Analysis of DsRed Mutants

SPACE AROUND THE FLUOROPHORE ACCELERATES FLUORESCENCE DEVELOPMENT

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Earlier mutagenesis of the red fluorescent protein drFP583, also called DsRed, resulted in a mutant named Fluorescent Timer (Terskikh, A., Fradkov, A., Ermakova, G., Zaraisky, A., Tan, P., Kajava, A. V., Zhao, X., Lukyanov, S., Matz, M., Kim, S., Weissman, I., and Siebert, P. (2000) Science 290, 1585–1588). Further mutagenesis generated variants with novel and improved fluorescent properties. The mutant called AC4 exhibits only green fluorescence. The mutant, called E5up (V105A), shows complete fluorophore maturation, eventually eliminating residual green fluorescence present in DsRed. Finally, the mutant, called E57 (V105A, I161T), matures faster than DsRed as demonstrated in vitro with purified protein and in vivo with recombinant protein expressed in Escherichia coli and Xenopus leavis. Comparative analysis of the mutants in the context of the crystal structure of DsRed suggests that mutants with free space around the fluorophore mature faster and more completely.

The green fluorescent protein (GFP) from the luminescent jellyfish Aequorea victoria has become one of the most popular instruments in molecular and cell biology as a transcriptional reporter, fusion tag, and biosensor (2). The discovery of new fluorescent proteins from nonluminescent Anthozoa species (3), in particular, the red-shifted fluorescent protein drFP583, also known as DsRed, opened new horizons for multicolor labeling and fluorescence resonance energy transfer applications. However, several major drawbacks, such as slow maturation and residual green fluorescence, need to be overcome for the efficient use of DsRed as an in vivo reporter.

Compared with GFP, we and others (4) noted a slow rate of fluorescence development for DsRed, which is a limiting factor for many practical applications. This difference is most pronounced at lower temperatures (close to room temperature), where GFP has maximal folding efficiency (5). In contrast, all Anthozoa proteins studied so far mature faster at higher temperatures (>30 °C) when compared with GFP. These temperature-dependent rates may be evolutionary adaptations to the animals’ natural habitat: corals live in the warm Indo Pacific waters, while Aequorea victoria live in the cold boreal waters. The need for faster maturing DsRed mutants is especially acute in studies involving heterologous expression systems maintained at low temperature (such as Drosophila or Xenopus). In addition, even after extensive maturation periods (i.e., several weeks) the wild-type DsRed protein has residual green fluorescence at 500 nm (4–5% of the major red fluorescence peak), which is a tangible source of problems in multicolor detection applications.

Here we report two mutants of DsRed with improved fluorescent properties: namely, faster maturation and the absence of residual green fluorescence. Moreover, based on the crystal structure of DsRed, we formulated the “space” hypothesis to explain faster development of red fluorescence.

EXPERIMENTAL PROCEDURES

Random mutagenesis was performed using the Diversy PCR Random Mutagenesis Kit (CLONTECH) according to manufacturer’s protocol in conditions optimal for three to four mutations per 1000 bp. PCR products were cloned into pQE-30/BamHI/HindIII expression vector. Escherichia coli DH5α (CLONTECH) were transformed by electroporation in 10% glycerol with ligation mixture (0.1-cm cuvette, 12.5 kV/cm, 200 μF, 25 farads) and were grown on LB agar plates with 100 μg/ml Amp and 0.1 mM IPTG at 37 °C overnight. Colonies (up to 2000–5000 per plate) were screened visually using a standard fluorescent microscope (Karl Zeiss) with the 31001 filter set (Chroma). The brightest colonies or colonies with different phenotypes were picked and further characterized. For protein expression, bacteria harboring the recombinant plasmid were grown on an Amax of 0.6 in LB containing 100 μg/ml Amp, induced with 1 mM IPTG, and incubated overnight at 37 °C. Bacteria were collected by centrifugation, resuspended in 1× PBS buffer, and lysed by sonication. Fluorescent protein was purified at 0–4 °C from the cleared lysate using TALON metal affinity resin (CLONTECH) according to the manufacturer’s protocol. Excitation and emission spectra were recorded using a PerkinElmer LS 50 B Luminescence Spectrometer.

E. coli agar plate culture: DH5α competent cells were transformed with plasmids pQE-E57, pQE-E5up, or pQE-DsRed, respectively. The transformants were grown overnight at 37 °C. Plates were then transferred to 4 °C for one and a half days. Two days after transformation, cells from a single colony were streaked out on a fresh LB/ampicillin plate and grown for 20–22 h at 37 °C. The pictures were taken under UV light. Microinjections of Xenopus embryos were performed as described previously (33).

