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ARTICLE *in* ANGEWANDTE CHEMIE INTERNATIONAL EDITION · JULY 2013

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Environment-Sensitive Fluorescent Turn-On Probes Targeting Hydrophobic Ligand-Binding Domains for Selective Protein Detection**

*Yu-De Zhuang, Po-Yi Chiang, Chia-Wen Wang, and Kui-Thong Tan**

Protein-specific detection or imaging is important in medical diagnosis to detect protein biomarkers, as well as in biology to investigate cellular processes. Small-molecule fluorescent turn-on probes that can detect specific proteins are particularly valuable as they allow for sensitive, simple, and specific detection with high signal-to-background ratios.[1] Currently most of the small-molecule fluorescent turn-on probes are designed for monitoring enzyme activities, for example, glycosidases, proteases, lactamases, and kinases.[2] Typically, their fluorescence turn-on mechanism is based on an enzymatic reaction with the chemical probes to convert a non-fluorescent substrate into a fluorescent product. On the other hand, the design of fluorescence probes for non-enzymatic proteins remains a challenging task. Currently, several fluorescence turn-on strategies such as hairpin peptide beacons,[3] aptamers,[4] supramolecular approaches,[5] polymer-conjugated nanoparticles,[6] and ligand-directed affinity labeling[7] have been reported to detect proteins through non-enzymatic reactions. Fluorescence signals can be turned-on upon recognition of the target proteins through charge–charge interaction, affinity labeling, or recognition of a specific peptide sequence. Although these fluorescent probes use novel strategies to detect non-enzymatic proteins, nevertheless, they suffer from either low selectivity and small fluorescent turn-on ratios, or are limited to sensing proteins on the cell surface. Thus, the development of a more general strategy to generate protein-specific fluorescent turn-on probes is necessary.

Herein, we introduce a novel strategy to generate fluorescent chemical probes for the selective detection of both enzymes and non-enzymatic proteins. Fluorescence turn-on can be achieved upon the binding of the fluorescent probes to the hydrophobic ligand-binding domain of the target protein. The fluorescent probes can be synthesized by incorporating an environment-sensitive fluorophore with a protein-specific small-molecule ligand (Figure 1a). Environment-sensitive fluorophores have emission properties that are highly sensitive to their immediate environment.[8] Typically they exhibit very weak fluorescence in polar and protic environments but show strong fluorescence and blue-shifted emission in hydrophobic surroundings. The rationale behind this new fluorescent probe design is that most of the ligand-binding sites in proteins are hydrophobic, and hydro-

