Crystal Structure of Venus, a Yellow Fluorescent Protein with Improved Maturation and Reduced Environmental Sensitivity*

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Yellow emission variants of green fluorescent protein (GFP) have been found useful in a variety of applications in biological systems due to their red-shifted emission spectrum and sensitivity to environmental parameters, such as pH and ionic strength. However, slow maturation properties and new requirements for more intense fluorescence necessitated further mutagenesis studies of these proteins. Venus, a new variant with improved maturation and brightness, as well as reduced environmental dependence, was recently developed by introducing five mutations into the well characterized variant, enhanced yellow fluorescent protein (EYFP). In this paper, we present the crystal structure of Venus at 2.2 Å resolution, which enabled us to correlate its novel features with these mutation points. The rearrangement of several side chains near the chromophore, initiated by the F46L mutation, was found to improve maturation at 37 °C by removing steric and energetic constraints, which may hinder folding of the polypeptide chain, and by accelerating the oxidation of the Cα-Cβ bond of Tyr66 during chromophore formation. M153T, V163A, and S175G were also found to improve the rate of maturation by creating regions of greater flexibility. F64L induced large conformational changes in the molecule, leading to the removal of halide sensitivity by preventing ion access to the binding site.

Green fluorescent protein (GFP),¹ originally isolated from the jellyfish Aequorea victoria, has been the subject of continued interest since its gene was first cloned in 1992 (1). The high stability of mature GFP over various environment conditions, the spontaneous autocatalytical generation of the fluorophore of GFP, and the possibility of spectral manipulation by mutagenesis, make GFP and its related proteins attractive tools for numerous biological applications (2).

Maturation of GFP proceeds in three major steps, beginning as the 238-residue single GFP polypeptide folds into its nearly native conformation. Residues 65–67 of the folded protein then undergo several chemical reactions necessary for chromophore formation, including cyclization and dehydration (3). The final rate-limiting step in the maturation process involves the oxidation of the Cα-Cβ bond of Tyr66 by aerial oxygen. This maturation process takes ~3 h at room temperature and its efficiency further decreases at 37 °C, hindering the use of GFP in some biological applications. To improve maturation and performance at 37 °C, mutations such as F99S, M153T, and V163A have been introduced, creating valuable GFP variants for a wider range of applications (4, 5).

Thorough understanding of the relationship between the protein sequence and its physico-chemical properties requires three-dimensional structure determination of GFP at atomic resolution. Crystal structures of both the monomeric and dimeric forms of GFP have been solved previously (6, 7). The wild-type GFP (wtGFP) chromophore, which exists in equilibrium between two ground states, produces two maxima in the light absorption spectrum. The near-UV absorption peak at 395 nm originates from the neutral chromophore, while the ionic chromophore absorbs at a longer wavelength and produces a smaller peak at 475 nm (3). Green light is emitted from wtGFP at 503 nm. Spectral properties of GFP were shown to be influenced by its dimerization state (8), which is characterized by a dissociation constant of ~100 μM (9).

In the case of YFP, a yellow variant of GFP, the main absorption maximum of 514 nm corresponds to the chromophore ionic form and is red-shifted relative to wtGFP. Unlike wtGFP, the neutral form does not emit fluorescence efficiently, and the higher energy peak is largely suppressed (10). The equilibrium between the neutral and ionic forms of the YFP chromophore can be shifted in favor of the latter form by the following two mutations: S65G, which prevents hydrogen bond formation between residues Gly65 and Glu222, and T203Y, which produces a smaller peak at 475 nm (3). Green light is emitted from YFP at 503 nm. Spectral properties of GFP were shown to be influenced by its dimerization state (8), which is characterized by a dissociation constant of ~100 μM (9).