RESULTS

E5up, Eliminating Residual Green Fluorescence—A number of mutants of the human codon-optimized DsRed gene (DsRed1, CLONTECH) were generated by random mutagenesis using the error-prone PCR technique (6). We initially characterized the mutant E5 (V105A, S197T), called Fluorescent Timer, for its ability to change the fluorescence color over time (1). The impact of each individual substitution on the fluorores-
cent properties of E5 was further studied by splitting the mutations. Mutants each containing only one of the two substitutions present in E5, called E5up (V105A) and E5down (S197T), were obtained by site-directed PCR mutagenesis (Table I). While the “down” mutation, S197T, produced the E5 phenotype (data not shown), the “up” mutation resulted in a new phenotype. Freshly isolated E5up mutant protein from E. coli shows a minimal green fluorescence in the first 24 h of maturation and no detectable green fluorescence after 48–72 h of maturation (Fig. 1B). Concordantly, the shoulder at 483 nm in the excitation peak observed for the wild-type protein (Fig. 1A) and in E5 (1) was diminished in fully matured E5up protein (Fig. 1B) (1, 3). In addition, E. coli expressing E5up showed increased brightness of red fluorescent colonies in comparison with those expressing wild-type DsRed (Fig. 2B). Position 105 mutated in E5up corresponds to position 106, occupied by tyrosine in GFP. Molecular modeling shows that, unlike GFP, DsRed has bulky amino acids surrounding position Val105 (3). Presumably, the substitution of a bulky aliphatic valine for a more compact alanine resulted in relaxation of the overall structure, which facilitates complete maturation of the red fluorophore (Fig. 3).

**E57, First Fast-maturing Mutant of DsRed**—To find faster maturing mutants, we used the E5 mutant as a template for a second round of random mutagenesis. We isolated a mutant named E57, which developed bright red fluorescence in bacteria much faster than wild-type and E5 did. As early as after overnight incubation colonies of E. coli transformants expressing E57 developed an intense pink hue when observed at normal daylight. In contrast, colonies expressing wild-type DsRed (Fig. 1A) and no detectable green fluorescence at any time (Fig. 1D). Remarkably, during screening we identified several mutants exhibiting intense green fluorescence. One of them, AG4 (Table I), showed only bright green fluorescence and had no detectable red fluorescence at any time (Fig. 1D). Substitution V71M in AG4, which completely abrogates fluorescence and had no detectable red fluorescence at any time (Fig. 1D). Substitution V71M in AG4, which completely abrogates fluorescence and had no detectable red fluorescence at any time (Fig. 1D). Substitution V71M in AG4, which completely abrogates fluorescence and had no detectable red fluorescence at any time (Fig. 1D). Substitution V71M in AG4, which completely abrogates fluorescence and had no detectable red fluorescence at any time (Fig. 1D).

**Summary of DsRed mutants**

| Clone  | Mutations        | Red t<sub>50</sub><sup>a</sup> h | Residual green fluorescence<sup>b</sup> | Description              |
|--------|------------------|----------------------------------|---------------------------------------|--------------------------|
| DsRed  | Wild-type        | 9–10                             | 5% after 24 h                         | Slow maturation          |
| E5up   | V105A, S197T     | 9–10                             | 14% after 43 h, 10% after few days    | Fluorescent Timer        |
| E57    | V105A            | 9–10                             | 3% after 24 h, 0% after several days  | No residual green fluorescence |
| AG4    | V71M, V105A, S197T | 3–4                         | 5% after 8 h, 4% after 24 h           | Fast maturation          |
|        |                  |                                  | 100% green at all times               | Green only, no detectable red fluorescence |

<sup>a</sup> As measured *in vitro*; green fluorescence λ<sub>em</sub> = 500 nm, red fluorescence λ<sub>em</sub> = 580 nm.

<sup>b</sup> Generation and properties of the Fluorescence Timer protein were described.

**Table I**

Fluorescence data measured for purified proteins following the course of *in vitro* maturation at 37 °C.

**Fig. 1.** Fluorescence excitation and emission spectra of matured (~2 weeks at 25 °C) DsRed (A), E5up (B), E57 (C), and AG4 (D). Note the absence of a 500 nm emission peak in E5up after prolonged maturation and a diminished peak at 483 nm in the excitation spectrum (marked by arrows).
ior in cells (i.e. nonspecific accumulation, precipitation, and interaction with cellular components). Thus, any observed difference in behavior of two proteins, one tagged by red-emitting variant of DsRed and another by AG4, should be unequivocally attributed to the proteins themselves and not to the difference of tags. Similar to DsRed AG4 is an obligate tetramer. Thus, one should be careful to perform the control experiment with AG4 in a different cell, to avoid a potential problem of producing mixed tetramers of DsRed and AG4.

