Novel Naphthalimide Fluorescent Sensors Selective for Certain Proteins on Basis of Non-covalent Interactions between Enzyme and Inhibitor

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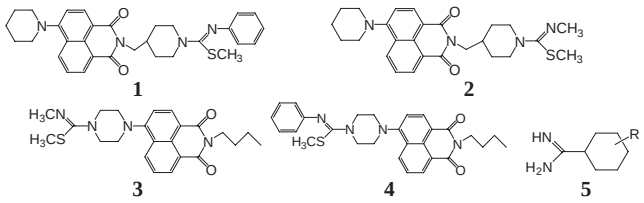
(Received February 15, 2005; CL-050202)

A non-covalent interactions' concept for proteins' fluorescent sensor of solvent-sensitive naphthalimide dyes was developed on basis of enzyme–inhibitor principle, which could selectively bind certain hydrolases with significant fluorescence enhancements and apparent blue-shifts in emission wavelength.

Selective binding of large biomolecules such as peptides, proteins, polysaccharides, or oligonucleic acids is becoming an important goal, because of its potential impact in the diagnostic, pharmaceutical, and pesticidal applications. Compared to the great progress in recognition of other large biomolecules, protein recognition is not yet well developed.[1,2] In current years, solvent-sensitive fluorescent dyes have been the hot topics as probes for proteins.[3–5] Reporting protein conformation changes and their activity using solvent-sensitive fluorescent dyes has important advantages complementary to those of FRET (the conventional approach), such as higher sensitivity, simpler operation and easier read-out.[6,7] However, the available dyes are mostly linked to the proteins either by covalent bond or by ion–dipole interaction, both of which might result in poorer selectivity and lower practicability.[8] Therefore, the selective recognition and sensing of proteins by non-covalent interactions is strongly required for further applications.

The unique binding interaction between the inhibitors and the corresponding enzymes is analogous to the interaction between lock and key. This specific interaction always occurs by non-covalent bonds at the hydrophobic regions, which is bound to alter the inhibitors' environment,[9,10] therefore, this phenomenon provides an opportunity to design an inhibitor analogue linked to a solvent-sensitive dye to bind the corresponding enzyme specifically by non-covalent bonds and to report the binding by fluorescence shifts. Since inhibition of certain enzymes by small molecules represents an important strategy for the treatment of a variety of human diseases (as well as plant diseases) and the conventional methods to evaluate the bioactivities of the inhibitors are both tedious and time-consuming, such fluorescent probes might be applied as more applicable and more convenient tools to detect and to screen new inhibitors through competitive binding.

Trehalase, a well-characterized hydrolase, was chosen to study the interaction between proteins and dyes. Trehalase (EC 3.2.1.28) is a very specific enzyme that hydrolyzes trehalose to two glucose units and is widely distributed in microorganisms, insects, plants, and animals. It plays important roles in many physiological regulations and thus it has been regarded as a novel target for rational design of pesticides.[11,12] Continuous efforts are now being devoted to the design and detection of more potent trehalase inhibitors. Some trehalase inhibitors with strong biological activities have been reported in our previous research.[13] The common structure of all these inhibitors ($R_1R_2NC(SR_3) = NR_4$, R_1, R_2, R_3, and R_4: H, alkyl, aryl) includes an isothiourea moiety as the activity center. Therefore we designed and synthesized four trehalase inhibitor analogues **1**–**4** with this activity center and with solvent-sensitive naphthalimides as fluorophores.



Scheme 1. The structures of molecules.

The emission spectra of **1**–**4** which were characterized in different solvents showed that all the dyes exhibited extraordinary high fluorescence intensity in non-polar environment, comparing to an extremely low intensity in polar solvent, accompanied by apparent red-shift of emission wavelength (see Supporting Information). Since the dyes were proved to respond the changes of the surrounding circumstance dramatically, further fluorescence detections were explored to evaluate the interaction between trehalase and the dyes. As controls, another two well-charaterized hydrolases, thrombin, and trypsin were tested for their effects on the dyes.

We observed that the interaction in solution between the dyes and the enzymes was rapid and tight (The same fluorescent changes were obtained at five seconds and at 12 h after the addition of proteins, respectively). Only **1** and **2** exhibited apparent trehalase selectivity and sensitivity (Table 1). When 1088.5 nM trehalase was added to the solution of the compounds (1 μM), more than 6-fold increase in intensity with 18-nm blue-shift in emission wavelength for **1**, and more than 20-fold increase in fluorescence intensity with 5-nm blue-shift in emission wavelength for **2** were observed, respectively. A control experiment in the same buffer solution with *N*-*n*-butyl-4-piperidinonaphthalimide which has the same fluorophore as **1** and **2** but does not have the recognition moiety to bind the enzymes revealed that the fluorescence of this compound was not influenced under the addition of the enzymes. This demonstrated that the enhancement in fluorescence intensity was indeed due to the interaction between the enzymes and the corresponding inhibitors.

