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Engineering a beta-turn in green fluorescent protein to a foreign loop

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A type I beta-turn in green fluorescent protein (GFP) was engineered to a foreign loop. Molecular dynamics simulation study showed that the addition of foreign loop into GFP did not have a negative influence on the conformation stability of GFP structure, but the GFP variant with the foreign loop sequence was completely misfolded in real folding conditions. The co-incorporation of the enhancing mutations for GFP folding made it possible to generate a foldable and active GFP variant with the foreign loop sequence, although the folding efficiency and specific activity of the GFP were negatively affected by the introduced loop.

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Introduction

Turns and loops are the regions in protein structures that connect two secondary structures. Their lengths generally lie within the range of 4–10 residues for connecting two adjacent secondary structures, but some loops can be as long as 40 residues for the connection of distant structures. In general, they play crucial roles in protein folding and stability, and have been a major target in protein engineering for the enhancement of protein folding and stability [1–3]. In addition, they are the most variable parts in a protein in terms of sequence and length with conformational flexibility, which made them the best candidates for the addition of a foreign activity [4,5].

Although a number of successful examples of turn/loop design and engineering were reported, it is not easy to perform the task efficiently in reality. A major problem is the limitations in the range of engineering of turn/loop sequences due to a misfolding problem. Despite their relatively high conformational flexibility, the variation of the turn/loop sequences often disturbs the formation of proper protein structure, leading to complete misfolding of the engineered protein. This effect becomes more prominent as the sequence and size of the new turn/loop become more varied compared to the original turn/loop, which limits the range of turn/loop variation. This misfolding problem in the turn/loop engineering requests a lot of experimental repeats of mutagenesis, folding and screening before acquiring a desired mutant protein. If the mutagenesis rate is too high, there are many cases that a desired mutant cannot be obtained despite such experimental repeats.

An approach to avoid the laborious experiments in turn/loop engineering may be the *in silico* test of a mutant protein with engineered turn/loop sequence for its folding. However, it is not possible to simulate a protein folding process directly yet, and indirect approaches such as *in silico* conformational stability test for the static protein are currently used to evaluate the foldability of an engineered mutant protein [6,7]. There is no direct correlation between protein folding and conformation stability, which often leads to the misfolding of a mutant protein although the protein successfully passed the *in silico* stability test. Therefore, current indirect *in silico* evaluation methods for protein folding cannot be efficiently employed in turn/loop engineering.

Here, we propose an approach to overcome the misfolding problem in the engineering of turn/loop sequence. The rationale is the neutralization of the negative effect of turns/loop engineering on protein folding by co-introducing the mutations that enhance the target protein folding process. Previously, we demonstrated that this approach could be efficiently used to solve the protein misfolding problem caused by the addition of harsh mutations such as unnatural amino acid incorporations, deletion mutagenesis and surface charge variations [8–10]. To test the strategy in the turn/loop engineering, a beta-turn in green fluorescent protein (GFP) was engineered to a foreign loop sequence with very different sequence and size with the co-introducing of the mutations that enhance the GFP folding. The engineered GFP was expressed in *Escherichia coli* and their *in vivo* folding efficiencies and fluorescent activities were experimentally estimated. Finally, the proteins were purified and their spectral properties were characterized. Fig. 1 shows the scheme of this study.

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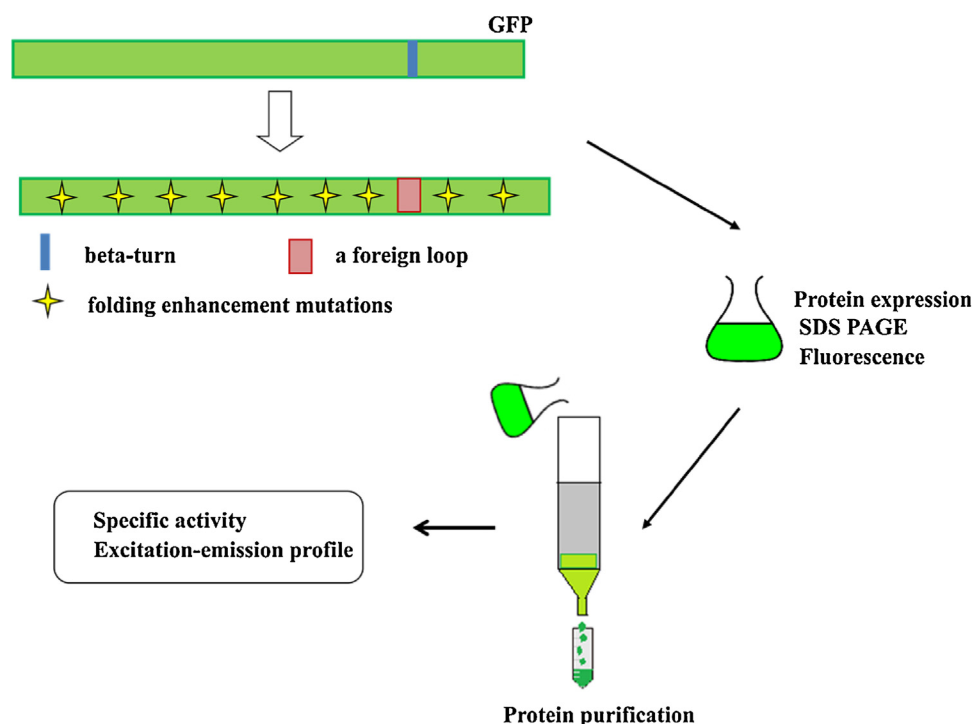

