Construction of Artificial Signal Transducers on a Lectin Surface by Post-Photoaffinity-Labeling Modification for Fluorescent Saccharide Biosensors

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Abstract: A new general method, post-photoaffinity-labeling modification (PPALM), for constructing fluorescent saccharide biosensors based on naturally occurring saccharide-binding proteins, lectins, is described in detail. An active-site-directed incorporation of a masked reactive site into a lectin was conducted by using a photoaffinity labeling technique followed by demasking and then chemical modification to yield a fluorescent lectin. Two photoaffinity labeling reagents were designed and synthesized in this study. The labeling reagent with a photoreactive site appended through a disulfide link to a mannoside unit was bound to the saccharide-binding pocket of the lectin concanavalin A (Con A). After light irradiation, the mannoside unit was cleaved by reduction. The unique thiol group thus produced was site-specifically modified with various fluorescent groups (dansyl, coumarin, or dimethylaminobenzoate derivatives) to afford fluorescent Con As. The labeling site was characterized by protease-catalyzed digestion followed by HPLC, MALDI-TOF MS, and tandem mass–mass spectrometry; these methods indicated that the photolabeling step is remarkably site specific. Strong fluorescence was observed in the engineered Con A with a fluorophore, and the emission changed sensitively upon saccharide complexation. The binding constants for various saccharides were determined by fluorescence titration and demonstrated that the binding selectivity and affinity of the engineered Con As are comparable to those of native Con A. The red shift of the emission maximum, the decrease in the fluorescence anisotropy of the dansyl unit, and the increase in the twisted intramolecular charge transfer emission caused by sugar binding to the engineered Con A explicitly indicate that the microenvironment of the appended fluorophores changes from a restricted and relatively hydrophobic environment into a rather freely mobile and hydrophilic environment.

Keywords: glycochemistry · lectins · photoaffinity labeling · protein engineering · saccharide biosensors

Introduction

In recent years, chemosensors based on organic scaffolds have been actively investigated for monitoring molecules of biological importance.[1] In contrast to the great progress in the detection of simple, small molecules such as ions, successful examples of chemosensors for complex biomolecules such as hormones, secondary messengers, and mono- or oligosaccharides are still limited.[2] This is mainly due to the lack of artificial receptors that can selectively bind biologically relevant molecules in aqueous solution. On the other hand, a biosensor is an ideal system for precisely monitoring ions and molecules of biological importance both in vivo and in vitro,[3] because the protein framework can provide a sophisticated molecular-recognition scaffold with high affinity and specificity. A key problem in this case is how to carry out the rational coupling of a signal-transduction device with molecular-recognition events on a protein scaffold. Unlike the situation with small, synthetic molecules, versatile methods for site-specific chemical manipulation of biomacromolecules are still poorly developed. Among the limited number of examples, Tsien,[4] Imperiali,[5] Berg[6] and their co-workers independently reported a fluorescence resonance energy transfer (FRET) type of biosensor for calcium or zinc cations that involved guest-induced peptide/protein folding. Hellinga and co-workers developed a fluorescent saccharide sensor equipped with an allosteric signal transducer.[7] The guest-induced change in the fluorescence depolarization was recently utilized by Thompson and co-workers.[8] Most of these successful strategies are based on genetic protein engineering or total/partial synthesis of proteins.

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Saccharides are important targets to be monitored quantitatively, not only for clinical usage but also for the further development of glycochemistry, because great advances in glycoscience and -technology have rapidly clarified many of the crucial roles of saccharide derivatives in biological phenomena such as cell signaling, organogenesis, fertilization, and inflammation.[9] Although an electrochemistry-based amperometric glucose sensor that uses glucose oxidase has now been commercialized for monitoring diabetes,[10] an optical sensing system for various saccharides is expected to be more advantageous and flexible.

To construct a fluorescent biosensor for saccharides, we recruited a naturally occurring saccharide-binding protein, lectin, as a protein scaffold.[11] Concanavalin A (Con A) from *Canavalia ensiformis*, the most extensively explored lectin, was utilized as a suitable model. Con A exists as a dimer at low pH values (<5.5) and as a tetramer at high pH values (>7).[12] Each monomer ($M_W \approx 26\,000$ Da) contains 237 amino acid residues with one sugar-binding site, as well as binding sites for the ions Mn^{2+} and Ca^{2+}.[13] Con A binds specifically to the α anomers of D-mannopyranoside and D-glucopyranoside.[14]

We recently reported a general semisynthetic method for constructing a fluorescent saccharide biosensor based on Con A.[15] Photoaffinity labeling of the sugar-binding pocket of Con A, cleavage of the ligand, and chemical modification with a fluorophore (post-photoaffinity-labeling modification, PPALM) affords a fluorescent Con A that can act as a saccharide biosensor. Here we describe several features of our PPALM technique for engineering fluorescent Con A. Detailed investigation of the specificity of the photoaffinity labeling site, the sugar-sensing mechanism of the fluorophore-appended Con A, and the introduction of other read-out modes, such as twisted intramolecular charge transfer emission, reveals that the present methodology is generally valuable for the rational design of fluorescent biosensors based on naturally occurring receptor proteins.

Results

Molecular design of the photoaffinity labeling reagents for Con A: We designed two PPALM reagents, **1a** and **1b**, which are shown in Figure 1a. The reagents needed to include three functionalities in the molecular structure, that is, 1) a high-affinity ligand (α-D-mannoside) to bind to Con A selectively, 2) a photoreactive group (trifluoromethylphenyl diazirine) to label Con A by photoirradiation, and 3) a suitable cleavage site (disulfide) to remove the ligand after photolabeling and to generate a chemoselective modification site[15a] (thiol group). It was previously reported that the strongest monosaccharide ligand for Con A is methyl-α-D-mannopyranoside.[16] Thus, a mannoside group attached through a disulfide link was employed as a high-affinity ligand. The disulfide bond could be reductively cleaved by (±)-dithiothreitol (DTT) or tris(2-carboxyethyl)phosphine (TCEP) treatment so that a reactive benzylthiol moiety is produced. As a photoreactive unit, trifluoromethylphenyldiazirine, which is conventionally used in these experiments, was selected for the design of the photoaffinity labeling reagents (**1a**, **b**) directed towards Con A.[17], [18], [19] The synthetic routes to these reagents are outlined in Figure S1 in the Supporting Information. Thioglycosylation of peracetyl-D-mannose (**4**) with thioacetic acid in the presence of $BF_3 \cdot OEt_2$ selectively gave peracetyl-α-D-1-thiomannoside (**5**). Deacetylation of **5** with LiOH followed by treatment with 2,2′-dithiodipyridine gave pyridyl-α-D-dithiomannoside **2**. The disulfide exchange reaction of **2** with the thiol derivatives of the corresponding labeling units (**3a**, **b**) afforded the photoaffinity labeling reagents **1a** and **1b**, respectively.