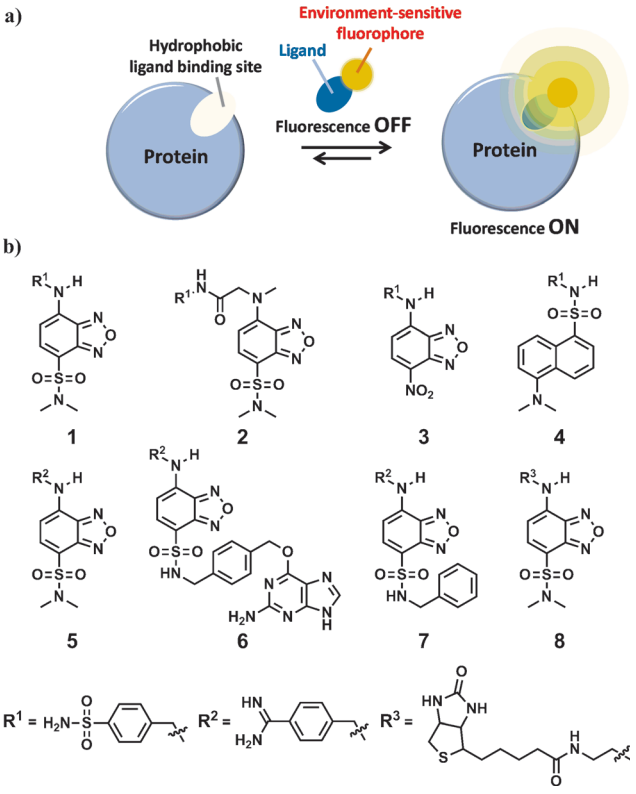

Figure 1. a) Illustration of the fluorescent turn-on probes for selective protein detection. The mechanism is based on the binding of the ligand to a specific hydrophobic site in a protein, whereby the adjacent hydrophobic environment can cause the environment-sensitive fluorophore to exhibit stronger fluorescence. In the absence of target protein, the fluorescent probe has low fluorescence. b) Chemical formulas of fluorescent probes **1**, **2**, **3**, and **4** for hCAII; **5**, **6**, and **7** for trypsin; **8** for avidin. Probe **1** is conjugated with the SBD fluorophore, while probes **3** and **4** are conjugated with NBD and dansyl fluorophores, respectively. R^1, R^2, and R^3 are the ligands for binding with hCAII, trypsin, and avidin.

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[**] We are grateful to the National Science Council (Grant No.: 100-2113-M007-018-MY2) and Ministry of Education (Grant No.: 101N2011E1), Taiwan (ROC) for financial support.
Supporting information for this article is available on the WWW under http://dx.doi.org/10.1002/anie.201302884.

phobic interactions constitute the principal thermodynamic driving force for the binding of small-molecule ligands to their proteins.[9] Therefore we expect that the binding of the ligand to the hydrophobic ligand-binding domain of the target protein would bring the environment-sensitive fluorophore closer to the hydrophobic pocket of the protein, and the proximity of the hydrophobic environment could cause the fluorophore to emit stronger fluorescence. In contrast, in the absence of target protein, the fluorescent probe would remain in the aqueous buffer and should exhibit only weak fluorescence. Although many examples have been reported, such as the conjugation of environment-sensitive fluorophores to peptides and proteins to study protein–protein interactions[10] and for the sensing of small molecules,[11] the application of small-molecule ligands conjugated to environment-sensitive fluorophores for the selective detection of proteins has not been described before.

