Bioorganic & Medicinal Chemistry Letters 21 (2011) 3021–3024

Contents lists available at ScienceDirect

Bioorganic & Medicinal Chemistry Letters

journal homepage: www.elsevier.com/locate/bmcl

Structure and characteristics of reassembled fluorescent protein, a new insight into the reassembly mechanisms

Masami Isogai [a], Yoshihiro Kawamoto [a], Kazuto Inahata [b], Harumi Fukada [b], Kenji Sugimoto [b,c], Toshiji Tada [a,c,*]

[a] Graduate School of Science, Osaka Prefecture University, 1-1 Gakuen-cho, Naka-ku, Sakai, Osaka 599-8531, Japan
[b] Graduate School of Life and Environmental Sciences, Osaka Prefecture University, 1-1 Gakuen-cho, Naka-ku, Sakai, Osaka 599-8531, Japan
[c] Live Cell Imaging Institute, Osaka Prefecture University, 1-1 Gakuen-cho, Naka-ku, Sakai, Osaka 599-8531, Japan

ARTICLE INFO

Article history:
Received 15 February 2011
Revised 8 March 2011
Accepted 10 March 2011
Available online 16 March 2011

Keywords:
BiFC
Venus
Crystal structure
Fluorescent protein
Thermal stability

ABSTRACT

Bimolecular fluorescence complementation (BiFC) assay has been used widely to visualize protein–protein interactions in cells. However, there is a problem that fluorescent protein fragments have an ability to associate with each other independent of an interaction between proteins fused to the fragments. To facilitate the BiFC assay, we have attempted to determine the structure and characteristics of reassembled fluorescent protein, Venus. The anion-exchange chromatography showed an oligomer and a monomer of reassembled Venus. Our results suggested that the oligomer was formed by β-strands swapping without any serious steric clashes and was converted to the monomer. Crystal structure of reassembled Venus had an 11-stranded β-barrel fold, typical of GFP-derived fluorescent proteins. Based on the structural features, we have mutated to β-strand 7 and measured T_m values. The results have revealed that the mutation influences the thermal stability of reassembled fluorescent complex.

Protein–protein interactions are crucial for all biologic process. Therefore, defining how each protein interacts with all possible partners in cells provides new insight into cellular functions of individual proteins. BiFC assay enables direct visualization of protein–protein interactions in living cells.[1,2] The basic principle of this technique is the formation of the fluorescent complex through the association of two non-fluorescent N- and C-terminal fragments of the fluorescent protein when they are brought together by an interaction between two proteins fused to the fragments. A number of fluorescent proteins have been also utilized to study post translational modifications, protein folding, protein aggregation, protein conformational change, and protein topology.[3,4] In particular, Venus generated from EYFP has been recently used in the BiFC assay, because of its fast chromophore maturation rate and bright yellow fluorescence that is relatively insensitive to changes in pH and ion concentrations.[5,6]

The crystal structure of Venus (whole Venus) was determined by X-ray crystallography at a resolusion of 2.2 Å.[7] The structure of whole Venus shows an 11-stranded β-barrel, typical of GFP-derived fluorescent proteins. Although the Venus-based BiFC assay provides information regarding when and where protein–protein interactions occur in the cell, background from spontaneous assembly of the fragments compromises their utility for detecting the interactions. The decrease of the affinity between the N- and C-terminal fragments may increases the signal-to-noise (S/N) ratio by suppressing the association with each other independent of an interaction between proteins fused to the fragments. Recently, it has been reported that a mutation in the Venus N-terminal fragment reduces the spontaneous self-assembly of the two nonfluorescent fragments and decreases background fluorescence in the Venus-based BiFC assay.[8]

There is no report for the structure and characteristics of reassembled Venus formed by the complementation between the N- and C-terminal fragments, nevertheless they should provide important information on the strategies to decrease background fluorescence in the BiFC assay. Thus, in order to facilitate the application of BiFC to research of protein–protein interactions, we have attempted to elucidate the structure and the physicochemical properties of reassembled Venus and its mutants. It was difficult to obtain the fragment of split fluorescent proteins expressed in *Escherichia coli* separately due to intrinsic folding problems and low solubility of these fragments in aqueous solutions (own unpublished results). Recombinant expression of fragment of split fluorescent proteins often results in low sample yield or most of them express in insolubility form. in *E. coli* cells.[9] Thus, we attempted to co-express two fragments of split fluorescent protein in *E. coli* and successfully overexpressed reassembled Venus in soluble form. Here, we describe the X-ray crystal structure of reassembled Venus at a resolusion of 2.1 Å and its characteristics

* Corresponding author. Tel.: +81 72 254 9819; fax: +81 72 254 9163.
E-mail address: tada@b.s.osakafu-u.ac.jp (T. Tada).