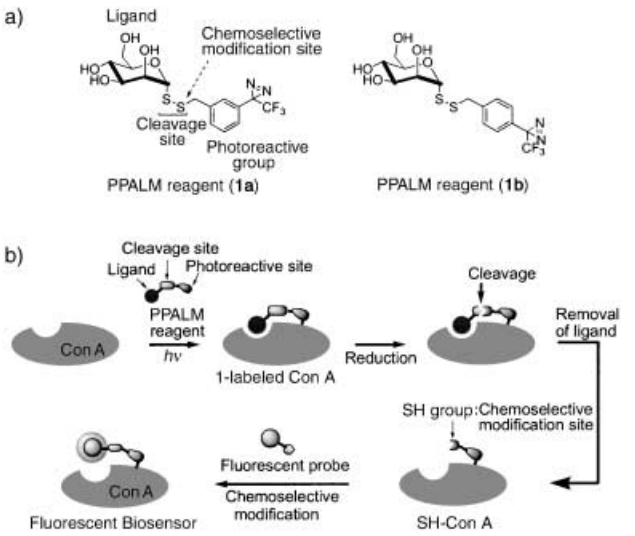

Figure 1. a) Molecular design and structure of PPALM reagents **1a** and **1b**. b) Representation of the PPALM scheme for semisynthesis of fluorescent biosensors.

Post-photoaffinity-labeling modification of Con A: The PPALM method is briefly illustrated in Figure 1b. When the affinity ligand **1** was bound to the binding pocket of Con A, photolabeling was carried out by UV-light irradiation ($330\text{ nm} < \lambda < 400\text{ nm}$ for trifluoromethylphenyldiazirine). The labeled Con A was purified by gel chromatography (Biogel P-30) and affinity column chromatography (Sephadex G-100).[20] As shown in Figure 2, three peaks were observed in the affinity chromatography results. Each of these was characterized by MALDI-TOF MS, the results of which allowed the first peak to be assigned to a homodimer of Con A labeled with one molecule of **1** and the second peak to be assigned to a heterodimer consisting of a monolabeled Con A and a nonlabeled Con A. The third peak was assigned to a homodimer of the nonlabeled Con A (that is, native Con A). For the structural and functional studies mentioned later, the heterodimer was mainly used because of its higher yield.[21] The yield of labeled Con A is greatly dependent on the molecular structure of the PPALM reagents: the yield is about 10% for **1a** and less than 1% for **1b**.

Subsequent treatment of the labeled Con A with DTT or TCEP gave a unique benzylthiol site (SH-Con A), which was chemoselectively modified with a haloacetylated fluorophore (Fluoro-Con A). These two steps proceeded almost quantitatively. The modified Con As (from labeling with **1a**) at all

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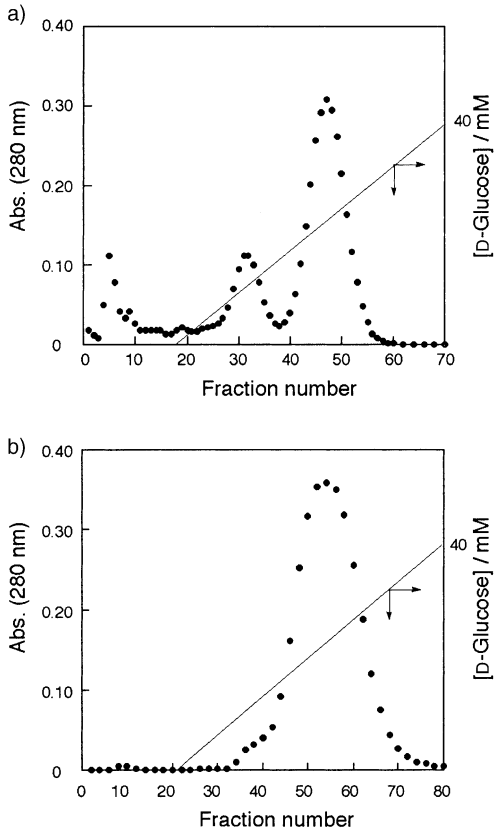

Figure 2. Fraction curves in the affinity purification of a) **1a**-labeled Con A; b) **1b**-labeled Con A.

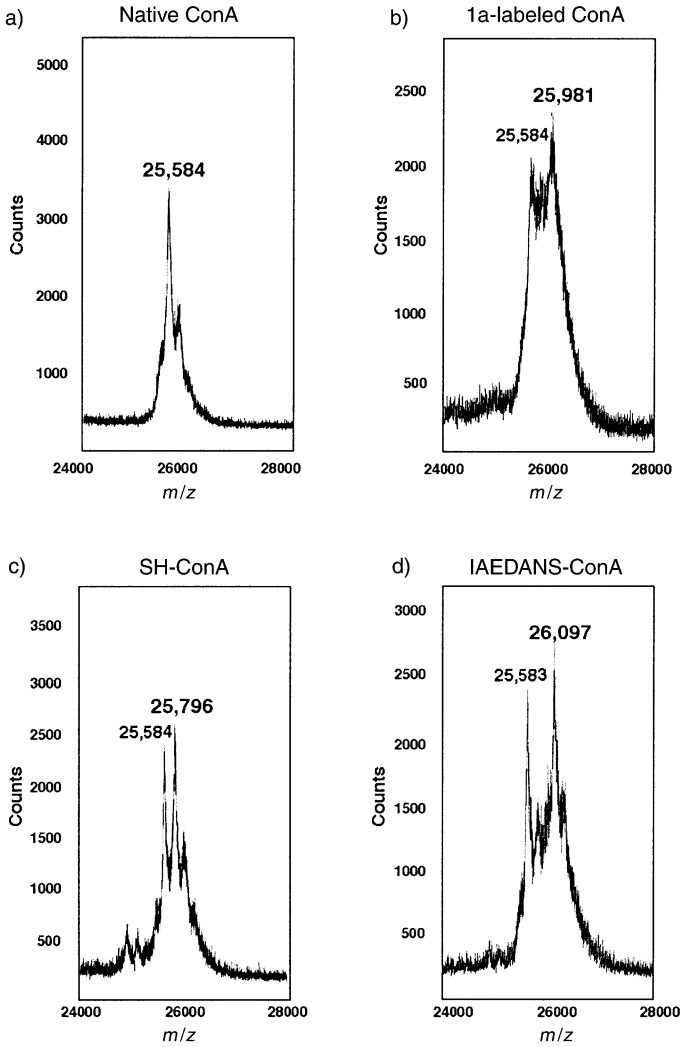

Figure 3. MALDI-TOF mass spectra of a) native Con A; b) **1a**-labeled Con A; c) SH-Con A; d) IAEDANS-Con A.

stages were characterized by MALDI-TOF MS (Figure 3). Mass peaks (m/z) were observed at 25 981 ± 10 for **1a**-labeled Con A, at 25 796 ± 11 for SH-Con A, and at 26 097 ± 6 for IAEDANS-Con A (see Scheme 1b for structure of IAEDANS); these data agree well with the sum of the M_W of native Con A (25 584 Da) and the corresponding residues.[22] The secondary structure of the modified Con As was monitored by CD spectroscopy. For the modified Con A at each stage (the **1a**-labeled, SH-, and IAEDANS-Con A), a negative Cotton peak at 218 nm, characteristic of a typical β sheet, was observed; this peak is comparable to that of native Con A,[23] a fact suggesting that the secondary structure of Con A is not significantly perturbed by the chemical modification.