Fig. 1. Schematic representation of the experimental studies to demonstrate that a turn in GFP can be engineered to a foreign loop with avoiding the misfolding problem.

Materials and methods

Materials

The reagents for the molecular cloning work were purchased from New England Biolabs (Tokyo, Japan) and the chemicals were obtained from Sigma chemicals (St. Louis, MO, USA). pET30b(+) from Novagen was used to express the target proteins. The cloning host *E. coli* strain DH5a was used for plasmid DNA preparation. *E. coli* BL21(DE3) was used as the host for the expression of the protein.

Cloning of the genes for GFP variants, protein expression, and protein purification

All the genes used in this study were synthesized commercially from GenScript USA, Inc. (USA) in the pUC57 vector with NdeI and XhoI restriction sites. The gene inserts were released from the cloning vector pUC57 by double digestion and cloned individually into the double digested expression plasmid pET30b(+). All the constructs had a C-terminal histidine tag for affinity purification. The recombinant vector containing the genes in this study was transformed into the *E. coli* DH5a. The positive clones containing the respective recombinant plasmids were recovered by plasmid extraction from the cloning host and transformed into *E. coli* BL21(DE) for overexpression of the target proteins.

The recombinant *E. coli* BL21(DE) was grown at 37 °C for 5 h after the protein was induced with 0.5 mM IPTG at an OD_{600} of 0.6–0.8. The expression was analyzed by SDS-PAGE (12% acrylamide gel) after lysing equal amounts of cells, using the commercial reagent from Novagen (BugBuster protein extraction kit).

For the protein purification, the cells with the induced recombinant proteins were collected by centrifugation at 5000 rpm for 10 min at 4 °C and lysed using a French press (Thermo Scientific). The soluble fraction was prepared by centrifuging at 20,000 rpm for 30 min at 4 °C. The soluble protein fractions were purified by Ni-NTA column chromatography (GE Healthcare Bio-Sciences, Sweden) by using the standard protocol. The elution fractions were dialyzed against phosphate buffered saline (PBS) pH 7.4 to remove the salts used during protein elution.

Fluorescence measurement

The fluorescence measurements of GFP variants were recorded on a Perkin Elmer/Wallac Victor 2 Multilabel Counter (1420-011) by measuring the fluorescence intensity by excitation at 485 nm and emission at 515 nm with excitation/emission slits of 5.0 nm. The whole cell fluorescence assay was performed by collecting the cell pellet from 200 ml of overexpressed culture and resuspending the same in 200 ml of PBS. The specific fluorescence activity was measured from equal amounts of protein for all the constructs used in this study and recorded on a Fluorometer as described above.

Molecular modeling and molecular dynamics simulation

The modeling of the GFP variant structures and molecular dynamics simulations were performed as described in our previous study [11]. Briefly, PDB ID 1EMA was used as a template for modeling the GFP mutant structures, *in silico* mutagenesis for the turn residues was performed using a Rosetta loop modeling protocol [12], and energy minimization was performed using the molecular dynamics simulation package GROMACS 4.5.544. Molecular dynamics simulations were performed using GROMACS (v 4.5.6)23 with a OPLS-AA/L all-atom force field.

Results

Redesign of a beta-turn in GFP to a loop

GFP is a beta-barrel shaped protein which consists of 11 beta-strands and a helix running across the axis of the beta-barrel [13]. There are seven beta-hairpins which are formed by connecting the beta-strands through turns and loops. The region that was chosen for the redesign study was a type I beta-turn with sequence KDDG

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