Design of generic biosensors based on green fluorescent proteins with allosteric sites by directed evolution

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Abstract Protein-engineering techniques have been adapted for the molecular design of biosensors that combine a molecular-recognition site with a signal-transduction function. The optical signal-transduction mechanism of green fluorescent protein (GFP) is most attractive, but hard to combine with a ligand-binding site. Here we describe a general method of creating entirely new molecular-recognition sites on GFPs. At the first step, a protein domain containing a desired molecular-binding site is inserted into a surface loop of GFP. Next, the insertional fusion protein is randomly mutated, and new allosteric proteins that undergo changes in fluorescence upon binding of target molecules are selected from the random library. We have tested this methodology by using TEM1 β-lactamase and its inhibitory protein as our model protein-ligand system. 'Allosteric GFP biosensors' constructed by this method may be used in a wide range of applications including biochemistry and cell biology.

Key words: Combinatorial protein design; Domain insertion; Protein engineering

1. Introduction

The green fluorescent protein (GFP) from the jellyfish *Aequorea victoria* is now one of the most widely used reporter proteins [1,2]. The fluorophore of GFP is intrinsic to the polypeptide chain and does not need to be introduced by covalent modification [2]. The remarkable properties of GFP were recently exploited to design optical sensors that act in living cells [3–6]. A calcium sensor based on calmodulin and two GFP mutants utilized the relatively large conformational change of calmodulin upon binding of calcium to induce fluorescence resonance energy transfer between a GFP donor and acceptor [3]. Unlike calmodulin, however, most proteins do not undergo dramatic structural changes upon ligand binding, and hence such molecular-recognition domains for various analytes cannot be used for the rational design of molecular sensors [6]. To overcome this limitation and to construct generic GFP-based sensors, we have adopted the screening method combined with the insertional gene fusion technique [7,8]. When a foreign epitope sequence was inserted into a surface loop of a certain enzyme, binding of an antibody to the inserted sequence activated the function of the enzyme [9,10]. Similarly, if GFP accommodates insertions of large domain sequences and if appropriate screening methods for their ligand sensitivity are available, it might be possible to select fusion proteins that show an increase in GFP fluorescence upon the binding of ligands (Fig. 1). In the present study, we have tested this methodology by using TEM1 β-lactamase (Bla) and β-lactamase-inhibitory protein (BLIP) [11] as our model protein-ligand system, since Bla undergoes essentially no conformational change upon binding of BLIP [12].

2. Materials and methods

2.1. Gene construction

A mutant GFP gene from pGFPuv [13] (Clontech) was subjected to site-directed insertional mutagenesis as follows. The N-terminal fragment (codons 1–172) of the GFP gene with an additional hexahistidine-encoding sequence was amplified by polymerase chain reaction (PCR) with a sense primer containing a *Nhe*I site and a reverse primer containing the *Hin*dIII-*Kpn*I sites. The PCR product was digested with *Nhe*I and *Kpn*I. Similarly the C-terminal fragment gene (codons 173–238) with *Kpn*I-*Eco*RI sites at the 5′ end and a *Sac*I site at the 3′ end was amplified and digested with *Kpn*I and *Sac*I. These fragments were simultaneously ligated into the *Nhe*I-*Sac*I backbone vector pEOR [14], resulting in the insertion of seven additional codons including the *Hin*dIII-*Kpn*I-*Eco*RI sites between Gln-172 and Asp-173 of GFP. A Bla gene (from pUC18) minus the signal sequence (codons 1–23) and the Bla stop codon was inserted in-frame into the GFP gene between the *Hin*dIII and *Eco*RI sites.

