Selection of Color-Changing and Intensity-Increasing Fluorogenic Probe as Protein-Specific Indicator Obtained via the 10BASEd-T

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Selection of color-changing and intensity-increasing fluorogenic probe as protein-specific indicator obtained via the 10BASE$_d$-T

Masumi Taki*, †, Hiroaki Inoue†, Kazuto Mochizuki†, Jay Yang§ and Yuji Ito‡

† Department of Engineering Science, Bioscience and Technology Program, The Graduate School of Informatics and Engineering, The University of Electro-Communications (UEC), 1-5-1 Chofugaoka, Chofu, Tokyo 182-8585, Japan
* E-mail: taki@pc.uec.ac.jp. Fax: +81-42-443-5501.

§ Department of Anesthesiology, University of Wisconsin, School of Medicine and Public Health, Madison, WI 53706 USA

‡ Department of Chemistry and Bioscience, Graduate School of Science and Engineering, Kagoshima University, 1-21-35 Korimoto, Kagoshima, Kagoshima 890-0065, Japan

ABSTRACT: To obtain a molecular probe for specific protein detection, we have synthesized fluorogenic probe library of vast diversity on bacteriophage T7 via the gp*10 based-t*hioetherificaion (10BASE$_d$-T). A remarkable color-changing and turning-on probe was selected from the library, and its physicochemical properties upon target-specific binding were obtained. Combination analyses of fluorescence emission titration, isothermal titration calorimetry (ITC), and quantitative saturation-transfer difference (STD) NMR measurements followed by *in silico* docking simulation, rationalized most plausible geometry of the ligand-protein interaction.

Rapid and readily-interpretable detection of a specific protein is crucial for broad range of disciplines, such as disease diagnostics[1-3] and environmental/cellular monitoring.[3-5] For this purpose, microenvironmentally-sensitive fluorogenic probes[3, 6] are frequently utilized. In particular, those that result in a color-changing and turning-on (CCTO) (i.e. shift in the emission spectrum with an increase in light intensity on target recognition) are desirable because of the ease of implementation for practical use and the readily-interpretable readout.[7] Among such probes, Prodan is one of the most predominantly-used fluorophores because of its remarkable CCTO property to report on the microenvironment together with its relatively small size.[8]

The mainstream approach for creating a target-protein detection probe using Prodan is to conjugate the fluorophore within a certain position of already-known ligands such as peptides.[9, 10] For instance, Imperiali *et al.* introduce Prodan at the penultimate position of the C-terminus of ribonuclease S-binding peptide, and successfully detect Pro-S protein by a fluorescence readout upon binding.[11] Nevertheless, the introduction of the fluorogenic probe often results in the loss or weakens the target-binding ability of the unmodified ligand. Ambiguous CCTO[10, 12] is another problem for the practical use, probably because geometry of Prodan on binding is not optimized and inappropriate. Moreover, these 'rationally-designed' probe development strategies are usually limited to well-known pairs of host proteins and guest ligands. A more general strategy for creating a CCTO probe is required to enable fluorogenic detection of a broad range of target proteins.

Meanwhile, we have recently reported on a method to create a modified-phage library through conjugation of artificial-molecule cores at designated cysteines in the randomized peptide region on a capsid protein (gp10) of T7 phage.[13, 14] This gp*10 based-t*hioetherificaion (10BASE$_d$-T) is carried out in a one-pot reaction without side reactions or loss of phage infectivity. Consequently, the cores can co-evolve during selection for target specific binders with the phage display technology. By using the 10BASE$_d$-T, here we conjugated Prodan with randomized peptide, to create a fluorogenic library with vast diversity (i.e. 10^9), and successfully found a target-specific probe with remarkable CCTO ability.