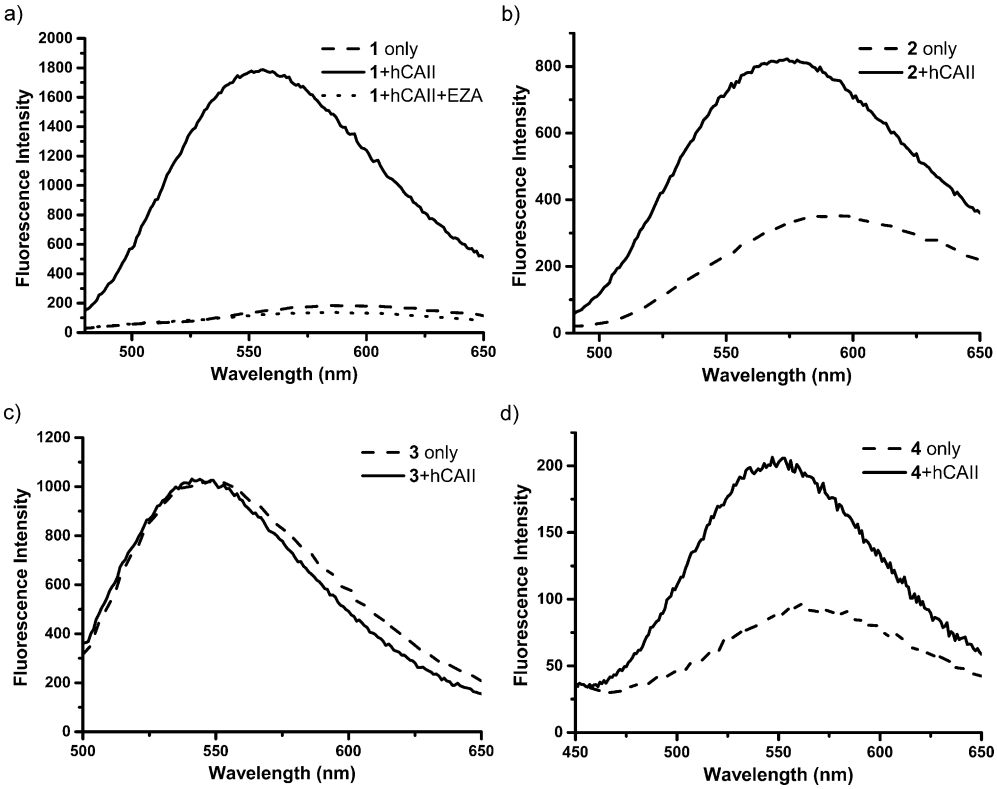

Figure 2. Detection of hCAII with environment-sensitive sulfonamide probes. a) Fluorescence spectra of 1 μM **1** in the absence (dashed line) and presence of 10 μM hCAII (solid line) in PBS buffer (1% DMSO), and after addition of 100 μM ethoxzolamide (EZA, dotted line). $\lambda_{ex}=430$ nm. b) Fluorescence response of **2** in the absence and presence of 10 μM hCAII. $\lambda_{ex}=448$ nm. c) Fluorescence spectra of **3** (1 μM) in the absence and presence of 10 μM hCAII. $\lambda_{ex}=460$ nm. d) Fluorescence spectra of **4** (1 μM) in the absence and presence of 10 μM hCAII. $\lambda_{ex}=340$ nm.

To generate environment-sensitive fluorescent turn-on probes for the selective detection of proteins, a small and highly environment-sensitive fluorophore, 4-sulfamonyl-7-aminobenzoxadiazole (SBD)[12] was incorporated with different ligands (Figure 1b). We further found that this fluorophore is a unique and optimal environment-sensitive fluorophore for this probe design because it gives remarkable fluorescence enhancement upon binding of the ligand to the target protein. Other environment-sensitive fluorophores, such as NBD and dansyl, gave no such fluorescence increase. In addition, SBD can be easily derivatized with different substituents at the 4-sulfamonyl moiety, which allows for optimization of the fluorescence enhancement with different substituents.

To test our fluorescent probe, human carbonic anhydrase II (hCAII), a monomeric soluble protein that consists of a hydrophobic binding site for many arylsulfonamide ligands, was selected as a target protein.[13] For the detection of hCAII, fluorescent probe **1** was prepared by reacting benzylamine sulfonamide with dimethyl-4-sulfamonyl-7-fluorobenzoxadiazole (DBD-F; Supporting Information, Scheme S1). The fluorescence was extremely weak when **1** was in aqueous PBS buffer, but this was dramatically enhanced (by 15-fold) upon the addition of hCAII (Figure 2a). The enhanced fluorescence of **1** can be quenched again by the addition of ethoxzolamide, a strongly competitive sulfonamide inhibitor for hCAII. In addition, such a fluorescence increase was not observed with the control probe containing no sulfonamide ligand moiety (Figure S1). These results show that the fluorescence increase is reversible and triggered only by the specific recognition of the arylsulfonamide ligand moiety to the hydrophobic pocket of hCAII.

We also compared the fluorescence spectra of **1** in PBS buffer alone and in the presence of hCAII. The maximum emission wavelength of **1** is blue-shifted about 39 nm from 589 nm in PBS buffer to 550 nm when hCAII is added. The blue-shifted emission spectrum of **1** overlays well with the spectrum from **1** dissolved in a hydrophobic solvent, acetonitrile (Figure S2). To further ascertain that the fluorescence enhancement of **1** is due to the hydrophobic environment of the arylsulfonamide binding site, we synthesized probe **2** by incorporating an amino acid sarcosine as a linker in between the arylsulfonamide ligand and the SBD fluorophore. In the presence of hCAII, **2** exhibits only a twofold increase in fluorescence (Figure 2b) and there is almost no fluorescence enhancement when the linker was extended even further (up to eight atoms; Figure S3). These results indicate that the blue-shifted emission and fluorescence increase can be attributed to the close proximity of the SBD fluorophore to

the hydrophobic binding domain. When probe **1** was incubated with an increasing concentration of hCAII, a gradual increase in fluorescence intensity with concentration was seen and the binding constant for hCAII with probe **1** was 4.0 ± 0.3 μM (Figure S4).