2.2. Selection of library

A screening vector pND101 derived from pEOR carries a kanamycin-resistance gene cassette from pUC4KIXX [15], the insertional mutant GFP gene (as above), and a BLIP gene [11] (a gift from W. Schroeder, University of Alberta, Canada) minus the signal sequence (codons 1–36) inserted between the P_{BAD} promoter [16] from *Escherichia coli* JM109 and the *rrnB* T_1T_2 terminator from pEOR. In this vector, the BLIP gene expression is inducible with L-arabinose and repressed with D-glucose. Random mutagenesis of the fusion GFP::Bla gene was performed using an error-prone PCR as described [8]. The PCR product was inserted in place of the GFP gene on pND101. The plasmid library was electroporated into *E. coli* JM109 cells that were plated on LB plates with 10 μg/ml kanamycin. One hundred colonies selected at random were transferred to two LB-kanamycin plates, one containing 0.2% arabinose to induce BLIP expression and the other containing 0.2% glucose. Under a hand-held ultraviolet lamp, colonies with the largest ratio of the fluorescence on the LB-arabinose plate to that on the LB-glucose plate were picked up and used as the template for the error-prone PCR in the next cycle. DNA sequencing was performed on an ABI Prism 377 DNA sequencer.

2.3. Protein purification and characterization

The sensor protein and BLIP with N-terminal hexahistidine sequences were overexpressed in JM109 *E. coli* cells under the control of the *tac* promoter and purified on a Ni-agarose column as previously described [17]. The sensor protein subjected to amino acid sequencing showed the peptide chain to begin with the expected sequences except for the N-terminal methionine to be cleaved. The fluorescence spectra of the sensor protein with various concentrations of BLIP were measured on a Shimadzu RF-502 spectrofluorophotometer at 25°C. The BLIP titration curve was fitted to the standard equation $\Delta F = \Delta F_{max}\ (1 + K_d/[\mathrm{BLIP}])^{-1}$, where ΔF is the change in

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Abbreviations: GFP, green fluorescent protein

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fluorescence emission intensity and K_d the dissociation constant. The penicillinase activity of GFP::Bla-1 was measured spectrophotometrically [18] at 25°C. The inhibition constant K_i, which expresses the affinity of the sensor protein for BLIP, was obtained from a competition experiment with benzylpenicillin [19].

3. Results and discussion

To construct a BLIP sensor, Bla was genetically inserted into GFP between the Gln-172 and Asp-173 sites (Fig. 2). We chose this insertion site for the following reasons. GFP consists of an 11-stranded β-barrel structure wrapped around a central helix [20,21]. The 172–173 site is located at a solvent-exposed loop between β-strands and tolerates 6–20 amino acid insertions without serious disturbance of the GFP structure and function [22]. Further, a suitable insertion site must not only accept large domain insertions, but also transduce signals sensitively from the inserted domains to the GFP fluorophore. The 172–173 site is far from the fluorophore (residues 65–67) in the primary sequence, but is three-dimensionally close to the fluorophore (Fig. 2), and thus is expected to be suitable for our purpose.

As a preliminary experiment, Bla was successfully fused to GFP by in-frame insertion. The purified fusion protein has both GFP fluorescence and Bla activity, but no change in the GFP spectrum upon addition of BLIP was observed. To convert the fusion protein into a BLIP sensor, we performed random mutagenesis and visual screening for BLIP sensitivity of the fluorescence of *E. coli* colonies (see Section 2). After two rounds of selection, we obtained a BLIP-sensitive clone and recovered the gene of the fusion protein, designated GFP::Bla-1. The GFP::Bla-1 carries two point mutations, M68I and V108I, in the inserted Bla but no mutation in the GFP region. The M68I mutation of wild-type Bla was known to decrease its thermal stability [23].

The in vitro characterization of purified GFP::Bla-1 demonstrated that this protein indeed acts as a BLIP sensor. The fluorescence spectrum of GFP::Bla-1 has two maxima at 395 and 475 nm (Fig. 3, inset), similar to those of the wild-type GFP [2]. The fluorescence intensity at 395 nm increased as a function of BLIP concentration in the range of 0.01–10 μM (Fig. 3), whereas no change in the spectrum was observed in the presence of a hydrophobic protein, BSA, at 10 μM. The GFP::Bla-1 retained penicillinase activity ($k_{cat}/K_m = 2\times10^6$ M^{-1} s^{-1}, compared with 5×10^7 M^{-1} s^{-1} for wild-type Bla [19]), which was inhibited by the addition of BLIP. The K_i

