DOI: 10.1002/cbic.200700364

Ratiometric Fluorescent Biosensor for Real-Time and Label-Free Monitoring of Fine Saccharide Metabolic Pathways

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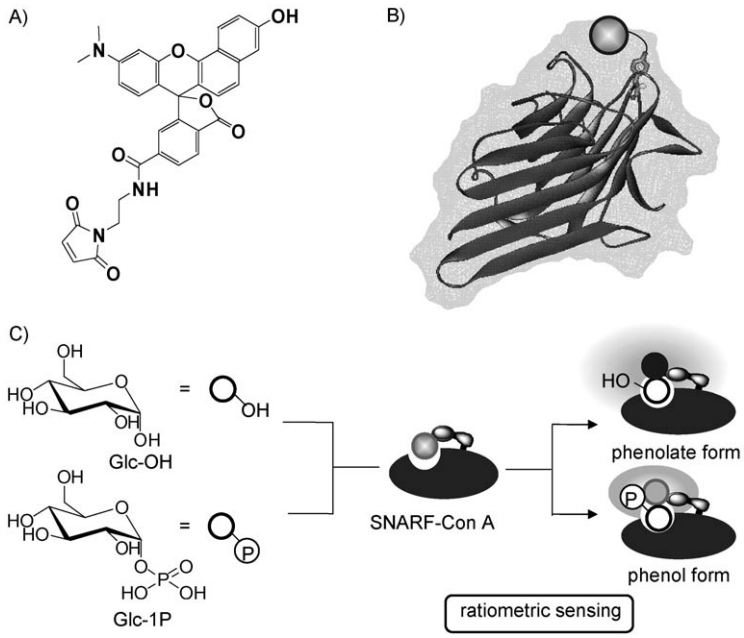
It is now recognized that saccharides and their derivatives, such as phosphorylated or sulfonylated saccharides, glycolipids and glycoproteins play crucial roles as materials, energy sources and intra- and intercellular messengers in various biological phenomena.[1] For instance, saccharometabolism is the main process for the accumulation and use of energy in living systems, and is also involved in the aging and the oxidative stress of living cells.[2] Therefore, a precise assay of such metabolic processes is important not only to gain an understanding of the fundamental glycobiology, but also for practical clinical applications.[3] It is difficult, however, to conveniently monitor these processes because the subtle structural changes at the anomeric position of saccharides cannot be distinguished well.[4] Here we describe a direct, label-free and real-time sensing method for the complicated saccharide conversion pathway that is involved in glycolysis by using a lectin-based ratiometric fluorescent biosensor that is capable of discriminating the fine anomeric moiety.



Scheme 1. A) Molecular structure of SNARF-mal. B) 3D structure of SNARF–Con A. C) Schematic illustration of the fluorescence sensing for subtle anomeric moiety of monosaccharides by using SNARF–Con A.

Because the anomeric residue is converted from a hydroxyl group to a phosphate or an alkoxyl functionality during the metabolic pathway,[5] the pH might be modulated in the proximity of the anomeric part. To sensitively detect such a micro-environmental pH change, we designed a semisynthetic lectin, a sugar-binding protein, that tethers seminaphthorhodafluor (SNARF),[6] a fluorescent pH indicator that shows a dual-emission change, to the vicinity of the sugar-binding pocket. Site-specific introduction of SNARF to concanavalin A (Con A; Scheme 1),[7] a mannose/glucose-binding protein, was conducted by post-photoaffinity-labeling modification (P-PALM)[8] to yield SNARF-modified Con A (SNARF–Con A), according to our established protocol (see Figure S1 in the Supporting Information). SNARF–Con A was characterized by UV–visible and fluorescence spectroscopies, and mass spectrometry (see Supporting Information). Prior to real-time and label-free monitoring of the saccharide conversion pathways, we initially evaluated the sensing capability of SNARF–Con A by fluorescence titration experiments with several saccharide derivatives such as phosphorylated saccharide, glycosylated nucleotide, monosaccharide and oligosaccharide under aqueous and neutral conditions (pH 7.5). Figure 1 A shows a typical fluorescence spectral change of SNARF–Con A upon addition of UDP-Glc. Clearly, the emission at 585 nm, which is due to the acidic phenol form of SNARF, increased, and the emission at 637 nm, which is due to its basic phenolate form, concurrently decreased with an emission isosbestic point at 610 nm (excitation wavelength: 534 nm).[6] The plot of the emission intensity ratio, R ($I_{585\,nm}/I_{637\,nm}$) showed good saturation behavior (differential $R=0.60$), and the Benesi–Hildebrand plot analysis afforded the binding constant ($\log K_a = 1.89$).[9] In the case of the other saccharide derivatives, the saturation curves that are shown in Figure 1 b gave binding constants for the various saccharides that are displayed in Table 1. A similar ratiometric change was observed for phosphorylated saccharides such as Glc-1P ($\log K_a = 1.73$) and Man-1P ($\log K_a = 2.38$). In contrast, a reciprocal spectral change occurred in the case of Glc-OH and Man-OH, both of which have a hydroxyl group at the anomeric position. That is, the emission of the basic phenolate form of the SNARF increased and the emission of the acidic phenol form concurrently decreased, which resulted in a decrease in the value of R (Glc-OH: $\log K_a = 3.04$, Man-OH: $\log K_a = 3.64$).[7b] On the other hand, such ratiometric changes never occurred for Me-α-Glc, Me-α-Man because the anomeric hydroxyl groups of these compounds are masked by methylation, although they are well known as the