Determination of the labeled site of the modified Con A: To determine the labeled site, the **1a**-labeled Con A was digested by lysyl endopeptidase (LEP), which can cleave peptide bonds on the C-terminal side of lysine residues.[24] Figure 4a shows the reverse-phase HPLC trace of the digested peptides for the labeled and native Con As. There are 12 lysine residues in Con A (the full amino acid sequence is given in the Supporting information) so 13 fragments were generated by the LEP digestion. In native Con A, eight peaks appeared in the HPLC trace, all of which were assigned by MALDI-TOF MS. Five other fragments (Ser31 – Lys35, Lys36, Thr37 – Lys39, Leu115 – Lys116, Asp136 – Lys138) were too short to be detected under the HPLC conditions used. In the case of the labeled Con A, eight peaks were also observed. However, by comparison with the HPLC chart of native Con A, it could be seen that one original peak (R_t = 74.5 min, assigned to fragment A4 by MALDI-TOF MS in the case of native Con A) disappeared and a new peak (R_t = 81.4 min) appeared instead. Consistently, MALDI-TOF MS analysis of the new peak showed that it can be ascribed to the peptide fragment A4 (Arg60 – Lys101)) plus one molecule of the denitrogenated ligand **1a**. Subsequently, the modified fragment A4 was collected by HPLC and subjected to trypsin-catalyzed digestion; the resultant mixture was analyzed by mass spectrometry. Fragment A4 involves two arginine residues so it was divided into one arginine and two peptides by trypsin. The longer peptide (Leu61 – Arg90) had a molecular weight identical to the native one, while the shorter peptide (Val91 – Lys101) showed the sum of the corresponding peptide and the denitrogenated ligand **1a** in its M_W (747.32 Da as a dication form). Precise sequencing of the shorter fragment was next conducted by the tandem mass – mass method (Figure 4b). Each difference in the mass peaks showed good agreement with the mass unit of the corresponding natural amino acid with the exception of the Tyr100. The difference mass of the Tyr100 site was determined to be 561.11 Da, a

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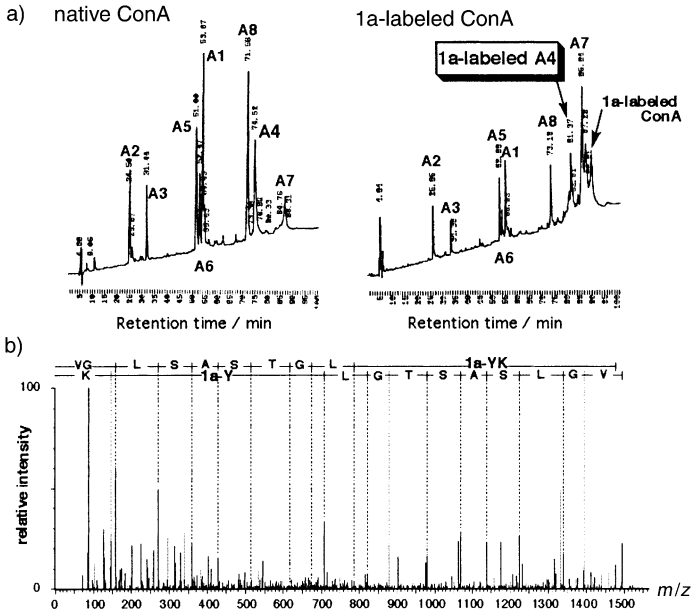

Figure 4. a) Reverse-phase HPLC trace of native and **1a**-labeled Con As after lysyl-endopeptidase digestion. b) Tandem mass–mass analysis of the **1a**-labeled fragment A4.

value that is completely coincident with the sum of the M_W of tyrosine and the denitrogenated labeling ligand **1a** (a calculated value of 561.1103 Da). Thus, the labeling site was assigned to Tyr100. It is clear that the photoaffinity labeling process of the present PPALM method is remarkably site selective on a protein surface.

Fluorescent sensing of saccharides by using fluoro-Con As: IAEDANS-Con A (from labeling with **1a**) prepared by the PPALM method with the iodoacetylated dansyl derivative showed a strong emission due to the dansyl fluorophore, an environmentally sensitive probe.[25] A spectral change in the emission of IAEDANS-Con A was observed on addition of methyl-α-D-mannoside (Me-α-Man), one of the strongest ligands for native Con A (Figure 5a). With increasing concentrations of Me-α-Man, the emission intensity at 484 nm decreased and the emission peak maximum was slightly red shifted (488 nm). This implies that the dansyl unit moves into a more polar microenvironment than the original position upon complexation of IAEDANS-Con A with Me-α-Man. It is noteworthy that the binding process of IAEDANS-Con A with Me-α-Man can be directly monitored by the fluorescence signal change. Fluorescence titration gave the typical saturation curve, which yielded a binding constant of 7.8×10^3 M^{-1} for Me-α-Man by the Benesi–Hildebrand plot analysis[26] (Figure 5b). This value is almost comparable to that of native Con A (1.1×10^4 M^{-1}) as determined by isothermal titration calorimetry.[12a]

We next conducted similar titration experiments for other saccharides (the structures are summarized in Scheme 1a and the Supporting Information) with IAEDANS-Con A. The saturation curves shown in Figure 6 gave the binding constants for various saccharides that are displayed in Table 1. Among the monosaccharides (Figure 6a) these show significant features: 1) Mannose derivatives showed a higher affinity