When compared with the green emission variants of GFP, the absorbance level of YFP and its derivatives display a strong environmental sensitivity. The ratio between the two absorbance peaks, and therefore fluorescence efficiency, are dependent upon pH and the halide ion concentration (10, 11). This sensitivity makes YFPs efficient pH and halide sensors (12, 13), but for applications requiring stable fluorescence in a wide range of environment conditions, it is necessary to have proteins that are insensitive to pH and halides. Griesbeck and colleagues (14) developed a less sensitive variant of yellow illumination.
fluorescent protein, citrine, which contained a Q69M mutation in addition to the four mutations used to create the original yellow variant EYFP (S65G/V68L/S72A/T203Y). This mutation helped decrease the pH and halide sensitivity and facilitated expression at 37 °C. With a relatively low value of $pK_a = 5.7$, citrine is particularly applicable in systems of lower pH and in a wide range of Cl$^-$ concentration.

More recently, Nagai et al. (15) introduced a new variant of yellow emission protein, called Venus, with reduced pH and halide dependence and an improved maturation rate. These properties were achieved in Venus by various mutations, which differ from those used in citrine (14). Instead of Q69M mutation used in citrine, Venus contains five other mutations (F46L, F64L, M153T, V163A, and S175G) in addition to the previously described four mutations characteristic to EYFP. The F46L mutation helps accelerate the oxidation step in chromophore formation, an effect which is specific to yellow emission proteins. The remaining four mutations are known to improve folding at 37 °C, and at least one was found to remove proton and chloride sensitivity (15) (Table I). The aim of our work was to solve the three-dimensional structure of Venus, shedding light on the structural basis of the observed molecular properties. We compared this crystal structure of Venus at 2.2 Å resolution with previously published structures of wtGFP (7) and EYFP (10) to pinpoint mutation-dependent structural changes. Our ultimate goal is to use systematic structural analysis of GFP mutants to provide future guidelines for rational design of improved variants.

**EXPERIMENTAL PROCEDURES**

**Protein Expression and Purification—**Venus was cloned into a pRSET, plasmid and expressed in the *Escherichia coli* strain BL21(DE3)pLysS. Cells were grown using standard culture medium (LB broth) in a shaker incubator at 37 °C until the optical density of 0.7 was reached. Upon induction with isopropyl-thio-β-D-galactopyranoside, the temperature was lowered to 25 °C, and protein was expressed for 7–8 h. Protein containing the N-terminal polyhistidine tag was purified using nickel affinity chromatography. Subsequently, ion exchange and gel filtration were employed to achieve satisfactory levels of purity. The N-terminal His$_6$ tag was cleaved using enterokinase.

**Structure Determination—**The protein solution was concentrated to 33 mg/ml in 20 mM Tris hydrochloride (pH 7.9), with 50 mM NaCl and 0.5 mM dithiothreitol. Yellow fluorescent rod-shaped crystals (P3 112), room temperature for 3 h. Protein containing the N-terminal polyhistidine tag was purified using nickel affinity chromatography. Subsequently, ion exchange and gel filtration were employed to achieve satisfactory levels of purity. The N-terminal His$_6$ tag was cleaved using enterokinase.

**RESULTS AND DISCUSSION**

**Chromophore Environment and Spectral Properties**

Venus was crystallized as a dimer with one molecule per asymmetric unit. The refined structure of Venus shows an 11-strand (labeled A to K from the N terminus) β-barrel, typical of GFP-derived fluorescent proteins. Calculated for α-carbons of residues 5–60 and 70–225, the root mean square deviation of a single Venus β-barrel relative to wtGFP and EYFP β-barrels is 0.32 and 0.35 Å, respectively. In all three structures compared, the cross-section taken perpendicularly to the long axis of the barrel is not perfectly circular but rather oval in shape. While the longer diameter of the oval cross-section of all three proteins is identical (23.8 Å; measured from distances of four C$_\alpha$ pairs), the shorter diameter differs between EYFP (22.3 Å) and Venus (21.9 Å). The difference in the oval shape between these yellow variants is closely related to the packing of interior amino acid residues and influences the integrity of dimer interface, as discussed below.