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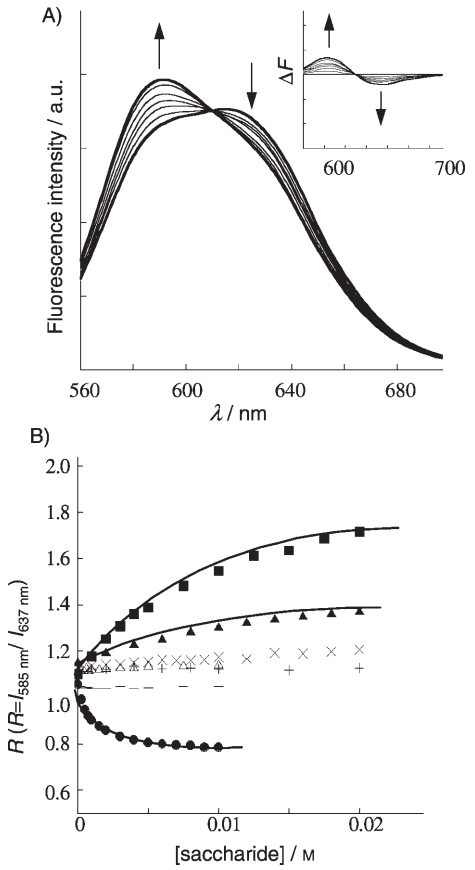

Figure 1. A) Fluorescence spectral change of SNARF–Con A upon the addition of UDP-Glc (0–20 mM). Inset: Differential fluorescence spectral change of SNARF–Con A upon the addition of UDP-Glc. B) Fluorescence titration plots of the emission intensity ratio (585 nm/637 nm) vs. the saccharide concentration. Glc-OH (●), Glc-1P (▲), UDP-Glc (■), Me-α-Glc (–), Isomal (△), Gal-OH (+), UDP-Gal (×)

Table 1. Comparison of the association constanst of SNARF–Con A with those of native Con A.

Saccharide	log *K*	
	SNARF–Con A[e] (Δ ratio[f])	native ConA[g]
Glc-OH	3.04 (–)	2.90
Man-OH	3.64 (–)	3.34
Gal-OH	–[a]	–[a]
Me-a-Glc	–[b]	3.48
Me-a-Man	–[b]	4.04
isomal	–[b]	3.23
13-Man2	–[b]	4.48
Man3	–[b]	5.40
Glc-1P	1.73 (+)	–[c]
Man-1P	2.38 (+)	–[c]
Glc-6P	–[a]	–[c]
Gal-1P	–[a]	–[c]
UDP-Glc	1.89 (+)	–[d]
UDP-Gal	–[a]	–[c]