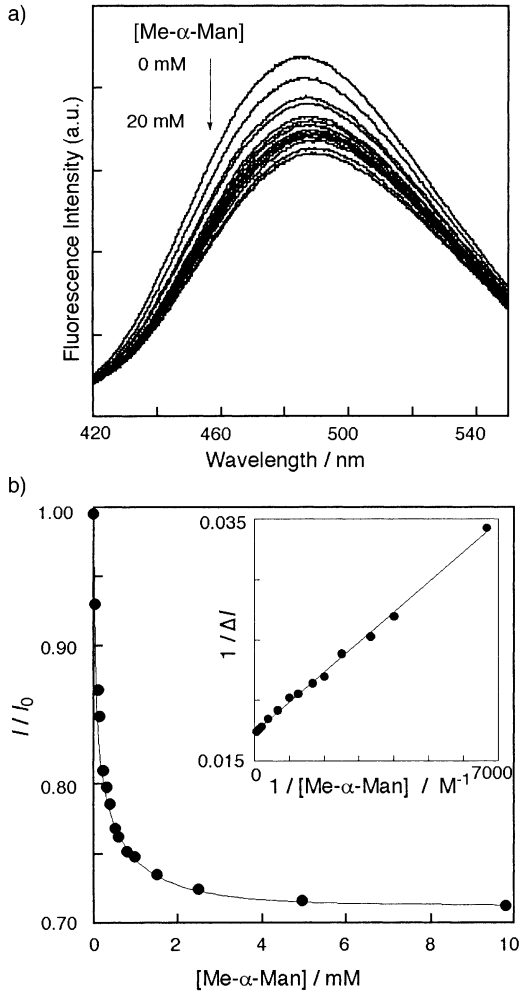

Figure 5. a) Spectral changes in the fluorescence of IAEDANS-Con A upon addition of Me-α-Man ($0 \rightarrow 20$ mM). [IAEDANS-Con A] = 5 μM, in 50 mM HEPES buffer (pH 7.0) containing 1 mM $CaCl_2$, 1 mM $MnCl_2$, and 0.1 M NaCl. Temperature = 15 ± 1 °C, λ_{ex} = 340 nm. b) The binding curve for IAEDNAS-Con A. Inset: Benesi–Hildebrand plot analysis. HEPES = 2-[4-(2-hydroxyethyl)-1-piperazinyl]ethanesulfonic acid.

than glucose derivatives (Me-α-Man > Me-α-Glc, Man > Glc); 2) the affinities of galactose, ribose, and arabinose were negligible; 3) the α configuration in the glycoside bond was preferable to the β configuration (Me-α-Glc > Me-β-Glc); and 4) L-glucose, a mirror image of native D-glucose, was practically not bound. The observed binding selectivity of IAEDANS-Con A was identical to that of native Con A,[16] a fact suggesting that the binding pocket of IAEDANS-Con A for the monosaccharide is not disturbed by the PPALM.

For a series of the oligosaccharides, the selectivity of IAEDANS-Con A observed by fluorescence titration was also in good agreement with that of native Con A as determined by isothermal titration calorimetry, that is, Man-tri > Man-bi > Isomal ≫ Cel, Lac (Figure 6b). Man and Glc at the nonreducing saccharide terminal were preferable to Gal, and there was also a preference for the α-glycoside anomer to the β-glycoside. It is noteworthy that the binding constant of IAEDANS-Con A for Man-tri was found to be identical with that of native Con A.[12a,b] Man-tri is known to be one of the main core structures in branched saccharides that attach to

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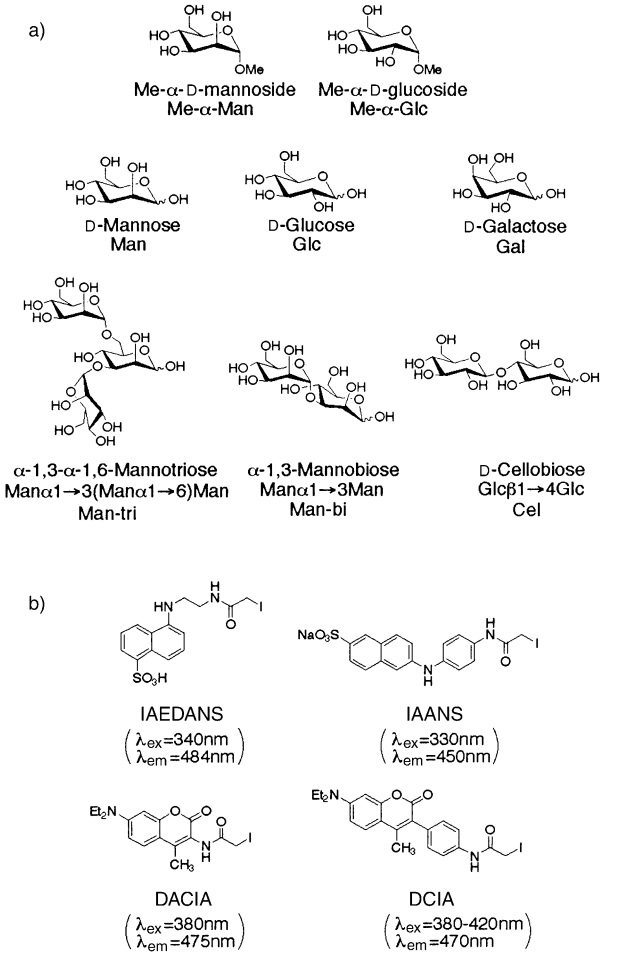

Scheme 1. a) Representative saccharide structures used in this study. The abbreviation is given below the saccharide name. b) Molecular structures of the fluorescent probes attached to SH-Con A. The wavelengths given in the parentheses are the excitation and emission wavelengths for each fluorophore.

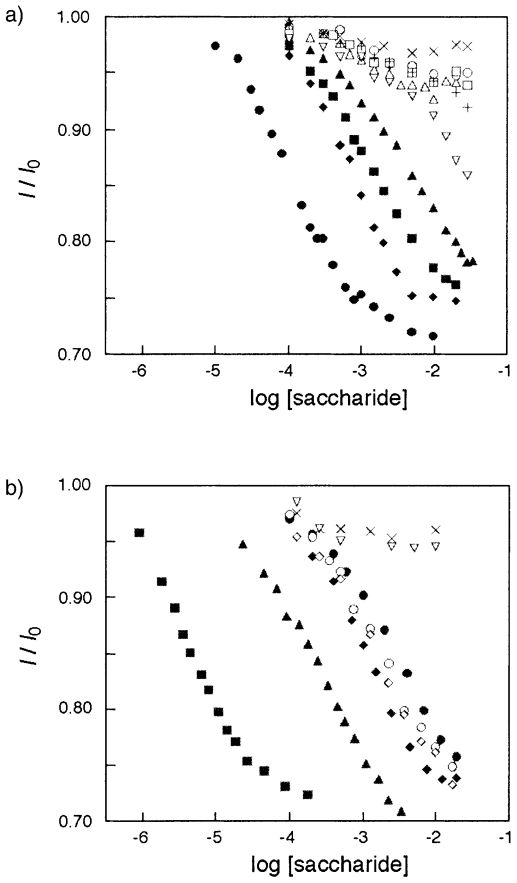

Figure 6. Fluorescence titration profile of the relative intensity (I/I_0) versus the saccharide concentration (log [saccharide]) for: a) IAEDANS-Con A (5 μM) with the monosaccharides Me-α-Man (●), Me-α-Glc (◆), Man (■), Glc (▲), Me-α-Glc (▽), L-Glc (▲), Ara (□), Me-α-Gal (+), Gal (○), and Rib (×); b) IAEDANS-Con A (0.2 μM) with the oligosaccharides Man-tri (■), Man-bi (▲), Pal (◆), Isomal (○), Isomalti (◇), Malti (●), Cel (×), and Lac (▽).

natural glycoproteins. Therefore, the ability to detect Man-tri is useful for quantitative estimation of such a glycoprotein family. This is the first example of fluorescence detection of Man-tri at μM concentrations, to the best of our knowledge. It was reported that the Con A surface in the proximity of the binding pocket is involved in Man-tri binding.[12c] The present data implied that the protein surface responsible for the sugar binding was structurally conserved even with the modification, in addition to the binding pocket. Thus, not only binding selectivity but also the affinity of native Con A for various saccharides is quantitatively retained in IAEDANS-Con A.

