the ratiometric sensing of saccharides.[30] Figure 4c shows a typical fluorescence change for AMCA–Fl–Con A upon the addition of 1,3- and 1,6-mannotriose (Man-3). It was found that the fluorescence of AMCA (450 nm) was not altered, whereas the fluorescence intensity of Fl (513 nm) decreased upon the addition of Man-3.

The fluorescence change of Fl can be attributed to the microenvironmental change of Fl caused by the sugar binding, because Fl was tethered at the proximity of the sugar-binding site of Con A. Since the AMCA emission was independent of the saccharide concentration, ratiometric sensing could be carried out using the AMCA emission as the internal standard. The emission ratio of Fl versus AMCA showed typical saturation behavior, meaning that the binding affinity is almost identical to that of native Con A.[27] A similar ratiometric fluorescence titration was conducted for other saccharides and glycoproteins. The evaluated selectivity and binding affinity are almost identical to the reported values of the native Con A determined by the ITC experiment, indicating that AMCA–Fl–Con A is a ratiometric fluorescent biosensor retaining the natural selectivity of Con A.[27]

Besides its detection ability in test tube experiments, AMCA–Fl–Con A can also be used to sense and image sugar derivatives localized on a cell surface by using the ratiometric technique. As a proof-of-principle experiment, the fluorescent imaging of MCF-7 cell line, a breast cancer cell covered with a high-mannose-type of saccharide, was conducted. Figure 5a shows photographs obtained by confocal laser scanning microscopy (CLSM). Upon treatment of the MCF-7 cells with AMCA–Fl–Con A, strong pinkish fluorescence due to AMCA was observed on the surface of the MCF-7 cells, relative to that of the bulk solution. This is reasonably explained by the fact that AMCA–Fl–Con A is concentrated on the MCF-7 cell surface by binding to the glycosides with high mannose content. This is strongly supported by the fluorescence spectra of the localized space of the cell. As shown in Fig. 5b, the fluorescence intensity coming from Fl was weaker on the cell surface compared to that from AMCA than the Fl intensity in the bulk solution.

Ratiometric fluorescence analysis must be more powerful if it is to be used inside a cell. To evaluate the applicability of AMCA–Fl–Con A for intracellular glucose imaging, AMCA–Fl–Con A was transported into HepG2 cells using a conventional surfactant procedure. CLSM study showed that a strong fluorescence appeared inside HepG2 cells. From the spectral analysis of the CLSM image, it is clear that the fluorescence is due to both AMCA (450 nm) and Fl (513 nm), indicating that AMCA–Fl–Con A was localized in the cytosol of HepG2. Figure 6a shows the ratiometric image from CLSM for various D-glucose (Glc) concentrations. With an increase in the Glc concentration outside the cell, a reddish-colored image changed into a yellow-to-bluish one in cells, implying that the relative fluorescence intensity coming from Fl gradually decreased (Fig. 6b). Such a ratiometric change did not occur under the similar experiment with D-galactose (Gal), which does not bind to Con A (Fig. 6c). These results indicate that AMCA–Fl–Con A located in the cytosol gradually bound Glc with the increase in the Glc concentration. This result clearly demonstrates that the semisynthetic AMCA–Fl–Con A, which tethers two distinct small fluorophores, responds to a Glc concentration change inside a HepG2 cell. Clearly, the present ratiometric biosensor system is more powerful for the imaging of the Glc concentration inside a cell than the intensity-based sensing systems that frequently give problematic artifacts due to photobleaching and/or the spatial redistribution of a single-modified probe protein. Such an in-cell glucose imaging will be useful for a cell-based screening assay of drugs for type II diabetes.[31]

3.4 Luminescent Saccharide Biosensor.[32] In addition to organic fluorophores, a metal-chelator may act as a second emission site in protein-based biosensors. It is generally con-