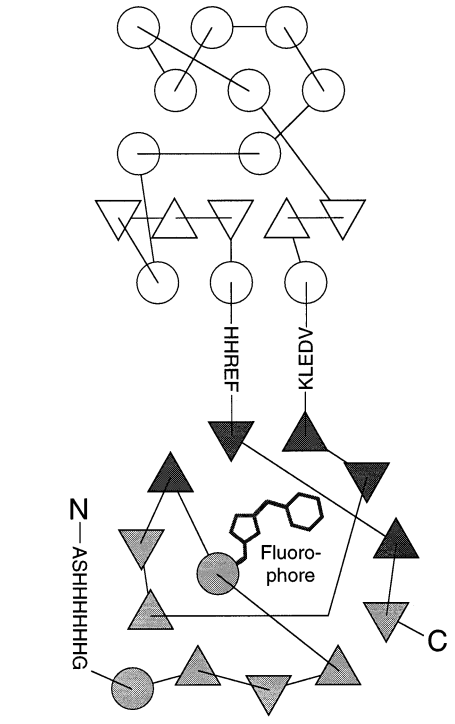

Fig. 2. Schematic topology diagram of the GFP::Bla fusion protein. Triangles represent β-strands and circles α-helices. The secondary structures of GFP [20,21] (indicated in gray and dark gray) and Bla [26,27] (white), amino acid sequences of the N-terminus and linkers between GFP and Bla, and the GFP fluorophore (dark gray) are shown. The dark gray triangles in GFP indicate β-strands bearing residues that directly interact with the fluorophore [20,21]. Short helices 3 and 4 of GFP are omitted from this figure.

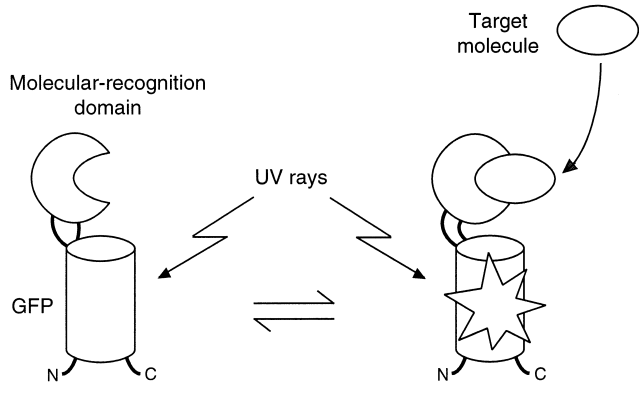

Fig. 1. Schematic representation of generic GFP-based sensors. A desired molecular-recognition domain is inserted into a loop of GFP. In the absence of the target molecule, conformational fluctuations of the inserted domain put stress on the GFP scaffold with a consequent reduction in fluorescence. The binding of a target molecule stabilizes the conformation of the inserted domain, resulting in restoration of the GFP fluorescence.

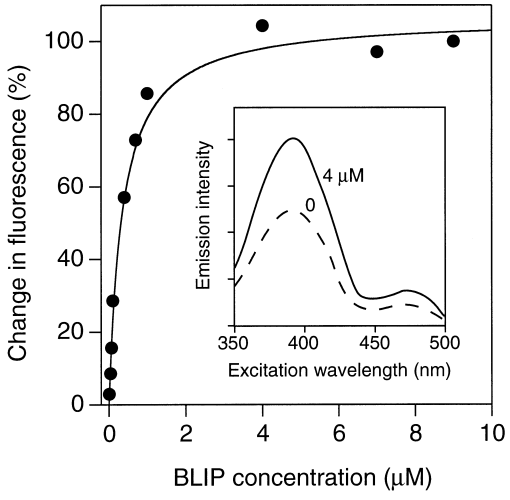

Fig. 3. BLIP titration curve of GFP::Bla-1 (0.3 μM). The differences in fluorescence intensity at 395 nm are normalized. The fitted curve corresponds to the dissociation constant given in the text. Inset shows the excitation spectrum in the presence (solid line) and absence (dashed line) of BLIP.

value is 0.2 μM, which is consistent with the dissociation constant (K_d = 0.35 μM) independently derived from the fluorescence measurements (Fig. 3). These results indicate that the change in fluorescence of GFP::Bla-1 is due to specific binding of BLIP.