The choice of an environment-sensitive fluorophore was crucial for the probes to give high fluorescence enhancement upon binding to the target protein. To ascertain whether other environment-sensitive fluorophores could be used in this probe, we attached the same arylsulfonamide to two well-known environment-sensitive fluorophores, NBD-Cl and dansyl-Cl, giving probes **3** and **4**. Interestingly **3**, which has a very similar structure to **1**, showed no change in fluorescence intensity in the presence of hCAII (Figure 2c). This is interesting because probe **1** and **3** share the same benzoxadiazole fluorophore skeleton, with the only difference being that **1** has a dimethyl-4-sulfamonyl moiety and **3** has a nitro group.[14] We also found that the dimethyl-4-sulfamonyl group on the benzoxadiazole skeleton is critical for high fluorescence enhancement, because probes where the dimethyl-4-sulfamonyl was changed to sulfonic acid exhibited no fluorescence enhancement (Figure S5). Currently, the effect of the dimethyl-4-sulfamonyl group on enhancing the fluorescence in the protein ligand-binding domain is not understood. We postulate that, as compared with the sulfonic and nitro moieties, the dimethyl-4-sulfamonyl SBD fluorophore is perhaps more susceptible to the hydrophobic microenvironment around the ligand-binding domain. On the other hand, **4** exhibited only a onefold fluorescence increase upon addition of hCAII (Figure 2d). These results suggest that the highly environment-sensitive SBD fluorophore is a good choice for our fluorescent sensor.

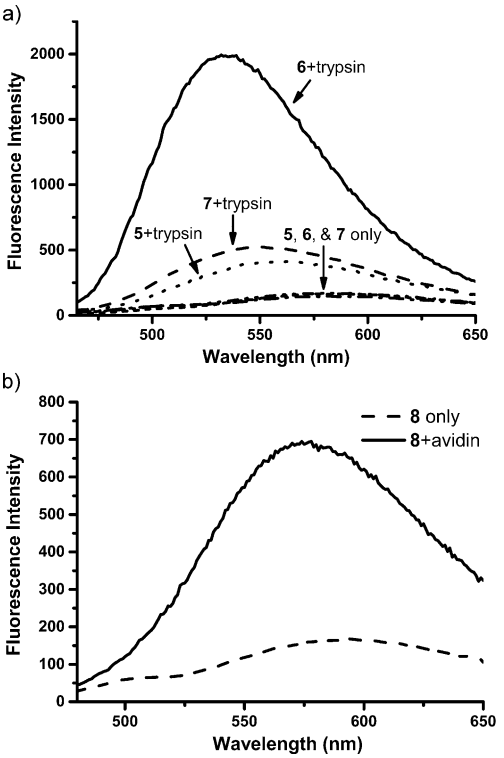

Figure 3. a) Fluorescence responses of probe **5**, **6**, and **7** (1 μM each) in the absence and presence of trypsin (100 μM). $\lambda_{ex} = 430$ nm and $\lambda_{em} = 560$ nm (for **5**), $\lambda_{em} = 530$ nm (for **6**), $\lambda_{em} = 550$ nm (for **7**). b) Fluorescence response of 1 μM probe **8** with 1 μM avidin. $\lambda_{ex} = 430$ nm, $\lambda_{em} = 569$ nm.