As a control sample, we prepared a Con A randomly modified with a dansyl group. Some lysine residues may have be modified because dansyl chloride was used as the reagent. After purification of the Con A modified randomly with an average of 1 molecule of dansyl unit per protein, a fluorescence titration with Me-α-Man was conducted. No significant changes in either its emission intensity or wavelength were observed (see Supporting Information). This control experiment indicates that the site-selective attachment of a dansyl group is crucial to the above-mentioned fluorescent response of IAEDANS-Con A.

The fluorescence depolarization measurements[8a] strongly supported the molecular mechanism of the fluorescence response of IAEDANS-Con A to saccharide binding. The fluorescence anisotropy ratio (r) was determined to be 0.40 without sugars, which is a typical value for the probe bound to proteins. Interestingly, the anisotropy ratio decreased to 0.35 on addition of Me-α-Man (see Supporting Information). The saturation curve showed good agreement with the curve obtained by the steady-state fluorescence titration and gave a binding constant of 7.6×10^3 M^{-1}, which is almost identical to the value from the fluorescence titration. 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 dansyl moiety is restricted in the binding pocket of Con A without sugars but the environment changes into one of rather higher mobility upon complexation with Me-α-Man. This is nicely consistent with our tentative explanation of the saccharide-induced fluorescence change in IAEDANS-Con A.

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Table 1. Comparison of the association constants of Fluro-Con As with those of native Con A.

Saccharide	K_a [M^{-1}]				
	native Con A	IAEDANS-Con A	IAANS-Con A	DACIA-Con A	DCIA-Con A
Me-α-Man	1.1×10^4[a]	7.8×10^3[b]	2.6×10^3[b]	3.7×10^3[b]	1.9×10^3[b]
Me-α-Glc	3.0×10^3[a]	1.6×10^3[b]	1.4×10^3[b]	1.2×10^3[b]	8.3×10^2[b]
Me-β-Glc	–[c]	$< 1.0 \times 10^2$[b]	–[d]	–[d]	–[d]
Me-α-Gal	–[c]	–[c]	–[d]	–[d]	–[d]
Man	2.2×10^3[a]	1.3×10^3[b]	–[d]	–[d]	–[d]
Glc	8.0×10^2[a]	1.0×10^3[b]	–[d]	–[d]	–[d]
L-Glc	–[c]	–[c]	–[d]	–[d]	–[d]
Gal	–[c]	–[c]	–[c]	–[c]	–[c]
Rib	–[e]	–[c]	–[d]	–[d]	–[d]
Ara	–[e]	–[c]	–[d]	–[d]	–[d]
Man-tri	2.5×10^5[a]	2.5×10^5[f]	6.5×10^5[f]	5.4×10^5[f]	1.2×10^5[f]
Man-bi	3.0×10^4[a]	5.8×10^3[b]	1.9×10^4[b]	6.9×10^3[b]	5.2×10^3[b]
Malti	–[e]	1.0×10^3[b]	–[d]	–[d]	–[d]
Pal	–[e]	1.6×10^3[b]	–[d]	–[d]	–[d]
Isomal	1.7×10^3[a]	1.3×10^3[b]	–[d]	–[d]	–[d]
Isomalti	–[e]	8.8×10^2[b]	–[d]	–[d]	–[d]
Cel	–[c]	–[c]	–[c]	–[c]	–[c]
Lac	–[e]	–[c]	–[d]	–[d]	–[d]

[a] Reported values detrermined by isothermal titration calorimetry.[12] [b] Determined by Benesi–Hildebrand plot analysis. [c] Cannot be determined because of the low affinity. [d] Titration experiments were not conducted. [e] Not reported previously. [f] Determined by nonlinear curve fitting analysis.

Other modified Con As with different fluorophores (coumarin derivatives DCIA and DACIA, or aminonaphthalenesulfonate derivative IAANS; shown in Scheme 1b) were prepared by the same procedure. These Fluoro-Con As (from labeling with **1a**) showed good fluorescent sensing affinity and selectivity in a similar way to IAEDANS-Con A (from labeling with **1a**), that is, they show sensitive fluorescent change with Me-α-Glc, Me-α-Man, Man-bi, and Man-tri, but do not respond to Gal and Cel (see Table 1). In addition to varying the monitoring wavelength (450–490 nm), it was an advantage to shift the excitation wavelength into the visible light region to avoid the undesirable interference by other impurities derived from proteins or DNA in the analyte solutions. In the present case, we could shift the excitation from 330 to 420 nm by changing the fluorophore without decreasing the sensing capability.

Twisted intramolecular charge transfer (TICT) mode for reading out the saccharide by DMAB-Con A: In addition to the environmentally sensitive probes (see above), a TICT emission probe can be engineered onto a Con A surface by using the PPALM method. Dimethylaminobenzoate (DMAB), a typical fluorophore for TICT, was converted into a bromoacetyl amide derivative, DMAB-Br (Figure 7a). DMAB-Br was appended to the benzylthiol site of SH-Con A (DMAB-Con A). Figure 7b shows difference fluorescence spectra of DMAB-Con A due to the addition of Me-α-Man. In contrast to the monotonous decrease in the fluorescence intensity for IAEDANS-Con A, an increase in the fluorescence at 435 nm was observed with a concurrent decrease in the fluorescence at 360 nm with an isosbestic emission point at 395 nm in the case of DMAB-Con A. Such a fluorescence change at two distinct wavelengths is, in principle, applicable to the ratiometric measurement, which enables the more precise (quantitative in some cases) estimation of analytes by reducing the influence of photobleaching, the effect of the probe concentration, and other environmental side effects.

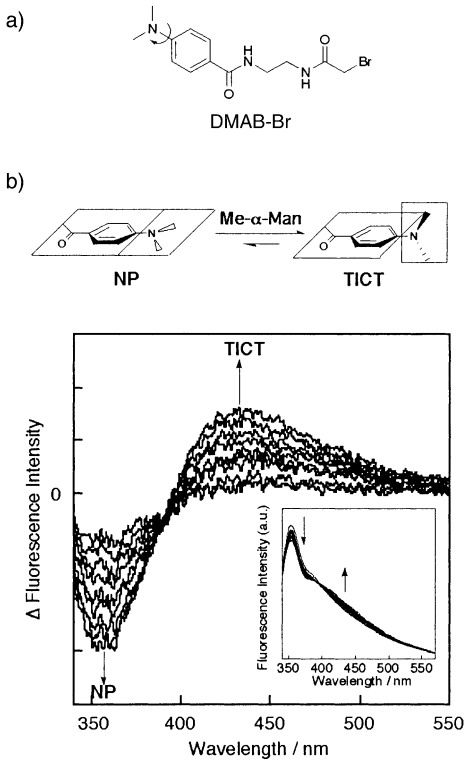

Figure 7. a) Structure of DMAB-Br. b) Differential spectral changes in the fluorescence of DMAB-Con A upon addition of Me-α-Man ($0 \rightarrow 10$ mM). [DMAB-Con A] = 1 μM in 50 mM HEPES buffer (pH 7.0) containing 1 mM $CaCl_2$, 1 mM $MnCl_2$, and 0.1 M NaCl. Temperature = 15 ± 1 °C, λ_{ex} = 310 nm. Inset: fluorescence spectral changes.

