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Key Amino Acid Residues Responsible for the Color of Green and Yellow Fluorescent Proteins from the Coral Polyp *Zoanthus* sp.

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Abstract—Site-directed mutagenesis was used to study the structural basis of color diversity of fluorescent proteins by the example of two closely related proteins from one organism (coral polyp *Zoanthus* sp.), one of which produces green and the other, yellow fluorescence. As a result, the following conversions of emission colors were performed: from yellow to green, from yellow to a dual color (yellow and green), and from green to yellow. The saltatory character of the spectral transitions and the manifestation of the dual-color fluorescence suggest that chemically different fluorophores are responsible for the green and yellow fluorescence. The simultaneous presence of three residues, Gly63, Lys65, and Asp68, is necessary for the efficient formation of the yellow rather than green fluorophore.

Key words: *green fluorescent protein, GFP, zFP506, zFP538, mutagenesis*

INTRODUCTION

The cloning of the gene for the green fluorescent protein from the jellyfish *Aequorea victoria* opened up wide possibilities of using this protein in biotechnology as a vital marker for gene expression [1-7].[2] The main special feature of this protein, which enables its use in various heterologous expression systems (from bacteria to mammals), is the ability to catalyze by itself the formation of the fluorophore group inside the protein globule without any exogenous organic cofactors [8-11]. The chromophore is formed by the cyclization of the protein backbone in fragment 65-67 (Ser-Tyr-Gly) followed by the dehydrogenation of the methylene bridge of the Tyr66 side chain. A structure with a system of conjugated double bonds results, which is capable of absorbing and emitting light in the visible spectral region (fluorescence maximum at 508 nm). It was shown by X-ray diffraction analysis that the polypeptide chain of GFP is packed in the form of so-called (β-barrel that is formed of 11 (β sheets wrapped around a single central α helix [10, 11]. The chromophore group is a part of this α helix; it is situated in the central part of the globule. Thus, the chromophore is entirely isolated from the surrounding solution and keeps contacts only with those amino acid residues whose side chains are buried inside the (β-barrel.

Numerous attempts to change the color of the GFP fluorescence by mutagenesis revealed only two amino-acid positions that can substantially affect the emission spectrum [12-14]. First, the substitution of a residue of another aromatic amino acid (Trp, Phe, or His) for Tyr66, which is included into the fluorophore structure, leads to a shift of fluorescence toward the blue region (up to 442 nm) [15]. Second, the substitution of Tyr or His for Ser203 causes a red shift of emission up to 529 nm [10].

Recently, we have cloned in our laboratory cDNAs that encode GFP-like proteins responsible for the fluorescence color of corals (class Anthozoa) [16, 17]. These proteins substantially differ in fluorescence color and can be subdivided into four groups, according to the position of the emission maximum in the blue-green (~485 nm), green (~505 nm), yellow (~540 nm), and red (>580 nm) spectral regions. It is obvious that the understanding of the relationship between the structure of a protein and the color of its fluorescence is of great scientific and applied significance.

In this study, we used site-directed mutagenesis to reveal the role of amino acid residues responsible for the color of the green and yellow fluorescent proteins from the coral polyp *Zoanthus* sp. (class Anthozoa).

RESULTS AND DISCUSSION

Previously [16], we cloned cDNAs of two highly homologous fluorescent proteins that are synthesized in one organism, the coral polyp *Zoanthus* sp. These pro-

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[2] Abbreviations: GFP, green fluorescent protein.

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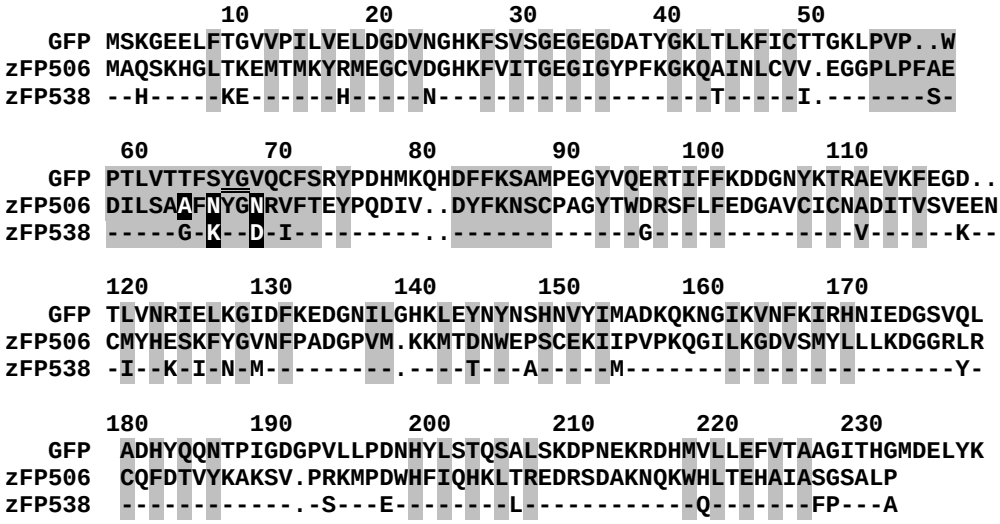