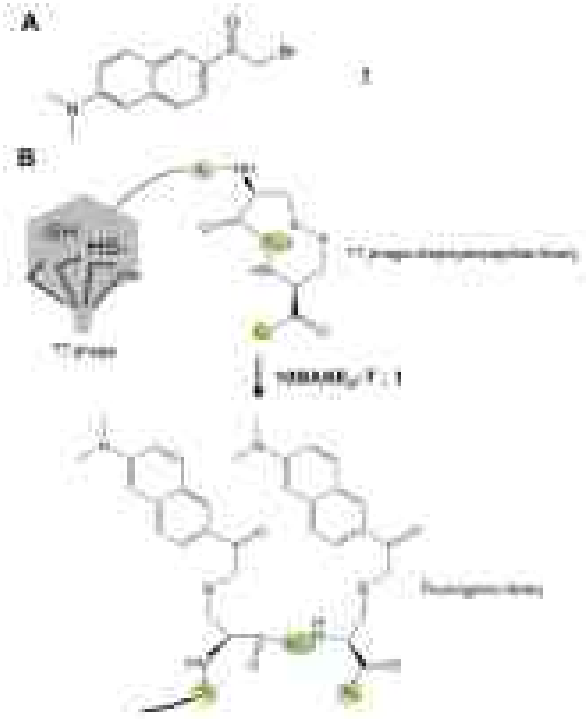

Figure 1. (A) Structure of fluorogenic synthon (Badan; a precursor of Prodan). (B) Construction of a fluorogenic library through the 10BASE$_d$-T. X represents randomized amino acid.

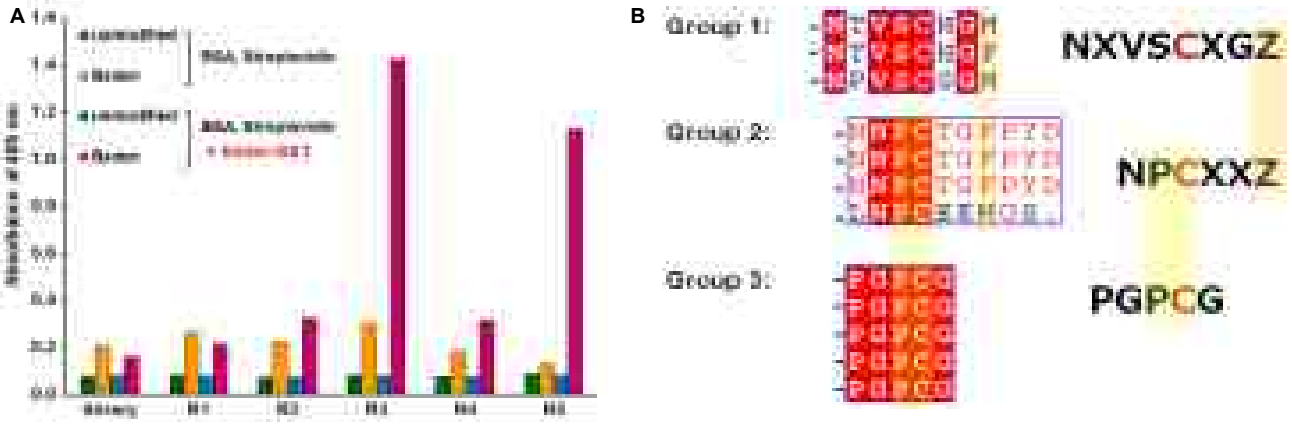

Figure 2. Biopanning against glutathione *S*-transferase (GST) using the fluorogenic library (-SGGG-X_3-C*-$X_{5\text{-}7}$-C*-X_3; Cys are conjugated with Prodan). (A) T7 phage polyclones after each round (R) of biopanning were reacted with Badan. Both the modified and unmodified T7 phage polyclones were subjected to enzyme-linked immunosorbent assay (ELISA). Mixture of streptavidin and bovine serum albumin (BSA) served as mock proteins. (B) Sequence alignment of GST-binding 12 monoclones with strong ELISA signals. Cysteine is alkylated with the fluorogenic core (i.e. Prodan). Z and X represent hydrophobic and any amino acids, respectively. T7 phage monoclones were chosen from the phage pool after 5 rounds of biopanning, and then subjected to DNA sequencing. They are categorized in three groups, and consensus sequences within each group were highlighted in red. Consensus sequences between the groups were colored in yellow and orange, respectively.