According to the literature,[27] the peak at 435 nm can be ascribed to the emission from the TICT excited state, and the peak at 360 nm is due to the emission from the normal planar

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(NP) excited state. It is generally considered that the TICT emission increases in a polar solvent rather than a less polar solvent because the TICT excited state is more charge-separated, relative to the NP excited state. Therefore, the increase in the TICT emission and the decrease in the NP emission induced by Me-α-Man indicate that the DMAB moiety shifts from a rather hydrophobic sugar-binding pocket to a bulk aqueous solution. This is absolutely consistent with the mechanism of the saccharide-induced fluorescence change in IAEDANS-Con A. Both emissions (due to TICT and NP) change with typical saturation behaviors, which give us the apparent binding constants as follows: 2.3×10^3 M^{-1} for Me-α-Man, 1.3×10^3 M^{-1} for Me-α-Glc, and a negligible value for Gal. The saccharide selectivity is again coincident with that of native Con A, similarly to the case of IAEDANS-Con A.

Discussion

The present results reveal that elaborate organic chemistry on a protein surface is a powerful tool for converting a lectin into a fluorescent saccharide sensor. As a pioneering example, Schultz and co-workers proposed a unique approach to conducting organic chemistry on an antibody cavity by a combination of affinity labeling with subsequent modification to produce a fluorescent antibody.[28] The reactive species of photolabeling reagents such as carbene or nitrene are so reactive that PPALM may be applied to a wide range of proteins which do not have such highly reactive amino acids around the ligand binding site. The photoaffinity labeling technique has been widely utilized so far in structural and functional biology to determine unknown enzyme active sites or protein/protein interaction surfaces.[17–19] The labeled proteins are usually fragmented into many peptides for the structural analysis. In contrast, the present PPALM method provides novel functionality to the target protein without fragmentation. Thus, the photoaffinity labeling method can be extended into protein engineering.

It is remarkable that the photoaffinity labeling reaction with **1a** is highly site-selective. As shown in Figure 8a, the crystal structure of Con A complexed with *p*-nitrophenyl-α-D-mannoside suggests that there are several nucleophilic amino acid residues around the saccharide-binding pocket.[29] These residues, such as Tyr12, Thr15, Tyr100, and His205, seem to be intrinsic candidates for a photolabeling reaction by the electrophilic trifluoromethyl carbene moiety. Among them, only Tyr100 was modified as shown in our peptide-mapping experiments in the case of **1a**. Importantly, it was reported that the hydroxy group of Tyr100 is not responsible for the sugar binding in native Con A. Tyr12 and Thr15 on the Con A surface, which can interact with the branched mannose unit of Man-tri, are considered to be on the opposite side to the hydrophobic fence consisting of Tyr100.[12c] Thus, the modification at Tyr100 does not strongly disturb the structure of the binding surface of Con A. These factors should contribute to the satisfactory retainment of the original selectivity and affinity for various saccharides in IAEDANS-Con A.

In contrast, another photolabeling reagent **1b** did not yield any substantial amount of labeled Con A. This is probably due to the fact that the *para* substitution is oriented to the outside of Con A and is thus far from the residues surrounding the binding pocket. On the other hand, we previously reported that *p*-azidophenyl-α-D-mannoside (*p*-APM) reacted with Con A in 20% yield and that the modification reaction is distributed to two sites, such as Tyr12 or Tyr100, both of which are positioned in the proximity of the sugar-binding pocket.[15b] Compared to **1a**, the linkage of *p*-APM between the mannose group and the phenylazide group is shorter by two bond lengths. Thus, the azide group can reach Tyr12, which is located on the more buried side, as well as Tyr100, so that photolabeling occurs at both positions. In contrast to *p*-APM, the photoreactive species of **1a** and **1b** is expected to be generated on the external protein surface, and thus it is reasonable that the photoaddition predominantly takes place

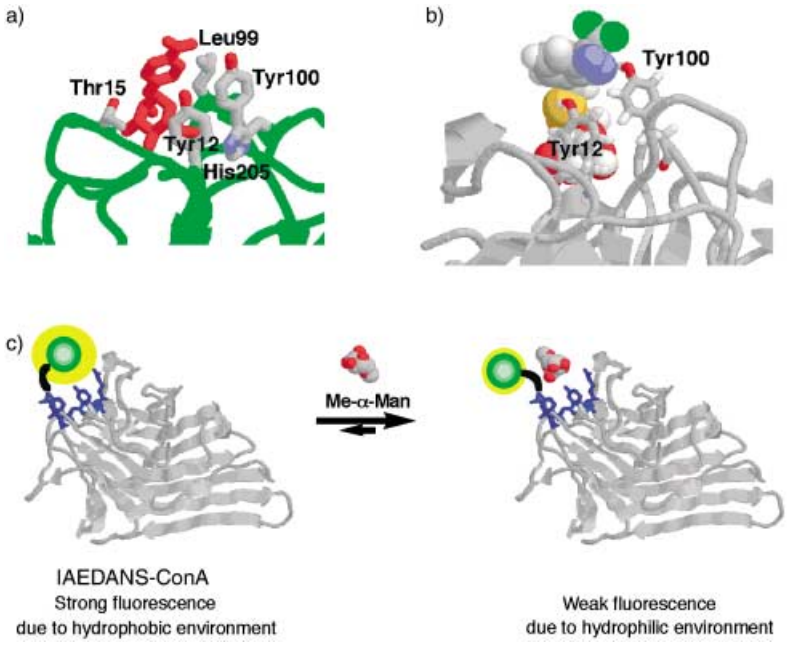

Figure 8. The molecular structures of the residues in the proximity of the saccharide-binding site of Con A: a) the crystal structure of the *p*-nitrophenyl-α-D-mannopyranoside complex (PDB file code: 1VAM); b) molecularly modeled structure of the complex with **1a**. c) Schematic illustration of the present sensing mode of IAEDANS-Con A.

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at the more solvent-exposed Tyr100. A molecular modeling study of the **1**-labeled Con A (Figure 8b) gave us the roughly estimated distance between the reactive center of **1** and Tyr100. In the case of *meta*-substituted **1a**, the average distance is in the range of 3.5 Å, which is close enough to be photolinked with Con A, whereas the distance is more than 5 Å in the complex with the linear *para* substituent **1b**. These results clearly demonstrate that fine tuning of the structure of the PPALM reagents will allow regulation of the modification position, which is crucial for the introduced novel function.