0960-894X/$ - see front matter © 2011 Elsevier Ltd. All rights reserved.
doi:10.1016/j.bmcl.2011.03.039

assessed by chromatography, MALDI-TOF MS (mass spectrometry), CD (circular dichroism) spectra. Thermal stability of reassembled Venus including three mutants, Y143F, Y145F, H148G, were evaluated by DSC (differential scanning calorimetry) analysis.

Reassembled Venus was expressed in *E. coli* as a polyhistidine fusion protein. The fluorescent fraction isolated by a Ni affinity column chromatography was separated into three peaks on the anion exchange chromatography[10] (Fig. 1a). The profiles of fluorescent spectra of these three peaks were identical to that of whole Venus.[11] The N- and C-terminal fragments of split fluorescent protein do not exhibit the absorption or fluorescence characteristics unless two fragments of split fluorescent protein completely associate to form a fluorescent complex having a β-barrel conformation.[12,13] Thus, three peaks should contain the fluorescent complex formed through the association of two non-fluorescent fragments at least as a parent compound.

Each peak was further purified by a size exclusion chromatography. The size exclusion chromatograms of peaks 1 and 2, respectively, showed a single peak. Based on its retention volume and MALDI-TOF MS data[14] (Table 1), peak 1 was elucidated to be a monomer of reassembled Venus. The structure of the species corresponding to peak 1 was confirmed by X-ray structure analysis. The retention volume indicated that peak 2 was also a monomer of reassembled Venus. However, the molecular weight of VN155 found in MS is approximately 180 Da larger than the calculated value (Table 1). These findings suggested that a part of reassembled Venus might be modified during incubating in *E. coli*.

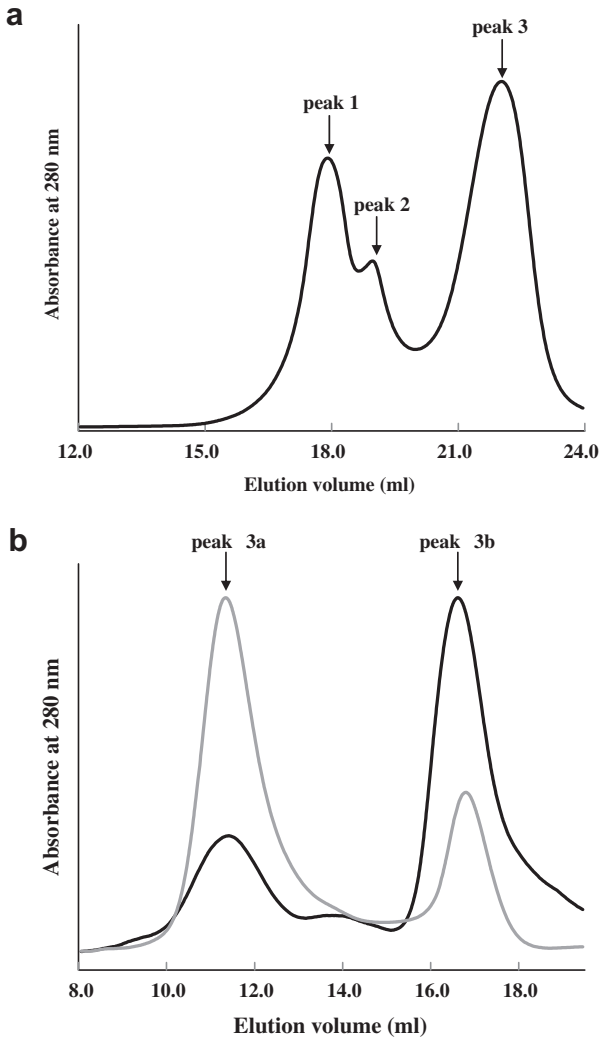

Figure 1. Chromatogram of reassembled Venus. (a) Anion exchange chromatogram of reassembled Venus. The elution points of three components of reassembled Venus are shown along the top. (b) Size exclusion chromatogram of peak 3 of reassembled Venus. The chromatogram of peak 3 incubated for 1 day at 4 °C is present in gray, and that of peak3 incubated for 3 days at 37 °C is present in black. For comparison, the absorbance is normalized by the maximum absorbance. The elution points of peak 3a and peak 3b are shown along the top.