The combination of domain insertion and directed evolution is expected to become a powerful tool for the construction of generic molecular sensors. The N- and C-terminal proximity observed in the large majority of globular proteins (see [24] and references cited therein) may make for easier insertions of molecular-recognition domains for various analytes into a surface loop of GFP. Furthermore, GFP is suitable for screening procedures, because the GFP signal can be easily detected, not only by simple visual inspection as described here, but also by fluorescence-activated cell sorting [25] and in vitro microplate assay [5]. The allosteric GFP biosensors thus may be applicable to the detection of many kinds of molecules, such as proteins, nucleic acids, hormones, drugs, metals and other small-molecular compounds, in complex mixtures both in vivo and in vitro.

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References

[1] Gerdes, H.-H. and Kaether, C. (1996) FEBS Lett. 389, 44–47.
[2] Tsien, R.Y. (1998) Annu. Rev. Biochem. 67, 509–544.
[3] Miyawaki, A., Llopis, J., Heim, R., McCaffery, J.M., Adams, J.A., Ikura, M. and Tsien, R.Y. (1997) Nature 388, 882–887.
[4] Siegel, M.S. and Isacoff, E.Y. (1997) Neuron 19, 735–741.
[5] Miesenböck, G., De Angelis, D.A. and Rothman, J.E. (1998) Nature 394, 192–195.
[6] Hellinga, H.W. and Marvin, J.S. (1998) Trends Biotechnol. 16, 183–189.
[7] Doi, N. and Yanagawa, H. (1998) Cell. Mol. Life Sci. 54, 394–404.
[8] Doi, N., Itaya, M., Yomo, T., Tokura, S. and Yanagawa, H. (1997) FEBS Lett. 402, 177–180.
[9] Brennan, C.A., Christianson, K., La Fleur, M.A. and Mandecki, W. (1995) Proc. Natl. Acad. Sci. USA 92, 5783–5787.
[10] Benito, A., Feliu, J.X. and Villaverde, A. (1996) J. Biol. Chem. 271, 21251–21256.
[11] Doran, J.L., Leskiw, B.K., Aippersbach, S. and Jensen, S.E. (1990) J. Bacteriol. 172, 4909–4918.
[12] Strynadka, N.C.J., Jensen, S.E., Alzari, P.M. and James, M.N.G. (1996) Nature Struct. Biol. 3, 290–297.
[13] Crameri, A., Whitehorn, E.A., Tate, E. and Stemmer, W.P.C. (1996) Nature Biotechnol. 14, 315–319.
[14] Prijambada, I.D., Yomo, T., Tanaka, F., Kawama, T., Yamamoto, K., Hasegawa, A., Shima, Y., Negoro, S. and Urabe, I. (1996) FEBS Lett. 382, 21–25.
[15] Barany, F. (1985) Gene 37, 111–123.
[16] Guzman, L.-M., Belin, D., Carson, M.J. and Beckwith, J. (1995) J. Bacteriol. 177, 4121–4130.
[17] Doi, N., Yomo, T., Itaya, M. and Yanagawa, H. (1998) FEBS Lett. 427, 51–54.
[18] Samuni, A. (1975) Anal. Biochem. 63, 17–26.
[19] Lenfant, F., Labia, R. and Masson, J.-M. (1991) J. Biol. Chem. 266, 17187–17194.
[20] Ormö, M., Cubitt, A.B., Kallio, K., Gross, L.A., Tsien, R.Y. and Remington, S.J. (1996) Science 273, 1392–1395.
[21] Yang, F., Moss, L.G. and Phillips Jr., G.N. (1996) Nature Biotechnol. 14, 1246–1251.
[22] Abedi, M.R., Caponigro, G. and Kamb, A. (1998) Nucleic Acids Res. 26, 623–630.
[23] Oliphant, A.R. and Struhl, K. (1989) Proc. Natl. Acad. Sci. USA 86, 9094–9098.
[24] Doi, N. and Yanagawa, H. (1998) FEBS Lett. 430, 150–153.
[25] Cormack, B.P., Valdivia, R.H. and Falkow, S. (1996) Gene 173, 33–38.
[26] Jelsch, C., Lenfant, F., Masson, J.-M. and Samama, J.-P. (1992) FEBS Lett. 299, 135–142.
[27] Jelsch, C., Mourey, L., Masson, J.-M. and Samama, J.-P. (1993) Proteins Struct. Funct. Genet. 16, 364–383.