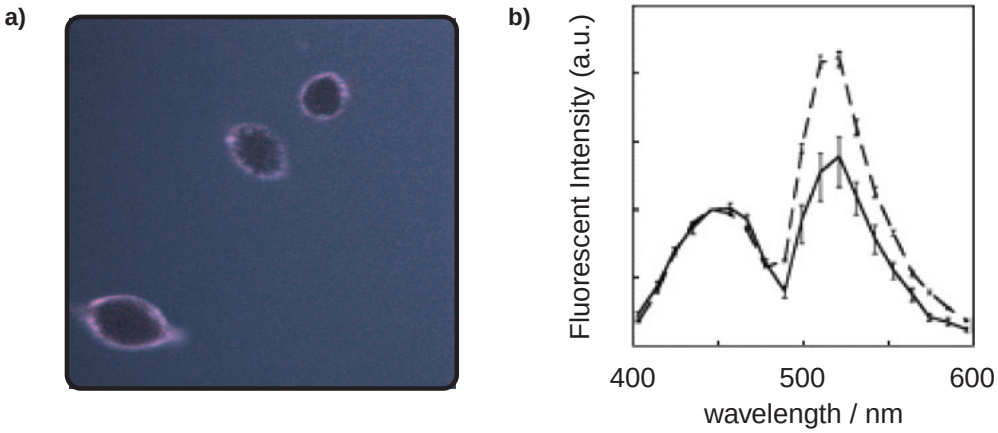

Fig. 5. a) CLSM images of MCF-7 cells. The images of the samples stained with AMCA–Fl–Con A by fluorescence channel. $\lambda_{ex} = 351$ nm. The red color and the blue color were assigned for AMCA and Fl, respectively. b) Fluorescence spectra of AMCA–Fl–Con A normalized at 446 nm on the cell surface (plane line) and in the bulk solution (dashed line). These spectra were collected from the cell image of Fig. 5a.

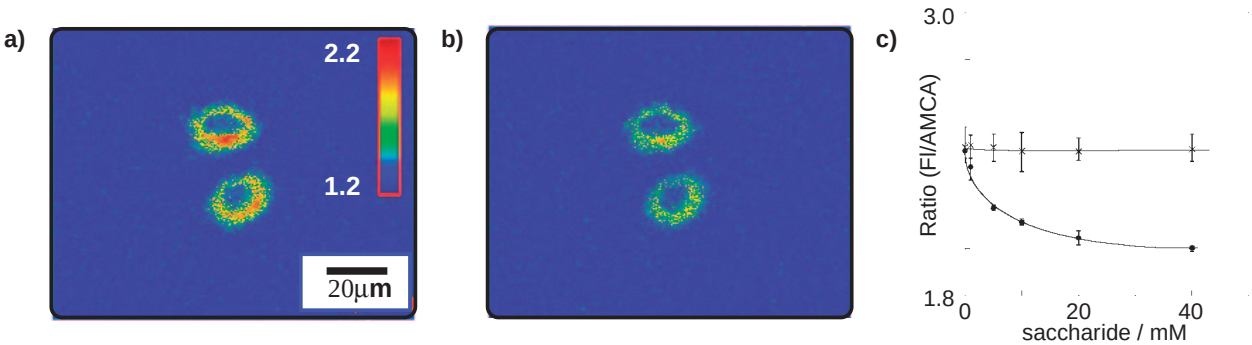

Fig. 6. a) b) CLSM images of intracellular ratiometric image of HepG2 cells proved by AMCA–Fl–Con A. Pseudocolor fluorescence ratio image of the extracellular concentration of Glc: 0 mM (a), 40 mM (b). $\lambda_{ex} = 351$ nm. c) The ratiometric intensity (Fl/AMCA) change depending on various extracellular saccharide concentrations (Glc (●) and Gal (×)).