The size exclusion chromatogram of peak 3 yielded two peaks after incubation for 1 day at 4 °C (Fig. 1b). Both peaks were fluorescent. MALDI-TOF MS analysis showed that the main components of peak 3 were VN155 and VC155 fragments of Venus. Thus, peak 3a emerged earlier and peak 3b emerged later were estimated to be an oligomer (octamer to decamer) and a monomer of reassembled Venus based on their retention volumes, respectively. This view was supported by CD spectrum[15] of peak 3b which was identical with that of peak 1. After incubation for 3 days at 37 °C, the area of peak 3a further decreased and that of peak 3b increased (Fig. 1b). On the other hand, the size exclusion chromatography of peak 1, after incubation for 3 days at 37 °C, did not give any other peaks except original peak. These findings suggest that the oligomer is converted to the monomer in an irreversible manner or the rate of conversion from the monomer to the oligomer is extremely slow.

The crystal structure of monomeric reassembled Venus was determined at 2.1 Å resolution.[16] There are four reassembled Venus molecules formed through the association of two VN155 and VC155 fragments in an asymmetric unit. Each molecule has an 11-stranded β-barrel fold including a chromophore in the middle, typical of GFP-derived fluorescent proteins (Fig. 2). The four molecules form two antiparallel dimers which have the same conformation. The dimer structure of reassembled Venus is quite similar to that found in the crystal structure of whole Venus[7], as indicated by the low rmsd value of 0.54 Å for the main chain atoms. GFPs are capable of forming antiparallel dimers; dimerization is facilitated at high concentration of protein and in high-salt concentration. The dimer structure may be stabilized in crystals. There is a slight but significant difference in the β-barrel fold between reassembled Venus and whole Venus. As compared with whole Venus, the seventh β-strand (β7) of reassembled Venus is shortened. Unlike whole Venus, two amino acid residues, Asn146 and Ser147, did not contribute to form β7. This means that the loop (amino acid residues from 138 to 147) was expanded and the formation of the β-barrel structure of reassembled Venus is partially insufficient. Based on this structural features, mutations in β7 of VN155, Y143F, Y145F and H148G (Fig. 3), were introduced to see if any change in the thermal stability of reassembled fluorescent complex is observed.[17]

DSC studies were carried out on whole Venus, reassembled Venus and mutants[18] (Fig. 4). The profiles were analyzed mainly in terms of the peak temperatures, because all of the samples showed irreversible transition. The thermogram of whole Venus shows a peak at 89.0 °C (T_m). On the other hand, the thermogram of monomeric reassembled Venus shows a peak at a lower temperature of 77.5 °C. The T_m value of the monomeric reassembled Venus did not fall at a lower concentration. It can therefore be

Table 1
TOF-MS of reassembled Venus

	Found		Calculated	
	VN155	VC155	VN155	VC155
Peak1	19920	10558	19925	10552
Peak2	20104	10553		
Peak3	19917	10552		

Comparison between the found and calculated masses of proteins.

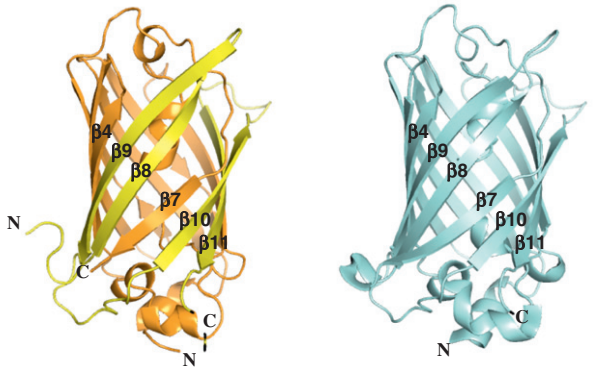

Figure 2. Comparison of reassembled Venus and whole Venus. Whole Venus is represented in cyan, VN155 and VC155 of reassembled Venus in orange and yellow, respectively.

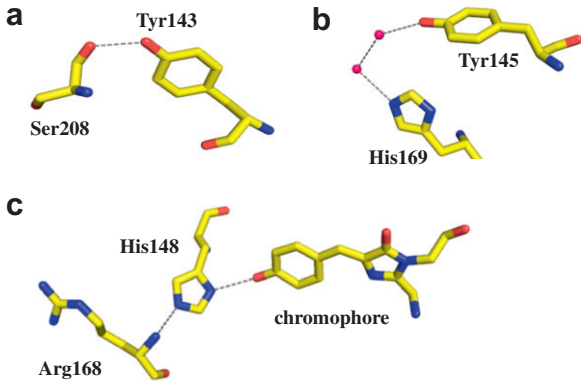

Figure 3. Close-up views of three amino acids around β7. Hydrogen bonds are indicated by dashed lines. Water molecules are drawn with pink spheres. (a) Tyr143. (b) Tyr145. (c) His148.