Starting form a commercially-available Prodan synthon (6-bromoacetyl-2-dimethylaminonaphthalene; also referred as Badan), the 10BASE$_d$-T was instantly performed as shown in Fig. 1 (for the details, see SI). In advance to performing selection using the library, we used T7 phage displaying a model peptide (-GSRVSCGGRDRPGCLSV),[13] and confirmed the appropriate thioetherification of Badan. After the 10BASE$_d$-T against the model phage, total proteins were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis followed by fluorescence imaging. A single fluorescent band could be seen at the appropriate molecular weight (~40 kDa) of the peptide-fused gp10 (Fig. S1, SI). This indicated that the Prodan moiety was site-specifically conjugated with the designated Cys in the model T7 phage-displayed peptide; no other T7 phage coat proteins including those required for infection was not alkylated.

As a target protein for the proof-of-concept study, we chose glutathione S-transferase (GST) because in our previous work we have already evolved tetramethylrhodamine (TMR) to GST-specific binders via the 10BASE$_d$-T[13]. Thus a direct comparison between the TMR- and Prodan-evolvers could be made. Prior to biopanning, Badan was reacted with T7 phage-displayed peptide library (-SGGG-X_3-C-$X_{5\text{-}7}$-C-X_3; where X represents any amino acids)[13, 15], which had been generated from a vector of mid-copy-number expression (5-15 peptide molecules per a phage; T7Select10), via the 10BASE$_d$-T as previously shown in Fig. 1B. To include the possibility that a doubly-alkylated peptide may improve the affinity toward the target protein, we conjugated two Prodan cores into the library peptide with two cysteines.[13] After the thioetherification, we examined the infectivity of the modified T7 phage library by plaque assay, and confirmed that the infectivity titre was fully retained (Fig. S2, SI).

Five rounds of biopanning were performed against biotinylated-GST, and enrichment of GST binders was assessed by enzyme-linked immunosorbent assay (ELISA). During the biopanning, increasing stringent conditions such as shortening the binding time and increasing the washing frequency were applied stepwise to each round. After the panning, the Prodan-conjugated polyclones on T7 phage showed the strongest binding to GST, whereas ones lacking the Prodan core structure did not (Fig. 2A). This suggests that the Prodan moiety played a crucial role in the peptide interaction with the target. Among 16 randomly chosen single T7 phage plaques, 15 clones had positive signals with 12 of them showing strongest binding on ELISA. The clones gave consensus displaying-peptide sequences which are categorized in three different groups: NXVSC*XGZ (**1**), NPC*XXZ (**2**), and PGPC*G (**3**) (C* and Z

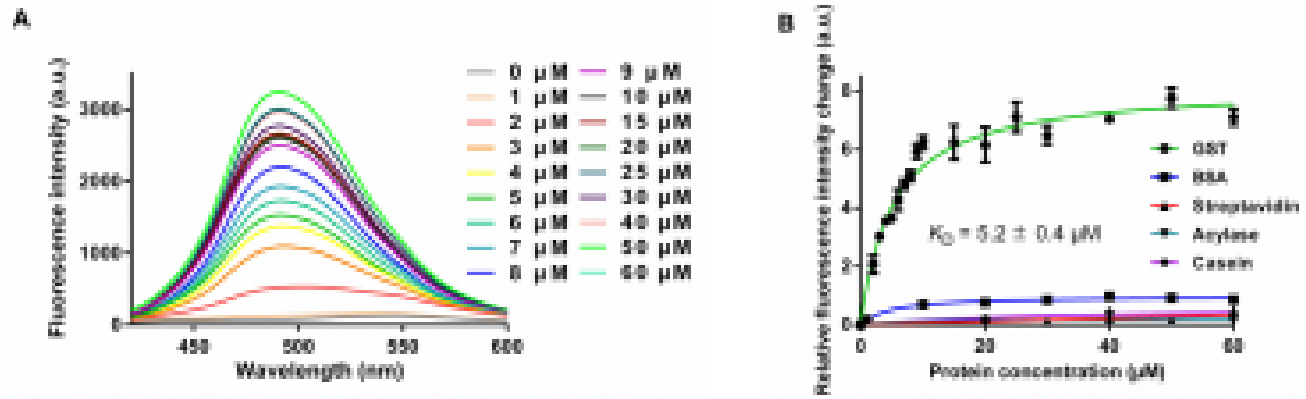

Figure 3. (A) Fluorescence emission titration upon GST-binding by using the best CCTO probe (NTVSC*HGF; **1**). (B) Determination of GST-binding affinity (dissociation constant; K_D) of the CCTO probe by relative fluorescence intensity change at 551 nm.