sidered that metal-based luminescence has advantages over the conventional fluorescence for several reasons, such as a longer lifetime of emission, a lower scattering of excited light, a lack of disturbance from fluorescent impurities, and absence of analogous properties in the biological sample. Therefore, a luminescence mode sensing system is anticipated to be promising particularly for application to complicated mixtures of biological samples.[33] A luminescent biosensor for saccharide using luminescence resonance energy transfer (LRET) was constructed by coupling P-PALM with the coordination chemistry. It has been reported previously that Con A has a binding site for metal ions such as Mn^{II} or Tb^{III} in proximity to the sugar-binding pocket.[34] When Tb^{III} chloride ($TbCl_3$) was added to Fl–Con A prepared by P-PALM (Fig. 7a), an additional peak at 513 nm was observed besides three main luminescence peaks due to Tb^{III} (490, 544, and 583 nm) in the phosphorescence mode under the experimental conditions (Fig. 7b). This indicated that a Tb^{III} complex was formed with Fl–Con A (Fl–Con A/Tb) and that the Fl emission appeared in the rather long time range by a LRET from Tb^{III}. Such a long-lived luminescence of Fl–Con A/Tb is advantageous over short-lived fluorescence, because fluorescent impurities, such as other contaminated fluorophores, protein emission, and scattered light due to the excitation, can be cancelled in luminescence spectra, yielding a simplified spectrum.

Thanks to the LRET emission of Fl, two distinct luminescent fluorophores (i.e., Tb^{III} and Fl), which are essential for ratiometric luminescence analysis, were found within one protein scaffold (Fl–Con A/Tb). Figure 7c shows a typical luminescent spectral change of Fl–Con A/Tb, with excitation at 280 nm (tryptophan residues in Con A), upon addition of a branched mannopentaose (Man-5). Clearly, the LRET peak at 513 nm decreased relative to the Tb luminescence at 544 nm with increasing Man-5 concentration. This implies that a ratiometric value of the intensity of the LRET peak over the Tb^{III} peak can be successfully utilized. In addition to sugars, glycoprotein sensing can be carried out by LRET of Fl–Con A/Tb with micromolar sensitivity for ribonuclease B (Ribo B), while mannose and the nonglycosylated protein (Ribo A) were less sensitively detected. Using this selectivity, a luminescent assay for an enzymatic trimming reaction on a glycoprotein surface was designed, where α-mannosidase, an exoglycosidase, and Ribo B were employed as a model enzyme and substrate, respectively. It should be noted that this simple method needs neither a tedious labeling process of saccharides nor fully equipped mass spectrometers. The present sensing method might be applicable to other enzymatic processes of glycoconjugates.