[a] Cannot be determined because of the low affinity($\log K < 1$). [b] Con A can bind but SNARF–Con A cannot respond. [c] Not reported previously. [d] Previously it was reported that Con A can bind, but the association constant was not determined.[9] [e] The averaged value of at least three independent titration experiments. [f] ratio $= I_{585nm}/I_{637nm}$. [g] The reported values were determined by isothermal titration calorimetry.[7b]

high-affinity ligands of Con A. Similarly, the oligosaccharide (Man-3, Man-2 and Isomal), in which the anomeric hydroxyl group is masked by the glycosylation, did not induce any change in the *R* value. In addition, a significant ratiometric change was not observed for the Gal derivatives (UDP-Gal, Gal-1P and Gal-OH), because these cannot be bound to Con A.[7b] These results strongly suggest that the ratiometric emission change of SNARF–Con A is influenced by the difference in the anomeric groups of sugars, as well as the binding affinity to Con A.[10]

The sensing mechanism of SNARF–Con A was examined by the UV–visible titration experiment and by fluorescence pH titration. The UV/Vis spectral change upon addition of UDP-Glc or Glc-OH is shown in Figure S3 a and b, respectively. The change induced by UDP-Glc is consistent with that induced by a pH shift from basic to acidic pH.[5] On the other hand, the UV/Vis spectral change by Glc-OH showed opposite pH shift, that is, from acidic to basic pH. This behavior is consistent with the fluorescence pH titration results that were obtained with or without these saccharides (UDP-Glc or Glc-OH, See Figure S4). The fluorescence pH titration of SNARF–Con A shows a shift in the apparent pKa toward more basic values in the presence of UDP-Glc, whereas the presence of Glc-OH causes an acidic pKa shift. As a result, the value of *R* was increased by UDP-Glc, and was decreased by Glc-OH at neutral pH (we can observe the difference between pH 6.0–8.0). It is clear that the ratiometric fluorescence change of SNARF–Con A can be attributed to the pKa shift of SNARF, which is located in the proximity of the binding pocket of Con A, and was induced by the anomeric residue of the bound saccharide.

By using this sophisticated biosensor, the real-time and label-free assays for the various saccharide conversion reactions that involve the anomeric residues of sugars were successfully developed. First, we attempted to monitor the fluorescence of the dephosphorylation process of the phosphorylated saccharide. Figure 2 A shows the real-time fluorescence spectral change of the reaction of Glc-1P with alkaline phosphatase (AP);[11] the difference spectra is shown in the inset of Figure 2 A. The emission at 585 nm decreased while the emission at 637 nm increased. These spectral changes can be explained as follows: before the addition of AP, SNARF–Con A mainly binds to Glc-1P, and the measured emission attributes to the acidic form of SNARF.[12] During the AP-catalyzed dephosphorylation, the substrate (Glc-1P) is consumed, and simultaneously the product (Glc-OH) is produced, so that Glc-OH replaces Glc-1P in the binding pocket of SNARF–Con A. Thus, the emission of SNARF–Con A changed from the acidic form to the basic form. As shown in Figure 2 B, this reaction is accelerated by an increase in the AP concentration, and the half lifetime of the reaction, which is determined by the differential *R* and is proportional to the amount of AP (Figure 2 B inset).[13]

This method was also applied to other enzymatic reactions by using glucokinase (Figure S5 a and b) and phosphor-glucomutase (Figure S5 c and d), both of which are involved in the saccharometabolism pathway. Glucokinase catalyzes the reaction to convert Glc-OH into Glc-6P in the upstream of glycolysis,[14] and phosphor-glucomutase converts Glc-1P into Glc-6P