Analysis of “Accelerator” Mutations in the Context of the DsRed Three-dimensional Protein Structure—The three-dimensional structure of wild-type DsRed (10, 11) shows that isoleucine 161 faces the fluorophore and contacts its phenolic moiety (e.g. Cβ-Cα distance is about 3.9 Å) (Figs. 3 and 4). After modeling and analysis of the mutants we predict that the volume of space occupied by the side chain in position 161 may affect the efficiency of the green-to-red maturation. In the course of this reaction, additional atoms of the central helix are involved into the rigid and flat moiety of the fluorophore. The comparative analysis of the DsRed and GFP structures shows that movement of the rigid fluorophore moiety relative to the helix is necessary for the maturation of the DsRed fluorophore (Fig. 3). Therefore, close packing of the residues that surround the fluorophore can hamper the reaction. The observed effect of I161T substitution from a bulky residue to a small residue agrees with this hypothesis. Interestingly, the corresponding position in GFP is occupied by an even bulkier side chain, that of phenylalanine (position 165 in GFP corresponds to position 161 in DsRed). Structural analysis of position 197 also supports our hypothesis that the DsRed fluorophore matures more rapidly, when its immediate environment within the folded protein is kept open and unencumbered by bulky side chains. Indeed, serine in position 197 of the wild-type DsRed protein is in direct contact with the phenolic moiety of the fluorophore (Fig. 4). Furthermore, our comparative analysis of the structures of GFP and DsRed shows that the fluorophore of DsRed is closer to the residue in position 197 (Fig. 3). It is reasonable to suggest that the kinetic barrier for the maturation reaction decreases in proportion to the volume of empty space near residue 197. Indeed, the E57 mutant, which has the fastest known maturation rate, has the large residue serine replaced by the smaller residue alanine in position 197. Another confirmation comes from the recent report where a serine to tyrosine substitution in position 197 of DsRed was described (12). In accordance with our theory, replacement of serine with the bigger tyrosine residue did not increase the rate of maturation; it affected only the overall folding efficiency (12).

FIG. 2. A, in vitro maturation kinetics: fluorescence emission of freshly purified proteins DsRed, E57, and E5up were recorded during maturation of the proteins at 37 °C. B, comparison of fluorescence between E. coli colonies expressing wt DsRed, E57, and E5up. C, wt DsRed and E57 mRNA were microinjected into Xenopus embryos at the two blastomere stages. Images were taken 15 h after microinjection at the early neurula stage.

FIG. 3. Stereo view of superposition of GFP (blue) (17) and DsRed (purple) (11) three-dimensional structures in the area of the fluorophores. The structures were superimposed based on Cα atoms of the central α-helices (positions 59–72) and residues Ser146 (red) versus His148 (blue), Glu195 versus Val198, Ile163 versus Phe165, Lys163 versus Ile167, Leu199 versus Ser205, and Ser197 versus Thr203, which surround the fluorophores. The fluorophore and residues, which are substituted in the DsRed mutants, are labeled and shown in ball-and-stick representations.

FIG. 4. Ribbon scheme of the three-dimensional structure of DsRed protein (11). Residues Ile163 and Ser197 are shown in yellow; the fluorophore moieties are in space-filling representation. The fluorophore and central α-helix are in purple.
DISCUSSION

Slow maturation of wild-type DsRed, residual green fluorescence, and lower brightness compared with GFP were pointed out as the major limitations for its use as a fluorescent reporter (13, 14). However, despite the efforts of several investigators (12, 14, 15) no mutants of DsRed protein with accelerated fluorescence development were reported. Here we describe three mutants of DsRed: E57 with improved maturation kinetics, E5up with eliminated residual green fluorescence, and AG4 with only green fluorescence but no detectable red fluorescence.

Serendipitous selection of the E5 variant (1) for the second round of mutagenesis was a key for the generation of improved red fluorescent proteins. Indeed, the rate of random mutagenesis used in this study (about 1 mutation per 1 kb) makes it unlikely, in the case of E57, to generate all three mutations simultaneously.

Precise determination of the relative brightness and quantum yield of wild-type DsRed was somewhat confusing with large discrepancies reported by various investigators (3, 4, 16). The generation of these data depend upon many different factors: source of the protein, maturation conditions, protein purity, reference standard for relative quantum yield calculations, protein concentration, and buffer system.3 It remains to be elucidated whether the increased fluorescence signal intensity obtained by expressing E5up and E57 in cells or whole organisms is due to a greater quantity of fluorescent protein, an increased brightness of the chromophore, or both. We believe that the direct in vivo comparison of protein performance in different model systems on the cellular and whole organism levels as shown in this work is more meaningful for practical applications than in vitro generated spectrofluorometric data. Eventually, the possibility of earlier detection of gene expression, increased fluorescence signal, and higher signal-to-noise ratio should be the most objective criteria for the usefulness of the new mutants as reporters for various biological applications.

Finally, based on the analysis of the three-dimensional structure of DsRed, we formulated the hypothesis where the space availability around the fluorophore is crucial for fast and complete maturation.

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Note Added in Proof—Recently another fast maturing mutant of the DsRed protein was described (18).

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