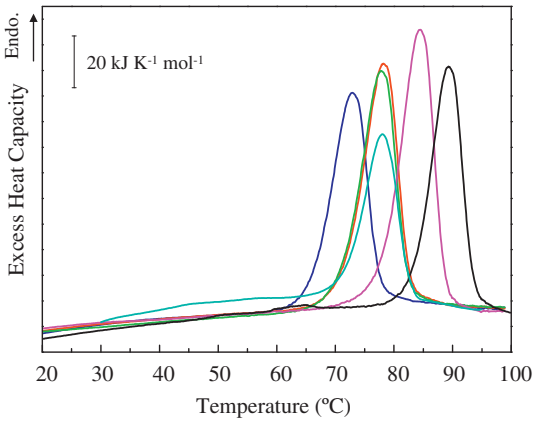

Figure 4. DSC of whole and reassembled Venus and its mutants. Heat capacity curves for whole Venus (black), peak 1 of reassembled Venus (red), peak 3 of reassembled Venus (cyan), Y143F (green), Y145F (pink), H148G (blue).

presumed that the dissociation and the thermal denaturation of monomeric reassembled Venus occur simultaneously at around T_m value.

The thermograms of three mutants of reassembled Venus, rV-Y143F, rV-Y145F and rV-H148G, showed T_m values of 77.1, 83.8 and 72.1 °C, respectively. The OH group of Tyr143 forms a hydrogen bond with the carbonyl O atom of Ser208 (Fig. 3a). Despite the hydrogen bond is lacking, T_m value of the rV-Y143F mutant was only slightly lower than that of reassembled Venus. Surprisingly, the substitution of Tyr145 with phenylalanine enhanced the thermal stability compared with monomeric reassembled Venus. Tyr145 and His169 are linked by the hydrogen bond network through two water molecules (Fig. 3b). The substitution seems to cause the release of one water molecule and increase the hydrophobic core with juxtaposed hydrophobic amino acids, Val61 and Ile167, or cause the rearrangement of those amino acids into alternative dense-packing structure. In contrast, the significant decrease in thermal stability was observed when His148 was substituted with glycine. This mutant seems to be useful for BiFC assay, because the lacking of the hydrogen bonds (Fig. 3c) may weaken the binding force between N- and C-fragments of Venus.

DSC measurement for the oligomer of reassembled Venus showed two peaks; one peak is identical to that for the monomer of reassembled Venus and has a maximum at 77.6 °C, and the other peak spreads through the range from 40 to 65 °C (Fig. 4 and Supplementary data Fig. S1). The size exclusion chromatography indicated that the oligomer was readily converted to the monomer. Thus, it can be considered that the conversion from the oligomer to the monomer occurs while temperature rises to ~65 °C.

In the coexpression of N- and C-fragments of fluorescent protein Venus using *E. coli*, there were two distinct thermodynamically stable and metastable forms, monomer and oligomer of reassembled Venus. Despite the overall structure of monomeric reassembled Venus was quite similar to that of whole Venus, the formation of oligomer could not be observed in the expression of whole Venus using *E. coli*. Oligomerization is a peculiar phenomenon found in only reassembled Venus. These findings indicate that oligomerization accompany a conformational change such as a β-strand swapping which has been reported in organization of dUTPase oligomers[19] Protein aggregation including oligomerization is said to occur by specific intramolecular associations involving the recognition of a sequence partner in another molecule rather than in the same molecule during the folding process. BiFC assay utilizes the formation of the fluorescent complex through the association of two non-fluorescent N- and C-terminal fragments of the fluorescent protein when they are brought together by an interaction between two target proteins fused to the fragments. The oligomerization seems not to be preferable for BiFC assay, because it will prevent the interaction between two target proteins and consequently increase background. The interface plays an important role in the complementary association of two non-fluorescent N- and C-terminal fragments. Our results have clearly showed that the mutation in β7 was susceptible to thermal stability of reassembled fluorescent complex. Thus, the substitution of amino acids in four β-strands (β8, β9, β10 and β11) of the C-fragment and three β-strands (β3, β4 and β7) of N-fragment is also expected to facilitate the application of BiFC to research of protein-protein interactions. The crystal structure of the monomer of reassembled Venus including water molecules provides new insights into decreasing background fluorescence in the BiFC assay.

Acknowledgment

We would like to thank the beamline staff at KEK-PF BL5A for assistance in the synchrotron experiments.