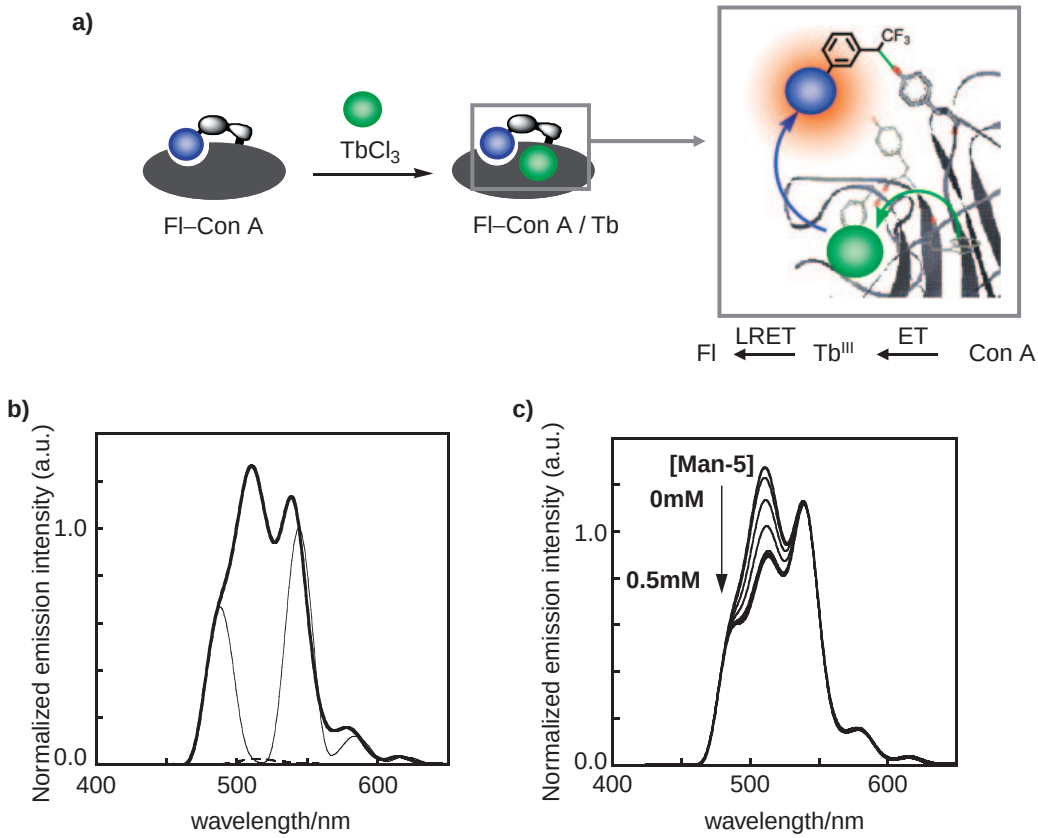

Fig. 7. a) Scheme for semisynthesis of the luminescent biosensor by Con A and the mechanism of LRET on Con A. b) Emission spectra of native Con A with $TbCl_3$ (light line) and Fl–Con A in the absence (dashed line) and presence (heavy line) of $TbCl_3$. c) Luminescent spectral changes of Fl–Con A/Tb upon addition of Man-5 (0–0.5 mM). [Fl–Con A] = 1.0 μM, [$TbCl_3$] = 50 μM in 10 mM HEPES buffer (pH 7.5) containing 5 mM $CaCl_2$, and 0.1 M NaCl. Temperature: 20 ± 1 °C, λ_{ex} = 280 nm.

3.5 Semi-Synthetic Fluorescent Biosensor.[35] Similar to the simple fluorescent probe, an artificial-sugar-binding site can be tethered to the proximity of the sugar-binding pocket using P-PALM strategy. It is expected that the introduction of an artificial-sugar-binding site into lectin will greatly enhance the binding affinity of the lectin toward specific saccharide having moieties intractive with both the lectin and the artificial receptor. As an artificial receptor to be attached to SH–Con A, APET–Br was designed and synthesized (Fig. 8a). The reagent had three functionalities in the molecular structure, that is, (i) a phenylboronic acid (PBA) to bind 1,2- or 1,3-diol derivatives,[36] (ii) a fluorescent tranducer motif to indicate a guest binding by photoinduced electron transfer (PET), and (iii) a bromoacetyl group to act as a reactive site for SH–Con A to afford APET–Con A (Fig. 8b).

As various saccharide titration was demonstrated, the fluorescence of the anthracene moiety in APET–Con A increased dependent on a specific saccharide (Fig. 8c). Such fluorescence intensification by sugars is direct evidence that the PBA site binds the saccharide, so that the PET mechanism works well on a Con A surface. Table 1 summarizes the association constants of engineered Con A for various saccharides, compared to native Con A. Most significantly, APET–Con A showed higher affinity and selectivity for specific oligosaccharides over monosaccharides. Maltitol, a disaccharide consisting of Glc and D-glucitol (Glucitol) connected by an α-1,4-linkage, was detected even in the sub-micromolar range by using APET–Con A. From fluorescence titration, an association constant ($\log K = 6.84$), which is approximately 3 orders of magnitude tighter binding than that of native Con A was determined. We found that the association constant for Maltitol is in good agreement with the value of the sum for each monosaccharide fragment, that is, Con A toward Me-α-Glc and PBA toward Glucitol, respectively. This result indicates that the cooperative binding of two sites, artificial receptor and native protein, occur in APET–Con A. Similarly, Maltotriitol, palatinose (Pal), isomaltose (Isomal), and Man-4, among many saccharides used in the titration experiment so far, were sensitively detected by using APET–Con A. Like Maltitol, these saccharides have two interaction sites for Con A and PBA. Interestingly, structure modulation of the artificial receptor part also controlled the saccharide selectivity of this semisynthetic Con A (Fig. 8d). When a tetramethylene spacer (APET-ape–Br, Fig. 8d) was inserted between Con A and PBA (APET-ape–Con A), the saccharides were more rigidly selected, that is, only Maltitol and Maltotriitol were sensed. Pal, Isomal and Man-4 were not detected in this case.[37]