represent Prodan-conjugated Cys and hydrophobic amino acids, respectively; Fig. 2B). These peptide sequences of Prodan-evolvers were completely different from that of TMR-evolved ones (i.e. ZC*XZDGZ; C* represents TMR-conjugated Cys),[13] except for the C-terminal GZ.

Next, we chemically synthesized peptides representing these three groups, and their specific-binding property was investigated by fluorescence titration experiment against GST. All of the Prodan evolvers (**1** – **3**) possessed solvatochromic fluorescence property as we expected. A remarkable CCTO from weak yellow to bright cyan was observed when NTVSC*HGF (**1**) was titrated against GST (Fig. 3A), whereas moderate CCTO could be seen for **2** and **3** (Fig. S3, SI). The GST binding ability of the best Prodan-evolver (**1**) was almost equal to that of the previously discovered TMR-evolved one;[13] the dissociation constant (K_D) of **1** was estimated to be 5.2 ± 0.4 μM (Fig. 3B), which is almost identical that obtained independently by isothermal titration calorimetry (ITC) measurement (i.e. 2.4 ± 0.1 μM; Fig. S4A, SI). In contrast to the GST-specific binding of Prodan-peptide conjugate (**1**), the surrounding peptide alone or Prodan core itself did not bind to GST (Fig. S4B and S4C, SI). Specificity of this probe was confirmed since **1** did not bind to a variety of different target-unrelated proteins (Fig. 3B and S5, SI). These results suggested that both structures of the fluorogenic core and the rest of the surrounding peptide were essential for the GST-specific binding. Thermodynamic parameters were also obtained from ITC,[14] and favorable enthalpy and entropy changes (ΔH and ΔS) were observed. These suggest that hydrogen bonding and van der Waals force, as well as hydrophobic effect, moderately contributed to the interaction between the Prodan-evolver **1** and GST.

Glutathione (GSH) is a well-known GST-binding small ligand.[16] We examined whether the Prodan-evolver **1** competes with GSH for the GST-binding. Interaction between **1** and GST was disrupted in the presence of GSH in a concentration-dependent manner, and the bright cyan fluorescence turned back to the weak yellow upon competition (Fig. S6, SI). This strongly suggested that **1** binds to the GSH-binding pocket of GST, in a similar manner of rationally-designed Prodan-conjugated-GSH which shows ambiguous CCTO.[10]

To obtain further insight of the site-specific binding of NTVSC*HGF (**1**) to GST, nuclear magnetic resonance (NMR) experiments were performed. First, ^{1}H-^{1}H COSY experiment was carried out, and we found that aromatic- and α/β-carbon-attached proton signals of the Prodan core and phenylalanine were broadened, and methyl protons of valine slightly shifted upon binding (Fig. S7, SI). This indicated that these hydrophobic moieties are important for the binding. Second, ligand-based epitope mapping was performed by saturation transfer difference (STD) NMR. To make it more quantitative, saturation time was varied in each measurement, and the relative STD intensities of these protons-of-interest were plotted against the saturation time (Fig. S8, SI).[17] Exponential curve fitting of the plot gave us relative saturation rate constant of each proton (k), which indicated the importance of these atoms upon binding. As summarized in Fig. 4, valine and the Prodan core strongly interacted with GST, whereas phenylalanine and histidine moderately and weakly, respectively. The importance of the above residues in this CCTO interaction with GST was also supported by the observation that the amino acids at the 3rd and 5th positions from the N-terminus were always valine and cysteine-Prodan (underlined), respectively, whereas the 6th histidine and 8th phenylalanine were variable (italicized) within the consensus sequence of NX**V**S**C****XG**Z*** discovered through 5-rounds of biopanning (Fig. 2B, group 1).