A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmcl.2011.03.039.

References and notes

1. Hu, C. D.; Chinenov, Y.; Kerppola, T. K. *Mol. Cell.* **2002**, *9*, 789.
2. Kerppola, T. K. *Nat. Protoc.* **2006**, *1*, 1278.
3. Hu, C. D.; Kerppola, T. K. *Nat. Biotechnol.* **2003**, *21*, 539.
4. Fan, J. Y.; Cui, Z. Q.; Wei, H. P.; Zhang, Z. P.; Zhou, Y. F.; Wang, Y. P.; Zhang, X. E. *Biochem. Biophys. Res. Commun.* **2008**, *367*, 47.
5. Nagai, T.; Ibata, K.; Park, E. S.; Kubota, M.; Mikoshiba, K.; Miyawaki, A. *Nat. Biotechnol.* **2002**, *20*, 87.
6. Shyu, Y. J.; Liu, H.; Deng, X.; Hu, C. D. *BioTechniques* **2006**, *40*, 61.
7. Rekas, A.; Alattia, J. R.; Nagai, T.; Miyawaki, A.; Ikura, M. *J. Biol. Chem.* **2002**, *277*, 50573.
8. Kodama, Y.; Hu, C. D. *Biotechniques* **2010**, *49*, 793.
9. Ottmann, C.; Weyand, M.; Wolf, A.; Kuhlmann, J.; Ottmann, C. *Biol. Chem.* **2009**, *390*, 81.
10. The anion exchange chromatography was performed using Mono Q™ 4.6/100 PE column (GE Healthcare) and the conditions are described below. Flow rate: 0.5 mL/min at 4 °C, gradient: 0–80% elution buffer in 25 column volumes, start buffer: 20 mM Tris–HCl, pH 8.0 + 20 mM NaCl, elution buffer: 20 mM Tris–HCl + 1.0 M NaCl.
11. Whole Venus, reassembled Venus and their mutants were diluted to 0.1 mg/mL with 20 mM sodium phosphate buffer, pH 7.0 for fluorescence spectral analysis. Fluorescence spectra were measured with a FP-175 fluorescence spectrophotometer (JASCO). Fluorescent proteins were excited at 485 nm, and emission spectra were recorded at a wavelength range from 500 to 600 nm at 20 °C.
12. Sakamoto, S.; Kudo, K. *J. Am. Chem. Soc.* **2008**, *130*, 9574.
13. Kent, K. P.; Childs, W.; Boxer, S. G. *J. Am. Chem. Soc.* **2008**, *130*, 9664.
14. The molecular mass of each component was measured on an autoflex II MALDI-TOF analyzer (Bruker Daltonics) using saturated α-cyano-4-hydroxycinnamic acid in 50% (v/v) acetonitrile and 0.1% (v/v) trifluoroacetic acid as a matrix.
15. Samples were diluted to 0.1 mg/mL with 20 mM sodium phosphate buffer, pH 7.0 for CD spectral analysis. CD experiments were performed on a J-820 spectropolarimeter (JASCO) with a Peltier PTC-423L thermo-unit (JASCO). The far-UV CD spectra (260-190 nm) were recorded using a 0.1 cm path length cell under constant nitrogen flush with a step size of 0.2 nm, bandwidth of 1 nm, and an averaging time of 2 s at 20 °C. The final spectra reported were an average of 10 scans.
16. Coordinates have been deposited with the Protein Data Bank with the following accession code: 3AKO.
17. Three mutants of VN155 (Y143F, Y145F, H148G) were constructed by site-directed mutagenesis of plasmids carrying VN155 (pET-16b) by PCR using *Pfu* Turbo (Stratagene). The sequence of mutants were verified by DNA sequencing with a dye terminator cycle sequencing kit (Beckman Coulter) and a CEQ2000 fragment analysis system (Beckman Coulter). Reassembled Venus consisting of VN155 mutants and VC155 (denoted as rV-Y143F, rV-Y145F, rV-H148G) were coexpressed and purified by the same procedures as those used for reassembled Venus.
18. Calorimetric experiments were carried out with a nanoDSC (TA instruments). Samples were prepared in concentrations of 0.5 and 1.0 mg/mL. The buffer used for the sample was 20 mM sodium phosphate, pH 7.0. Experiments were performed over a temperature range of 25–95 °C at a scan rate of 1 °C/min and excess pressure of 2.8 atm.
19. Takács, E.; Barabás, O.; Petoukhov, M. V.; Svergun, D. I.; Vértessy, B. G. *FEBS Lett.* **2009**, *583*, 865.