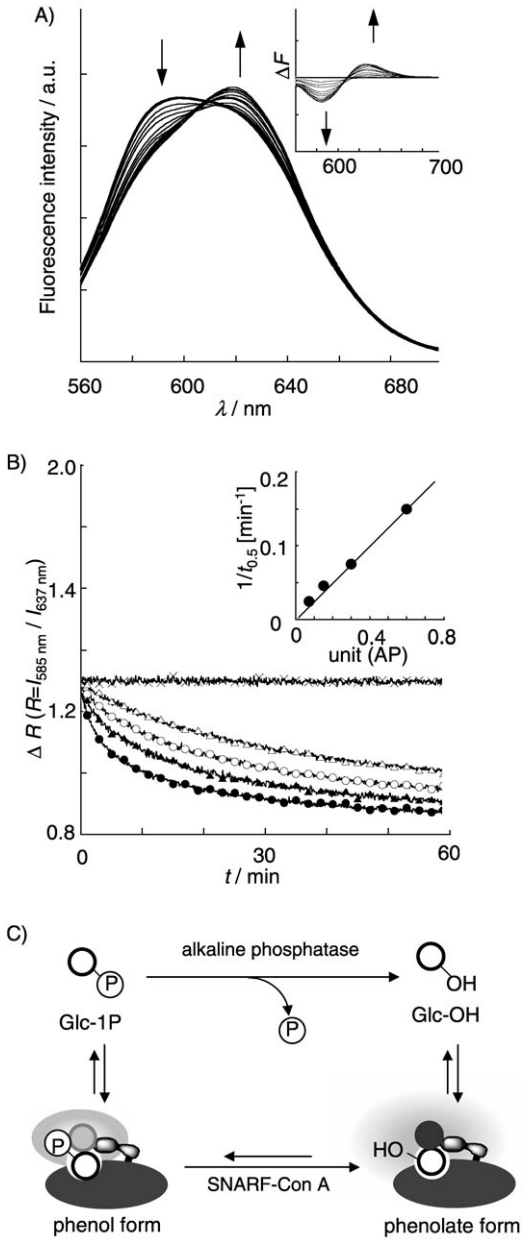

Figure 2. A) Real-time spectral change of the alkaline phosphatase (AP)-catalyzed dephosphorylation. Inset: Differential fluorescence spectral change of SNARF–Con A during dephosphorylating reaction. B) Real-time trace of the AP-catalyzed dephosphorylation as monitored by the emission ratio (637 nm/585 nm) of SNARF–Con A (excited at 534 nm) with various concentration of AP [0 (×), 0.075 (△), 0.15 (○), 0.3 (▲), 0.6 (●) unit]. Inset: apparent rate constant of the AP-catalyzed dephosphorylation as a function of the amount of AP. C) Schematic illustration of the ratiometric fluorescence detection of the AP-catalyzed dephosphorylation by using SNARF–Con A

in glycogenesis.[15] When glucokinase and ATP/Mg^{2+} were added to the solution of Glc-OH in the presence of SNARF–Con A, the emission ratio increased by reflecting the consumption of Glc-OH during phosphorylation. On the other hand, the value of *R* of SNARF–Con A linearly decreased upon addition of phosphor-glucomutase to the solution of Glc-1P. In these cases, the reaction rate was again confirmed to be proportional to the amount of the corresponding enzymes by our monitoring method, which strongly indicates that the present technique can be used to follow these enzymatic reactions quantitatively.

In conclusion, we have developed a ratiometric fluorescent biosensor for the discrimination of the fine saccharide structure, and for label-free monitoring of various saccharide conversion reactions that are involved in biological saccharometabolism. Compared to the conventional coupled enzyme assay that requires indirect and multistep reactions,[16] the present method is simple, direct and can be appreciated in real-time, even for the subtle but significant structural conversion at the anomeric position of saccharides.

Experimental Section

Con A was labeled with SNARF-mal and purified according to our previously reported method.[8c] The fractions that contain the labeled Con A were collected and used in the following study. The synthesis of SNARF-mal and the preparation of SNARF–Con A are described in the Supporting Information. In the fluorescence and UV-titration experiments, saccharide solution was added to SNARF–Con A (fluorescence titration: 0.75 μM, UV titration: 5.7 or 6.9 μM) in 10 mM Tris buffer solution (pH 7.5) at 20 °C. In less than 20 s, the equilibrium for the binding of SNARF–Con A with sugars was reached. Thus, after 20 s, the fluorescence spectra were measured. The titration curves were analyzed by a Benesi–Hildebrand plot to determine the association constants for various saccharides. In the AP assay, Glc-1P (10 mM) in Tris buffer (pH 7.5, 0.1 M) was mixed with various units of AP (EC 3.1.3.1) in the presence of SNARF–Con A (0.5 μM). The fluorescence spectrum changes were traced to determine the fluorescence ratio (585/637 nm; λ_{ex} = 534 nm). The details of other experimental procedures (fluorescence pH titration, enzyme assay (glucokinase (EC 2.7.1.2) and phosphor-glucomutase (EC 5.4.2.2)) also are shown in the Supporting Information.

Keywords: biosensors · fluorescent probes · lectin · ratiometric sensing · saccharometabolism

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Received: July 2, 2007
Published online on December 5, 2007