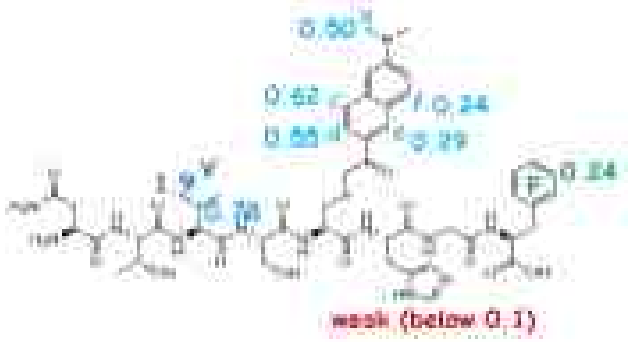

Figure 4. Quantitative epitope mapping of the Prodan-evolver NTVSC*HGF (**1**) upon GST binding on the basis of STD-NMR measurement. Relative saturation rate constant of each proton (k; shown here) was obtained from relative STD signal intensity (STD) at each saturation time (t) and maximum STD signal intensity (STD_{max}) by using an equation of $STD = STD_{max} \times (1-e^{kt})$ shown in Fig. S9.

To rationalize the binding geometry, we performed a protein-ligand docking simulation using AutoDock.[18, 19] Among the nine different geometries generated in a grid around the GSH-binding pocket, only the best model with a lowest binding energy (Fig. S9, SI) was consistent with the STD-NMR results. The simulation suggested that the Prodan core plays a dominant role for the binding; it was buried inside the deep hydrophobic pocket and shielded from aqueous environment, which could have caused the remarkable CCTO observed.

In conclusion, we demonstrated creation of a fluorogenic library with vast diversity in T7 phage, and successfully found GST-specific CCTO probes. In pioneering works of fluorogenic sensing without color-change, Winter *et al.*[20] and Ito *et al.*[21, 22] elegantly selected nitrobenzoxadiazole (NBD)-conjugated antibodies and peptides, respectively, from random library pools. Nevertheless, in most cases, selected target-specific binders do not increase fluorescence intensity upon target recognition and are not turning-on probe molecules.[20, 22] Such inability may be because the position of the NBD may not be completely optimized, resulting in fluorescence quenching via electron transfer from NBD to aromatic amino acid(s) within the hydrophobic pocket of the target protein.[23] In contrast, Prodan-based probes could yield a remarkable color-change regardless of fluorescence-intensity change, enhancing the possibility of obtaining practical indicators with an unambiguous readout. We envision that a similar approach to that described here will create CCTO probes for many protein targets since Prodan, one of the smallest probes possessing a neutral charge, can be buried into different hydrophobic pockets of these targets. We are now trying to confirm the generality/limitation of this probe-discovery system by changing target proteins and/or derivatizations of the CCTO core structures.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information (SI), including Supplementary figures, and Materials and methods, is available free of charge on the ACS Publications website as a PDF file.

AUTHOR INFORMATION

Corresponding Author

* E-mail: taki@pc.uec.ac.jp. Fax: +81-42-443-5501.
Phone: +81-42-443-5980.

Notes

The authors declare no competing financial interest.

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REFERENCES

(1) Bilitewski, U. *Anal. Chim. Acta* **2006**, *568*, 232-247.

(2) Zhang, L.; Lei, J.; Liu, L.; Li, C.; Ju, H. *Anal. Chem.* **2013**, *85*, 11077-11082.

(3) Li, X.; Gao, X.; Shi, W.; Ma, H. *Chem. Rev.* **2013**, *114*, 590-659.

(4) Urano, Y.; Asanuma, D.; Hama, Y.; Koyama, Y.; Barrett, T.; Kamiya, M.; Nagano, T.; Watanabe, T.; Hasegawa, A.; Choyke, P. L.; Kobayashi, H. *Nat. Med.* **2009**, *15*, 104-109.