These results indicate that the rational coupling of an artificial receptor with a native receptor protein can produce a novel semisynthetic biosensor with sophisticated function. The cooperative action between an artificial and a native receptor is essential for such evolution. For the last several decades, host–guest chemistry has produced a number of artificial receptors[38] and biochemical studies have already unveiled various natural

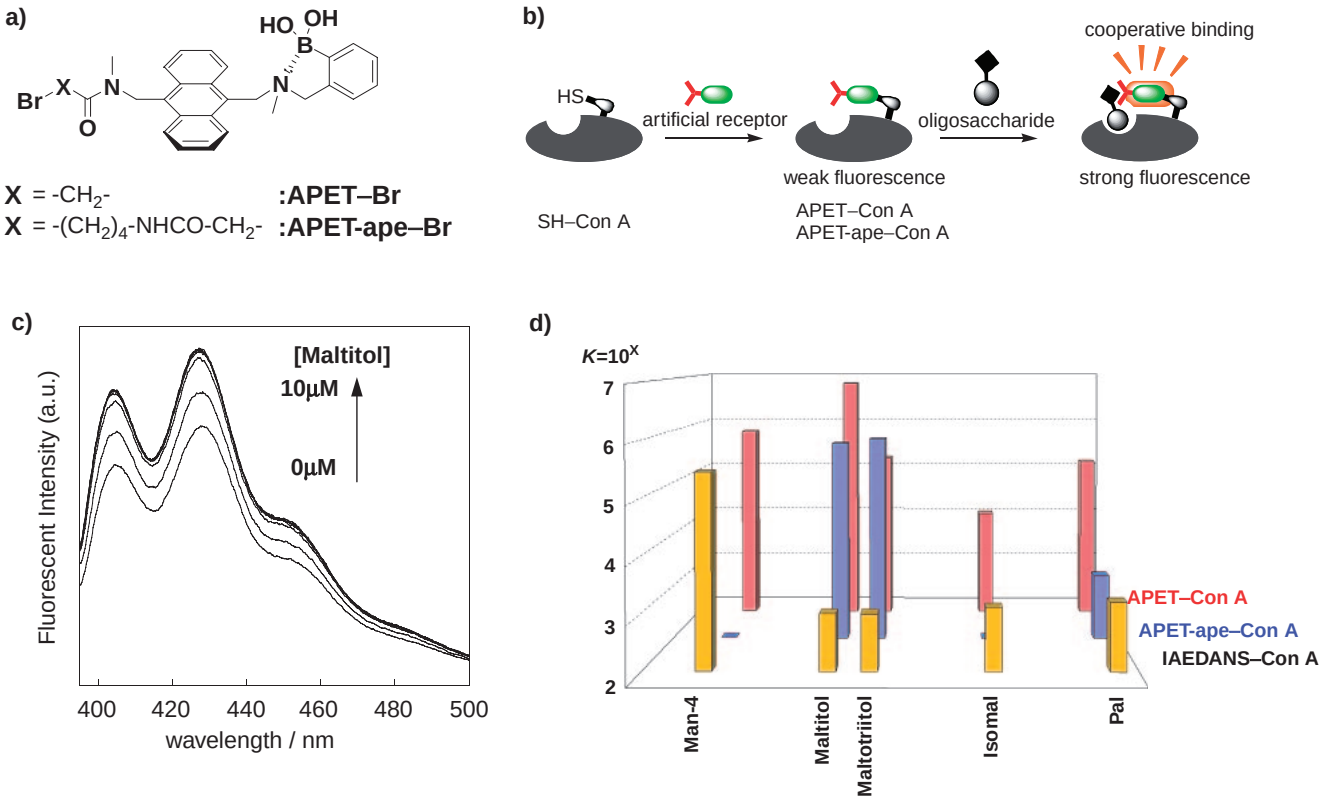

Fig. 8. a) Molecular structure of a APET–Br and APET-ape–Br. b) Scheme for semisynthesis of the oligosaccharide selective biosensor. c) Fluorescence spectral change of APET–Con A upon addition of Maltitol (0–10 μM). [APET–Con A] = 0.7 μM, in 50 mM HEPES buffer (pH 7.5) containing 5 mM $CaCl_2$ and 0.1 M NaCl. Temperature: 15 ± 1 °C, λ_{ex} = 375 nm. d) Summary of he binding constants evaluated by fluorescent change of various biosensors.