Fig. 1. A comparison of amino-acid sequences of fluorescent proteins GFP, zFP506, and zFP538. The amino-acid residues in the sequence of zFP538 that coincide with the corresponding residues of zFP506 are designated by hyphens. Gaps introduced to align sequences are designated by dots. The residue numbering corresponds to the sequence of GFP. The amino-acid residues, the side chains of which are buried inside the protein globule [10, 11], are shown on a gray background. The residues Tyr66 and Gly67, which form the chromophore of GFP, are underlined. Residues mutated in this study are shown by white against a black background.

teins clearly differ in their spectra. One of them, zFP506, fluoresced in the green region of the spectrum (506 nm), and the other, zFP538, in the yellow region (538 nm). A high similarity in their amino acid sequences (87% identity) makes the proteins a convenient model for the identification of the amino-acid residues responsible for the fluorescence color.

The amino-acid sequences of GFP, zFP506, and zFP538 are compared in Fig. 1. If the spatial structure of fluorescent proteins from corals is assumed to be similar to the GFP β-barrel (recently, the red fluorescent protein drFP583 was reported to have a structure similar to β-barrel [18, 19]), then, of 31 amino-acid residues that distinguish zFP506 from zFP538, only eight residues are buried in the globule. Four of the eight residues lie in the immediate vicinity of amino acid residues in positions 66 and 67 that form the chromophore. We presumed that three of them (residues 63, 65, and 68) might determine the difference in the fluorescence colors of zFP506 and zFP538.

First, we examined how the substitution of Lys65 of the yellow protein zFP538 affects its fluorescence spectrum (this experiment was part of a large project on the mutagenesis of several fluorescent proteins in position 65). Using a degenerated primer for the PCR, we tested seven amino acid substitutions in this position (Leu, Ile, Met, His, Gln, Asn, and Lys). Approximately 100 clones were visually examined for the presence and color of fluorescence using a fluorescent microscope. We found that about 50 clones exhibited a green fluorescence of different intensities, and only six clones exhibited a yellow fluorescence. All of the six clones were found to have Lys in position 65. Of green clones, we characterized in detail the brightest variant, which contained the K65M substitution. This mutant was almost indistinguishable from zFP506 in both the form and position of maxima in the excitation and emission spectra and the fluorescence brightness (Fig. 2a).

Since the special feature of Lys is the presence of a positive charge in the side chain, we examined whether another positively charged amino acid residue, Arg, could be substituted for Lys65 with the retention of yellow fluorescence. A zFP538-K65R mutant was obtained, which did not mature to the stage of fluorescent protein when being expressed in *E. coli.*

Thus, Lys65 in zFP538 turned out to be the key amino acid residue necessary for the manifestation of yellow rather than green fluorescence. This result was surprising since Lys in this position is not unique to zFP538: another known fluorescent protein, amFP486, with the emission maximum in the blue-green (486 nm) region, has Lys65 [16]. Consequently, one can conclude that only the combined effect of Lys65 and some other residues leads to the characteristic shift of the fluorescence spectrum toward the long-wavelength region.

Then, we focused our attention on Asp68, which is unique to zFP538 (other known fluorescent proteins carry Asn, Ser, or Val in position 68 [16]). The D68N substitution led to a two-peak emission band with maxima in both the green (506 nm) and yellow (537 nm) spectral regions (Fig. 2b). Therefore, Asp68 in zFP538