(5) Urano, Y.; Sakabe, M.; Kosaka, N.; Ogawa, M.; Mitsunaga, M.; Asanuma, D.; Kamiya, M.; Young, M. R.; Nagano, T.; Choyke, P. L.; Kobayashi, H. *Sci. Transl. Med.* **2011**, *3*, 110ra119.

(6) Orlowski, R.; Banasiewicz, M.; Clermont, G.; Castet, F.; Nazir, R.; Blanchard-Desce, M.; Gryko, D. T. *Phys. Chem. Chem. Phys.* **2015**, *17*, 23724-23731.

(7) Klymchenko, A. S.; Mely, Y. *Prog. Mol. Biol. Transl. Sci.* **2013**, *113*, 35-58.

(8) Sinkeldam, R. W.; Greco, N. J.; Tor, Y. *Chem. Rev.* **2010**, *110*, 2579-2619.

(9) Chen, H.; Chung, N. N.; Lemieux, C.; Zelent, B.; Vanderkooi, J. M.; Gryczynski, I.; Wilkes, B. C.; Schiller, P. W. *Biopolymers* **2005**, *80*, 325-331.

(10) Svensson, R.; Greno, C.; Johansson, A. S.; Mannervik, B.; Morgenstern, R. *Anal. Biochem.* **2002**, *311*, 171-178.

(11) Nitz, M.; Mezo, A. R.; Ali, M. H.; Imperiali, B. *Chem. Commun. (Camb)* **2002**, 1912-1913.

(12) Harikumar, K. G.; Pinon, D. I.; Wessels, W. S.; Prendergast, F. G.; Miller, L. J. *J. Biol. Chem.* **2002**, *277*, 18552-18560.

(13) Fukunaga, K.; Hatanaka, T.; Ito, Y.; Taki, M. *Mol. BioSyst.* **2013**, *9*, 2988-2991.

(14) Fukunaga, K.; Hatanaka, T.; Ito, Y.; Minami, M.; Taki, M. *Chem. Commun. (Camb)* **2014**, *50*, 3921-3923.

(15) Hatanaka, T.; Ohzono, S.; Park, M.; Sakamoto, K.; Tsukamoto, S.; Sugita, R.; Ishitobi, H.; Mori, T.; Ito, O.; Sorajo, K.; Sugimura, K.; Ham, S.; Ito, Y. *J. Biol. Chem.* **2012**, *287*, 43126-43136.

(16) Stebbins, C. E.; Russo, A. A.; Schneider, C.; Rosen, N.; Hartl, F. U.; Pavletich, N. P. *Cell* **1997**, *89*, 239-250.

(17) Viegas, A.; Manso, J.; Nobrega, F. L.; Cabrita, E. J. *J. Chem. Educ.* **2011**, *88*, 990-994.

(18) Morris, G. M.; Goodsell, D. S.; Halliday, R. S.; Huey, R.; Hart, W. E.; Belew, R. K.; Olson, A. J. *J. Comput. Chem.***1998**, *19*, 1639-1662.

(19) Morris, G. M.; Huey, R.; Lindstrom, W.; Sanner, M. F.; Belew, R. K.; Goodsell, D. S.; Olson, A. J. *J. Comput. Chem.* **2009**, *30*, 2785-2791.

(20) Jespers, L.; Bonnert, T. P.; Winter, G. *Protein Eng. Des. Sel.* **2004**, *17*, 709-713.

(21) Wang, W.; Uzawa, T.; Tochio, N.; Hamatsu, J.; Hirano, Y.; Tada, S.; Saneyoshi, H.; Kigawa, T.; Hayashi, N.; Ito, Y.; Taiji, M.; Aigaki, T.; Ito, Y. *Chem. Commun. (Camb)* **2014**, *50*, 2962-2964.

(22) Manandhar, Y.; Bahadur, K. C.; Wang, W.; Uzawa, T.; Aigaki, T.; Ito, Y. *Biotechnol. Lett.* **2015**, *37*, 619-625.

(23) Taki, M.; Hohsaka, T.; Murakami, H.; Taira, K.; Sisido, M. *J. Am. Chem. Soc.* **2002**, *124*, 14586-14590.

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