In addition, the 3D structure suggests that the saccharide-binding pocket is partially hydrophobic because it is surrounded by the hydrophobic fence consisting of Tyr12, Tyr100, and Leu99 (Figure 8a). Thus, it is reasonable that the hydrophobic fluorophore appended to the proximity of the binding pocket is preferably located in the rather hydrophobic position without saccharides. The fluorophore moves to the more hydrophilic microenvironment upon sugar binding, as suggested by the results in which the emission is lessened in its intensity and red-shifted upon sugar binding. The decrease in the fluorescence anisotropy upon saccharide binding strongly supported the tentative explanation mentioned above. That is, the mobility of the fluorophore is restricted in the saccharide-binding pocket, and the sugar-induced relocation causes a partial recovery of the freedom in motion. This is also nicely coincident with the increase in the TICT emission induced by Me-α-Man complexation to DMAB-Con A, along with the concurrent decrease in the NP emission. In the control experiment with randomly modified Con A, no significant fluorescence changes occurred. Therefore, the signal-transduction modes displayed in the present examples are based on the response caused by the microenvironmental change in the fluorescent probes induced by sugar binding (Figure 8c). It is concluded that the active-site-directed incorporation of the fluorophores effectively coupled the sugar-binding event to the fluorescent signal change.

Various fluorophore-labeled Con As can be prepared from SH-Con A by simple mixing with the haloalkylated species. The binding constants for Me-α-Man evaluated by the corresponding Fluoro-Con A (from labeling with **1a**) are varied: 7.8×10^3 (IAEDANS), 3.7×10^3 (DACIA), 2.6×10^3 (IAANS), and 1.9×10^3 M^{-1} (DCIA). Apparently, the more hydrophobic and bulky fluorophores suppressed more strongly the binding affinity to Me-α-Man. This might be ascribed to the partial blocking of the binding pocket of Con A by the bulky fluorophore or to the fluorophore-induced disorder of the binding pocket. However, the considerable suppression of the affinity by the attached fluorophore was not observed in the case of oligosaccharide sensing, which may be due to the fact that the protein surface, as well as the binding pocket, is greatly involved in the oligosaccharide binding. The present PPALM strategy has the advantage that one can conveniently and rapidly attach various kinds of fluorophores to Con A. This facilitates the screening for satisfactory sensitivity, the modulation of the excitation, the monitoring of the wavelength, and the selection of an appropriate transduction mode, such as environmentally sensitive probes and TICT emission probes, to meet each objective in biosensing.

Conclusion

We have successfully proposed a simple method called PPALM (post-photoaffinity-labeling modification) for converting a lectin protein into a fluorescent saccharide sensor. The resultant engineered Con A showed a fluorescent response to a series of saccharides with superior selectivity and affinity identical to that of native Con A. The high site specificity of the attachment of the probe and the chemical flexibility for selection of a suitable fluorophore are advantageous in the PPALM method. It was reported in the literature that the binding pocket of naturally occurring lectins is rather hydrophobic in many cases.[11a],[30] Therefore, this strategy is anticipated to be widely applicable to other saccharide-binding proteins, simply by exchanging the sugar (that is, the ligand) part of the PPALM reagent **1**, to produce other fluorescent biosensors with distinct saccharide specificities. These will advance the quantitative estimation of various saccharides and facilitate glycoscience and -technology. Furthermore, many other naturally occurring receptor proteins might become intriguing targets for PPALM, which will extend the research field of biosensors.

Experimental Section

General methods: CH_3CN and MeOH were distilled under a nitrogen atmosphere prior to use. CH_3CN was dried over calcium hydride. MeOH was dried over Mg and I_2. Unless otherwise stated, all of the air- or moisture-sensitive reactions were performed under a nitrogen atmosphere. ^1H NMR spectra were recorded either on Bruker AC250-P (250 MHz) or Bruker DRX-600 (600MHz) spectrometer. ESI-TOF MS was measured with a PerSeptive Mariner apparatus. MALDI-TOF MS were recorded on a Perkin–Elmer Voyager DE-RP apparatus, with 2,5-dihydroxybenzoic acid, α-cyano-4-hydroxycinnamic acid (CHCA), or sinapinic acid (SA) as the matrix. UV-Vis spectra were obtained on a Hitachi U-3000 spectrometer. CD spectra were recorded on a Jasco J-720 spectrometer. Fluorescence spectra were recorded on a Hitachi F-4500 spectrometer. Synthetic methods and characterization data for **1a**, **1b**, and DMAB-Br are described in the Supporting Information. Con A was purchased from Funakoshi and used without further purification. All of the fluorescent probes were purchased from Molecular Probes. The other chemical reagents were purchased from Aldrich or Tokyo Chemical Industry.

Photoaffinity labeling of Con A with the labeling reagent 1a[31]: Con A (30 mg, 1.2 μmol/monomer-based) was dissolved in 10 mM acetate buffer (2 mL; pH 5.0) containing 1 mM $CaCl_2$, 1 mM $MnCl_2$, and 0.2 M NaCl. The labeling reagent **1a** (2.0 mg, 4 equiv to 1 sugar-binding site of Con A) in DMF (80 μL) was slowly added to the Con A solution at 0 °C. This solution was stored for 30 min at 0 °C in the dark and then gently bubbled with nitrogen gas to remove dioxygen; this was followed by centrifugation (10000 rpm, 5 min) at 4 °C. The supernatant was transferred into a UV cell (1 cm) and photoirradiated for 40 min at 10 °C by a 500 W high-pressure Hg lamp (USHIO Optical ModuleX) with a glass filter that cuts out light of wavelengths below 320 and above 380 nm. After removal of a trace amount of precipitate by centrifugation (10000 rpm, 5 min), the supernatant was subjected to gel filtration chromatography (Biogel P-30, 2×20 cm), eluted with the buffer A (10 mM acetate buffer (pH 5.0), 1 mM $CaCl_2$, 1 mM $MnCl_2$) at 4 °C. The protein fractions were collected and concentrated by ultrafiltration (YM10 filters, Amicon). The concentrated protein solution was subsequently purified by affinity chromatography (Sephadex G100, 2×30 cm), eluted with a linear gradient of buffer A with $0 \rightarrow 40$ mM D-glucose. Three peaks were observed in the trace. Each fraction was characterized by MALDI-TOF MS. The first peak (m/z 25981) was assigned to a homodimer of Con A labeled with 1 molecule of **1a**, the second peak was assigned to a heterodimer consisting of monolabeled Con A and nonlabeled Con A. The third peak (m/z 25583) was assigned to

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a homodimer of nonlabeled Con A (that is, native Con A). (It has been reported that Con A exists as a dimer at pH 5.0.) The fractions which made up the second peak was collected and concentrated.