Table 1. Parameters of fluorescence spectra of the sensors **1–4** (1 μM): emission wavelength (λ_{em}) and relative intensity (I/I_0) at the emission wavelength

		λ_{em}	λ_{em0}	I/I_0
1	Trehalase	526	544	6.541
	Thrombin	542	544	1.205
	Trypsin	540	543	0.929
2	Trehalase	537	542	20.201
	Thrombin	532	541	6.390
	Trypsin	542	542	0.917
3	Trehalase	539	541	1.160
	Thrombin	538	539	1.181
	Trypsin	540	541	1.043
4	Trehalase	523	525	0.913
	Thrombin	526	525	1.211
	Trypsin	526	526	0.978

λ_{em0}, I_0: Measured with no addition of enzymes. λ_{em}, I: Measured in the presence of trehalase (1088.5 nM), thrombin (1100.5 nM), trypsin (1000 nM) respectively. Measured at 25 °C in DMSO–H_2O 10:90 (V:V), 0.01 M Tris-HCl, pH 7.5, with **1**: $\lambda_{ex} = 425$ nm; **2**: $\lambda_{ex} = 451$ nm; **3**: $\lambda_{ex} = 399$ nm; **4**: $\lambda_{ex} = 400$ nm.

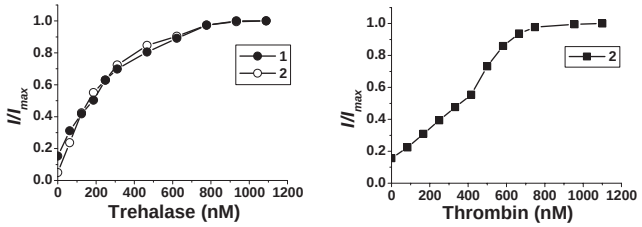


Figure 1. Continuous fluorescence titration (I/I_{max}) of **1** and **2** (1 μM) with trehalase and thrombin. Measured at 25 °C in DMSO–H_2O 10:90 (V:V), 0.01 M Tris-HCl, pH 7.5, with **1**: $\lambda_{ex} = 425$ nm; **2**: $\lambda_{ex} = 451$ nm.

However, **3** and **4** showed no change under this condition, which suggested that **3** and **4** exhibited lower binding affinities to trehalase comparing to the dyes **1** and **2**. We titrated **1** and **2** with trehalase solutions and the resulting fluorescence emission spectra were shown in Figure 1. It was obvious that the fluorescence intensity of **1** and **2** increased gradually and inclined to reach a plateau with the increase of trehalase. From these binding curves, we were able to evaluate the trehalase stability constants (k_S) to be about 4.488×10^6 and $5.872 \times 10^6\ M^{-1}$ for **1** and **2**, respectively.[14]

It was interesting that **2** also exhibited thrombin sensitivity (more than 6 fold increase in fluorescence intensity and 9-nm blue-shift of emission wavelength when 1100.5 nM thrombin was added, and k_S was about $2.518 \times 10^6\ M^{-1}$), while the other dyes did not. This might be deduced that the structure of **2** might be similar to a kind of potent thrombin inhibitors with a general structure **5** and thus exhibit thrombin binding ability.[15] Our results further showed that all the dyes exhibited no response to trypsin, a similar enzyme to thrombin. This clearly demonstrated that the binding between the enzymes and the dyes was selective.

Among all the compounds, **1** highly selectively bound trehalase, and **2** selectively bound trehalase and thrombin, while **3** and **4** did not respond to any of the three enzymes. The results prove that it is possible for selective or specifical sensing of certain proteins by solvent-sensitive dyes based on enzyme-inhibitor interactions. The key of this recognition is to rational design specific inhibitor analogues.

In summary, we developed novel proteins' fluorescent sensors of solvent-sensitive naphthalimide dyes on basis of enzyme–inhibitor interactions. The binding affinities to the enzymes of the inhibitors might be directly correlated with their inhibition efficiencies, therefore, the addition of the drugs with higher (compare to the sensors) inhibition efficiencies (which mostly mean higher binding affinities) to the mixture of the sensors and the corresponding enzymes might result in fluorescence quenching through competitive binding. Thus such fluorescent probes might be applied to detect and screen new inhibitors instead of the conventional methods. The results open the possibility of using easily obtainable solvent-sensitive dyes for enzymatic applications and for new drug and pesticides development.

We are grateful to the National Key Project for Basic Research (2003CB114400) and the National Natural Science Foundation of China for financial support of this research.

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16 The detailed synthesis and characteration data as well as the emission spectra of the compounds were enclosed as Supporting Information.

Published on the web (Advance View) April 9, 2005; DOI 10.1246/cl.2005.696