To demonstrate the modular nature of our probe for protein detection, we replaced the arylsulfonamide on the SBD fluorophore to benzamidine for the detection of trypsin. It was reported that the binding pocket of benzamidine for trypsin is also hydrophobic,[15] therefore we expected that its binding to trypsin would also place the SBD fluorophore in close proximity to the hydrophobic pocket, causing an increase in fluorescence. Probe **5**, which consists of the dimethyl substituent, was prepared initially for the detection of trypsin. In contrast to the large fluorescence enhancement of **1** with hCAII, probe **5** showed only 2.5-fold fluorescence increase upon addition of trypsin. On the other hand, probe **6** which consists of a benzylguanine substituent at the 4-sulfamonyl moiety showed a dramatic 17-fold fluorescence increase (Figure 3a). Titration of **5** and **6** with an increasing concentration of trypsin reveals that **6** has a stronger binding affinity to trypsin ($K_d = 10.6 \pm 1.3$ μM), while **5** has $K_d = 20.6 \pm 2.6$ μM (Figure S6). Probe **7**, which has a benzyl substituent, was synthesized to help understand the high fluorescence enhancement of probe **6**. In the presence of trypsin, **7** exhibits a fluorescence increase of 4.5-fold with a binding affinity of 5.7 ± 0.8 μM. The stronger binding affinity of **6** and **7** is in agreement with a previous study, which reported that besides the binding of the benzamidine group in the trypsin S1 pocket, a secondary sulfonamide moiety in the molecule can also interact favorably with the amino acids located in the S3/S4 hydrophobic pocket.[16] We therefore postulated that this additional interaction results in the better positioning of the SBD fluorophore in the trypsin binding pocket with the larger size of the benzylguanine group providing a more hydrophobic environment and therefore exhibiting higher fluorescence enhancement. The fluorescence of **6** can be quenched by the addition of an excess amount of free benzamidine (Figure S7), indicating that the fluorescent increase is produced by the specific recognition of the benzamidine ligand by the hydrophobic pocket of trypsin. Similar to the situation with hCAII, two other trypsin probes constructed by attaching benzamidine to NBD-Cl and dansyl-Cl do not exhibit fluorescence enhancement upon addition of trypsin (Figure S8), again indicating that SBD fluorophore is an optimal environment-sensitive fluorophore for our strategy.

Based on the results for trypsin detection, where three different substituents introduced at the 4-sulfamonyl moiety exhibited several fold fluorescence enhancement, we replaced the dimethyl substituent of probe **1** with benzyl and benzylguanine to see if high fluorescence turn-on could be achieved in hCAII as well. In the presence of hCAII, both probes showed fluorescence enhancement of about fivefold (Figure S9). This indicates that the SBD fluorophore is a versatile environment-sensitive fluorophore and different substituents can be tolerated at the 4-sulfamonyl moiety, which could lead to a variety of applications in diagnostics and imaging of proteins. For example, an SBD fluorescent probe incorpo-

rated with a benzylguanine moiety could be site-specifically conjugated to a self-labeling protein, SNAP-tag,[17] to study protein ligand interaction in a specific subcellular compartment of living cells.[18]

Finally, fluorescent probe **8** was synthesized for the detection of avidin, a non-enzymatic protein which consists of a hydrophobic binding pocket for biotin.[19] In the presence of avidin, the fluorescence intensity of **8** was enhanced by 4.5-fold (Figure 3b; Figure S10). The lower fluorescence enhancement for **8** was attributed to the ethylene linker between the SBD fluorophore and biotin, which distances the fluorophore from the hydrophobic biotin binding domain. We believe that an avidin probe without the linker would give a better fluorescence increase.

We then proceeded to study the selectivity of our environment-sensitive fluorescent probe by incubating nine other non-targeted proteins with **1**, **6**, and **8**. As shown in Figure 4a, **1** shows exceptional selectivity toward its target protein hCAII. With the exception of BSA, which shows a 1.7-fold increase in fluorescence, all the other non-targeted proteins exhibited no fluorescence change with probe **1** at 10 μM. For a selectivity test of the trypsin probe **6**, the same set of proteins at 100 μM were used. At this high protein concentration, we observed some nonspecific fluorescence increases from BSA (fourfold), concanavalin A (twofold) and β-casein (threefold), and virtually no change of fluorescence intensity for the other proteins (Figure 4b). For avidin probe **8**, fluorescence enhancement occurred only when avidin was added, but not with the other nine non-targeted proteins (Figure S11). The excellent selectivity of our fluorescent probes toward their target proteins shows its potential for clinical diagnostics and cellular imaging for specific detection of non-enzymatic proteins.