Preparation of SH-Con A: DTT (final concentration: 5 mM) was added to a solution of **1a**-labeled Con A (from the fractions that made up the second peak in the affinity column; 20 μM, 10 mL) in 100 mM phosphate buffer (pH 8.0), and the reaction mixture was incubated for 20 h at 4 °C under a nitrogen atmosphere. The reaction solution was purified by gel filtration chromatography (Biogel P-30, 2 × 20 cm) eluted with 100 mM phosphate buffer (pH 7.2) to give SH-Con A (12 μM, 16 mL) in quantitative yield. The thiol content in the SH-Con A was determined by the Ellman method,[32] which showed that one Con A dimer has one SH group. MALDI-TOF MS (SA) demonstrated the cleavage of the thiomannose unit: *m*/*z* calcd: 25787; found: 25796. The SH-Con A was quickly subjected to the next modification.

Preparation of Fluoro-Con A: Iodoacetyl-fluorophore (in the case of IAEDANS: 0.22 mg, 5×10^{-7} mol, 10 equiv to 1 SH group) in DMF (100 μL) was slowly added to a solution of SH-Con A (20 μM, 5 mL) in 100 mM phosphate buffer (pH 7.2) at 0 °C, and the reaction mixture was incubated for 12 h at 4 °C in the dark. After incubation, the solution was submitted to gel filtration chromatography (Biogel P-30, 1 × 15 cm), eluted with the buffer B (50 mM HEPES buffer (pH 7.0), 1 mM $CaCl_2$, 1 mM $MnCl_2$, and 0.1 M NaCl). The protein fraction was collected, concentrated by ultrafiltration, and dialyzed against buffer B to afford Fluoro-Con A in quantitative yield (in the case of IAEDANS-Con A: 12 μM, 8 mL). For IAEDANS-Con A, the labeling efficiency was estimated from the ratio of [IAEDANS]/([Con A]/2) to be 0.75–0.90. IAEDANS-Con A was identified by MALDI-TOF MS (SA): *m*/*z* calcd: 26093; found: 26097. The same procedure was employed for modification with the other fluorophores and the overall labeling efficiency of SH group was shown to be 0.7–0.9. IAEDANS: $\varepsilon_{336} = 5700\ M^{-1}cm^{-1}$; IAANS: $\varepsilon_{327} = 26000\ M^{-1}cm^{-1}$; DCIA: $\varepsilon_{384} = 33000\ M^{-1}cm^{-1}$; DACIA: $\varepsilon_{384} = 31000\ M^{-1}cm^{-1}$; DMAB: $\varepsilon_{300} = 22500\ M^{-1}cm^{-1}$.

Preparation of the randomly modified Con A: A solution of dansyl chloride (0.24 mg, 8.9×10^{-7} mol) in acetone (0.1 mL) was added to a solution of Con A (15 mg, 5.9×10^{-7} mol) in 0.1 M $NaHCO_3$ buffer (pH 8.4; 15 mL). The mixed solution was stored for 24 h at 4 °C in the dark. The solution was dialyzed against 10 mM acetate buffer (pH 5.0) and then 50 mM HEPES buffer (pH 7.0) containing 1 mM $MnCl_2$, 1 mM $CaCl_2$, and 0.1 M NaCl to yield the randomly dansyl-modified Con A. The UV/Vis spectrum showed that one Con A monomer had one dansyl group.

Lysyl-endopeptidase digestion: A solution of the **1a**-labeled Con A (from the fractions making up the first peak in the affinity purification; 100 μM, 500 μL) in 50 mM tris(hydroxymethyl)aminomethane (Tris)/HCl buffer (pH 9.0) containing 3 M urea was treated with lysyl-endopeptidase at 37 °C for 15 h at the enzyme/substrate ratio of 1:50 (w/w). The digestion reaction was stopped by addition of trifluoroacetic acid (final concentration: 0.1% (v/v)). The digested peptides were separated by HPLC and the collected fractions were analyzed by MALDI-TOF MS (SA or CHCA).

Peptide sequencing by tandem mass–mass analysis: The sample including fragment A4 was treated with trypsin (in Tris/HCl buffer, pH 8.0) at 35 °C for 20 h. The resultant mixture was purified through a ZipTip C18 (Millipore), eluted with 70% aqueous acetonitrile containing 1% formic acid). The used eluent was subjected to ESI-TOF MS (Q-Tof 2, Micromass, UK). The target peak (747.32/2+) was sequenced by the tandem mass–mass method and analyzed by BioLynx software (manual mode).

UV/Vis, fluorescence, and CD spectroscopies: UV/Vis, fluorescence, and CD spectra of the modified Con As were obtained by using 5 μM protein concentration as determined by a Bradford assay in 100 mM phosphate buffer (pH 7.0) at 25 °C. For the measurement of the fluorescence spectra the slit widths of the excitation and emission were set to 10 nm, and an excitation wavelength of $\lambda_{ex} = 280$ nm was used for characterization of labeled- and SH-Con A. In the measurement of the CD spectra the following conditions were used: sensitivity: 200 mdeg; resolution: 0.5 nm; bandwidth: 1.0 nm; response: 0.5 s; scan speed: 100 nm/min.

Fluorescence titration of saccharide[33]: Saccharide stock solution was added dropwise to 0.2–5 μM Fluoro-Con A in 50 mM HEPES buffer (pH 7.0) containing 1 mM $CaCl_2$, 1 mM $MnCl_2$, and 0.1 M NaCl at 15 ± 1 °C. The fluorescence spectra were measured. The slit widths for the excitation and emission were set to 10 nm and 5 nm, respectively. The excitation and emission wavelengths used for each Fluoro-Con A are described in the corresponding figures. The fluorescence titration curves were analyzed with a Benesi–Hildebrand plot or nonlinear curve-fitting analysis to yield the association constants (K_a).

Fluorescence depolarization measurement[34]: The fluorescence anisotropy ratio (r) of IAEDANS-Con A was evaluated from Equation (1) (Weber equation), where $I_\parallel$ and $I_\perp$ are the intensities of fluorescence observed through polarizers parallel and perpendicular to the polarization of the exciting light, respectively.

$$r = (I_\parallel - I_\perp)/(I_\parallel + 2I_\perp) \quad (1)$$

The fluorescence intensity was measured with a fluorescence spectrophotometer (Hitachi F-4500) equipped with polarizer.

Molecular modeling: Model building and molecular dynamics calculations were performed with the Insight II/Discover program packaged in the context of Molecular Simulations Inc. All calculations were performed with the consistent valence force field. The structure shown in Figure 8 is a minimized low-energy conformation selected from 50000 dynamics interactions at 450 K. The minimization was performed until a maximum derivative of 0.001 was achieved. During the dynamics run and the minimization, the Con A and the mannopyranoside ring were restrained according to the crystallographic structure of the Con A–*p*-nitrophenyl-α-D-mannoside complex published by Kanellopoulos and co-workers.[29]

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