receptor proteins.[39] Therefore, it is rationally envisioned that the designed hybrid of these two families will facilitate the generation of semisynthetic receptors with enhanced affinity and selectivity, not only toward a variety of simple carbohydrates, but also for other biological substances, such as glycolipids, glycopeptides/proteins, bioactive hormones, etc.

4. One-Pot and Sequential Organic Chemistry on an Enzyme Surface[40]

As mentioned above, P-PALM is a valuable method based on photoaffinity labeling to incorporate a unique chemoselective tag proximal to the protein active site. This is beneficial from the viewpoint that the active site-directed introduction of a chemical tag to naturally occurring proteins can be carried out without the use of genetically engineered proteins. However, thiol chemistry used is not sufficiently general, because this method is not applicable to many proteins that have cysteine residues. Another drawback of P-PALM is that tedious and time-consuming purification procedures are needed to remove the reagents and other by-products, because of the low yield of photo-affinity labeling step (about 20%). A simpler method is most preferable.

We developed a one-pot sequential chemistry method based on the affinity-labeling modification using epoxide, followed by the hydrazone/oxime exchange reaction[41] on a protein surface, called post-affinity-labeling modification (P-ALM). In a proof-of-principle experiment, human carbonic anhydrase II (hCAII)[42] was used as a model protein. In the design of the P-ALM reagents (Fig. 9a), benzenesulfonamide, which is a typical inhibitor of hCAII, and an epoxide moiety, which is a suitable electrophile for hCAII, were used for targeting the active site of hCAII and for reacting with hCAII, respectively. These two moieties are linked by a hydrazone unit with various spacer lengths.

As shown in Fig. 9c, the sequential reaction on the surface of hCAII was conducted. Affinity labeling was directed to the modification site close to the active site of hCAII, and the hydrazone linkage between the ligand and the reaction site was subsequently replaced with an oxime bond (Fig. 9c). These two reactions proceed in a sequential manner under mild conditions, so that a one-pot modification can be carried out. It was confirmed that the labeling reaction was almost complete under suitable conditions, and the labeling site was determined as one of the two His exposed on the protein surface that are closely connected to the active site. Examination of the optimal conditions of the hydrazone/oxime exchange reaction using aminoxyacetic acid clarified the following facts: (i) the reaction efficiently proceeds under acidic pH conditions, due to acceleration of the proton-assisted exchange reaction, (ii) an excess amount of the aminoxy groups is needed for good conversion, and (iii) the reaction yield is not very sensitive to the reaction temperature. Interestingly, when fluorescent aminoxy derivatives, such as EDANS or FITC (Fig. 9b), were employed in the second step, a fluorescent probe was successfully introduced to hCAII.