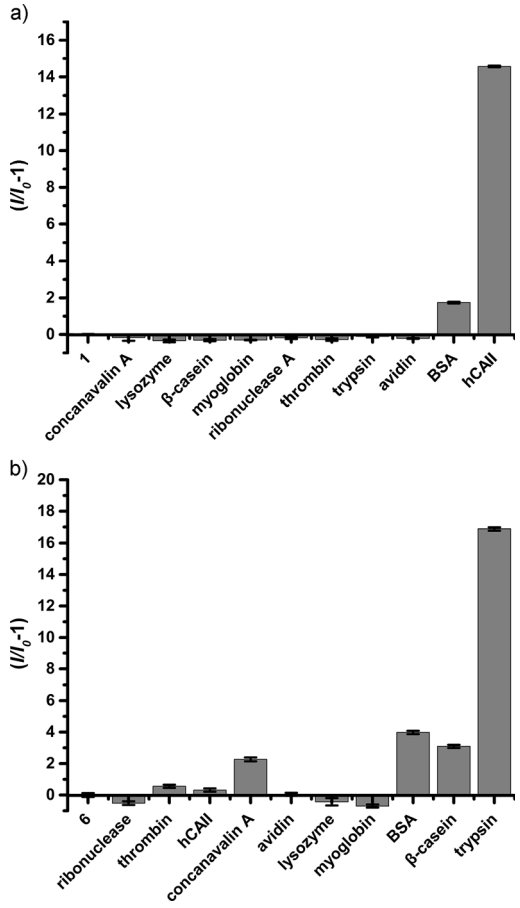

Figure 4. Selectivity test of probes **1** and **6** with nine other non-targeted proteins. a) hCAII probe **1** (1 μM) was tested with non-targeted proteins at 10 μM. b) Trypsin probe **6** (1 μM) was tested with non-target proteins at 100 μM. Bars represent relative fluorescence intensity at 550 nm (for **1**) or 530 nm (for **6**). Error bars were calculated from three independent experiments.

In summary, based on the fact that most ligand-binding sites are hydrophobic and that the emission properties of environment-sensitive fluorophores differ between aqueous and hydrophobic environments, we have established new fluorescent turn-on probes by using the SBD fluorophore conjugated to a protein-specific ligand for the detection of native enzymes and non-enzymatic proteins with diverse functions and structures. This probe is modular and versatile as demonstrated by the selective detection of hCAII, trypsin, and avidin. Because most ligand-binding sites have a hydrophobic environment, we expect this approach to be a general strategy to detect enzymes and non-enzymatic proteins. Herein, we found that the SBD fluorophore is an ideal environment-sensitive fluorophore for the current probe design, because ligands conjugated with NBD or dansyl fluorophores exhibited no significant fluorescent enhancement.

As compared with other fluorescent turn-on strategies to detect non-enzymatic proteins, our fluorescent probes show remarkable fluorescence enhancement and high protein selectivity. Preparation of our fluorescent probes requires only simple synthetic steps and can be generated in large quantities at low cost (see Supporting Information for synthetic details). Because these fluorescent probes have smaller molecular size and neutral charge, we believe that they could easily diffuse into cells for imaging and to study various intracellular receptors, for example, the estrogen receptor.[20] Finally, we believe that this novel fluorescent turn-on probe will be useful for a wide range of applications, such as diagnosis and molecular imaging where high fluorescent turn-on ratios and simple detection methods are required.

Received: April 8, 2013
Revised: May 27, 2013
Published online: June 18, 2013

Keywords: environment-sensitive fluorophores · fluorescent probes · non-enzymatic proteins · protein detection · protein modifications

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