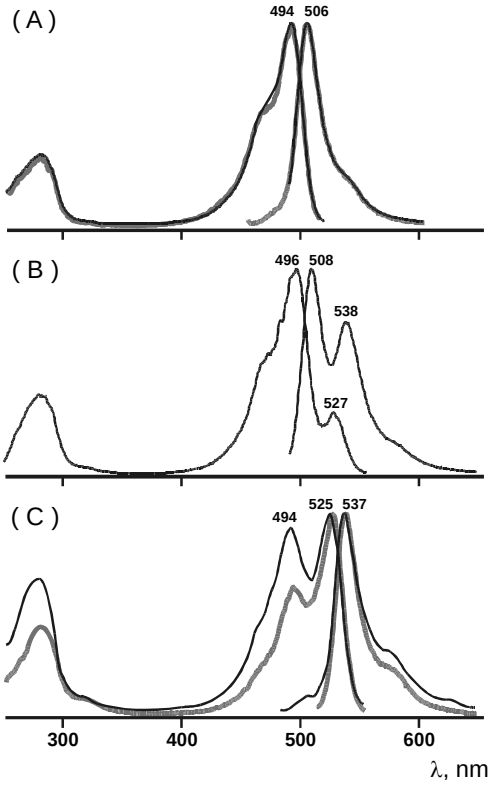

Fig. 2. Excitation and emission fluorescence spectra of proteins zFP506, zFP538 and their mutants with the changed fluorescence color: (a) green mutant zFP538-K65M (black lines) compared with the green protein zFP506 (thick gray lines); (b) green-yellow mutant zFP538-D68N; (c) yellow mutant zFP506-A63G/N65K/N68D (black lines) as compared with the yellow protein zFP538 (thick gray lines). In each pair of spectra, the shortest-wavelength spectrum is the excitation spectrum, and the longest-wavelength spectrum is the emission spectrum.

is important but not absolutely necessary for the manifestation of yellow fluorescence.

And, finally, we tried to transform the green fluorescent protein zFP506 to a yellow protein. However, the presence of Lys65 and Asp68 was insufficient for this: the zFP506-N65K/N68D mutant fluoresced in the green spectral region and differed from the wild-type green protein only by a weaker brightness (data not shown). Only the triple mutant zFP506-A63G/N65K/N68D acquired a yellow fluorescence with the emission maximum at 537 nm (Fig. 2c). The excitation spectrum of this mutant somewhat differed from that of the yellow protein zFP538: the minor peak at 494 nm became more pronounced.

Thus, we were able to change the fluorescence color for the zFP506 and zFP538 proteins from yellow to green, from yellow to a dual color (yellow and green), and from green to yellow. Note that these spectral transitions are saltatory and no fluorescence peaks appear in the intermediate regions of the spectrum.

The zFP538-D68N mutant that exhibits a two-peak emission spectrum is of particular interest. Previously, similar mutants with fluorescence maxima in the green and red spectral regions were described for two red fluorescent proteins, asFP595 and drFP583 [20-24]. Structural studies showed that chemically different chromophore groups, which also differ from the chromophore of GFP, are responsible for the red fluorescence of these proteins [25, 26]. The two-peak fluorescence spectrum of the asFP595 and drFP583 mutants was explained by the fact that some mutations hinder the formation of a native red chromophore, and a portion of the protein molecules form a GFP-like green chromophore. Our results suggest that chemically different fluorophores are responsible for the green and yellow fluorescence of zFP506 and zFP538. We may hypothesize that the positive charge of Lys65 and the negative charge of Asp68 are involved in catalysis during the formation of the yellow chromophore, whereas Gly63 provides the necessary mobility to the protein chain.

EXPERIMENTAL

The following preparations and reagents were used: Tris, sodium chloride, EDTA, and nucleotide triphosphates from Sigma (United States); restriction endonucleases from New England Biolabs (United States); and an Advantage KlenTaq mixture of polymerases for PCR from Clontech (United States).

Site-directed mutagenesis was carried out by the PCR using primers providing the site-directed substitution by the method described in [27]. Mutant DNA sequences were cloned into the expression vector pQE30 (Qiagen) at the *Bam* HI and *Sail* restriction sites. The introduction of site-directed nucleotide substitutions and the absence of random substitutions were checked by sequencing on a CEQ 2000 DNA Analysis System (Beckman, United States). The recombinant proteins carried the sequence MRHHHHHHGS instead of the first residue Met, which makes possible a rapid purification of these proteins by metal affinity chromatography.

For producing large amounts of fluorescent proteins, *E. coli* strains carrying the recombinant plasmids were grown at 37°C in a volume of 50 ml to an optical density of about 0.6. Then, the expression was induced by the addition of isopropyl β-D-thiogalactopyranoside to a concentration of 0.2 mM. The culture was incubated overnight at 37°C. On the next day, cells were harvested by centrifugation, resuspended in a buffer containing 100 mM NaCl and 20 mM Tris-HCl, pH 8.0, and destroyed by sonication. After centrifugation of the sample, fluorescent proteins were isolated from the soluble fraction using a TALON metal affinity resin (Clontech) according to manufacturer's recommendations.

The elution from the sorbent was performed by a 20 mM Tris-HCl, pH 8.0 buffer containing 100 mM NaCl and 100 mM EDTA. The degree of protein purification was no less than 90%, as indicated by gel electrophoresis.

Spectra were measured on an LS50B spectrofluorimeter (Perkin-Elmer). Prior to measurements, protein samples were diluted with 20 mM Tris-HCl, pH 8.0, buffer containing 100 mM NaCl to a concentration of about 50 μg/ml.

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