For the labeled hCAII, the enzymatic activity was nearly

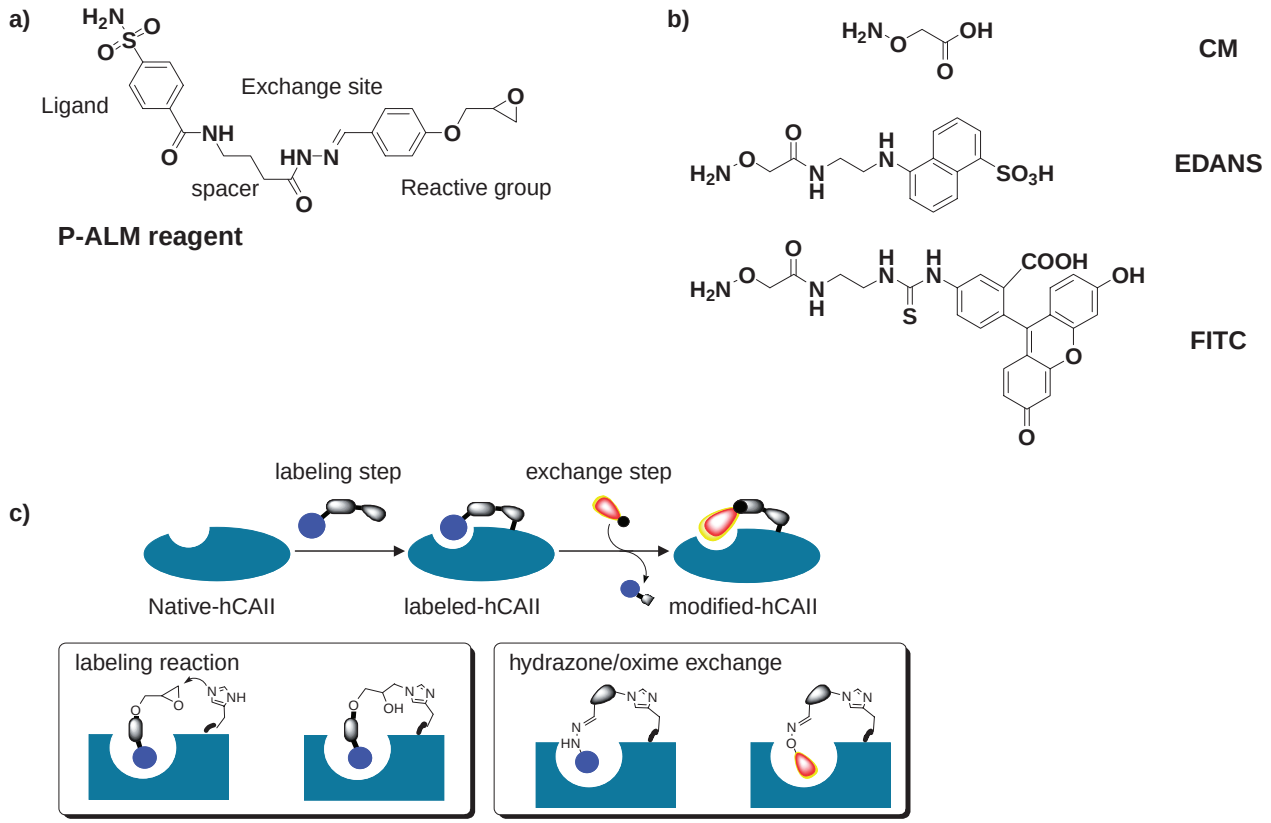

Fig. 9. Molecular structure of a) P-ALM reagent and b) aminoxy derivatives. c) P-ALM scheme for the selective modification of a protein (hCAII).

suppressed so that the catalytic parameters could not be determined. This is because the benzenesulfonamide part of the P-ALM reagent efficiently masked the active site. In the case of modified hCA, in contrast, the suppressed activity was nearly identical to that of the native hCAII, clearly indicating that the closed active site is re-opened by the hydrazone/oxime exchange reaction. These results indicate that the present P-ALM method can maintain the active site of hCAII while an artifical molecule such as fluorescent probe is attached in proximity to the active site. This unique advantage was successfully utilized for the development of a fluorescent biosensor on the basis of the hCAII scaffold. Actually, a typical fluorescence spectral change for EDANS–hCAII (and also FITC–hCAII) was induced by the addition of the sulfonamide family, and both the inhibitor affinity and the selectivity were practically identical with that for native hCAII.

Most significantly, these features can be extended to various proteins including the disulfide bond or cysteine-containing protein by using hydrazone/oxime exchange reaction. In addition, this method can be conducted in not only a test tube but also inside or on the surface of a living cell with appropriate improvements in the future.

5. Conclusion

In this account, we presented two original methods for protein engineering, which were based on synthetic chemistry, and demonstrated that the rationally designed incorporation of various unnatural molecules is powerful for controlling and modulating natural protein functions. We believe that step-by-step developments in various areas should afford more sophisticated methods and tools that are more applicable to protein. Our efforts are now under way.

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