The residues surrounding the Venus chromophore are similar to those surrounding the chromophore of EYFP (Fig. 1A). More specifically, there are no significant changes between Venus and EYFP in the distances between the chromophore and residues Thr$^{62}$, Leu$^{68}$, Gln$^{69}$, Gln$^{94}$, Arg$^{96}$, Asn$^{146}$, Arg$^{168}$, Gln$^{163}$, Tyr$^{203}$, and Ser$^{205}$. Electron density studies of Venus show an 11° angle between the planes of the chromophore and Tyr$^{203}$, while the same angle measured in EYFP is 11.5–12.3°, making the plane of the Venus chromophore slightly more parallel than that of EYFP. This small difference may account for a minor change in the absorption spectrum of Venus relative to EYFP, as seen at a pH range of 4.6–8.6 and a 50 mM NaCl concentration. Although the position of the main absorption maximum remains unchanged (516 nm) in both Venus and EYFP, a smaller peak resulting from the neutral (protonated) chromophore is shifted in Venus by 20 nm producing a peak at 413 nm versus 393 nm in EYFP. The slightly more parallel orientation of the aromatic rings of Tyr$^{68}$ and Tyr$^{203}$ might also help improve the π–π interaction, thus reducing the excited state energy of the neutral chromophore. If this is the case, we can hypothesize that at lower pH, which is favorable to chromophore protonation, the angle between the rings would be smaller than 11°.
One of the changes observed around the Venus chromophore is a 0.3-Å increase in the distance between the Cα-Cβ bond and the OE1 atom of the Glu222 side chain, compared with EYFP. A result of this increased distance is a 0.5-Å shift of the chromophore away from the protein surface and the Glu 222 side chain. Following this shift, an increased rate of oxidation is observed, attributed to enhanced oxygen access to the oxidation site in Venus. Other factors facilitating this reaction will be discussed under “Venus-specific Mutations.”

Another difference in the Venus chromophore area, when compared with that of EYFP, is the presence of a shorter hydrogen bond (by 0.2 Å) between the chromophore phenolate and His148. This may result from a slight rotation in the His148 imidazole, making it less parallel to the β-barrel surface. However, this effect may also be due to the higher pH at which the crystals were grown (pH 8.5 for Venus, pH 6.9 for EYFP). The previously observed pH effect on S65T suggests that at a lower pH this distance becomes larger and hydrogen bonds are broken, consistent with our observations of Venus as well as past studies of EYFP (20).

The mutual position of the two chromophores within the Venus dimer differs from that of wtGFP and EYFP. The chromophore-chromophore distance in the Venus dimer is shorter than that of others by approximately 2 Å when measured using the phenol oxygens (Fig. 3 A). These differences may be attributed mainly to the different orientation of subunits in the dimeric structure, coupled with the overall closer intersubunit contact in Venus than in EYFP (Fig. 2 and Table III).

**Dimer Structure and Halide Sensitivity**

The subunit orientations in the Venus dimer (Fig. 1, B and C) are similar to those in the wtGFP dimer, but differ from those of EYFP. When the backbone chain of one Venus subunit is superimposed on that of wtGFP, the second subunits display a similar orientation with respect to the superimposed subunits. In contrast, following superimposition of the first subunit of Venus on that of EYFP, the second subunits display a significantly different orientation (Fig. 2A). The root mean square deviation for the aforementioned second subunits is 1.15 Å between Venus and wtGFP and 4.97 Å between Venus and EYFP. By using the angular parameters described in Table III, we can quantitatively define the unique orientation of the two subunits within the dimeric fluorescent proteins. The “tilt” angle between two subunits of Venus is similar to that of wtGFP, but differs from that of EYFP (Table III). These differences and similarities in domain orientations, depicted in Fig. 2A, clearly show the differences in the position of one subunit when the other subunit is superimposed.

Comparison of the buried surface areas for wtGFP, EYFP, and Venus indicates that the dimerization interface differs significantly between the three proteins (Table III). While the residues involved in the dimer interactions are essentially identical between Venus and wtGFP, significant differences are observed between Venus and EYFP. In both Venus and wtGFP, Glu142, Tyr146, Asn149, Arg168, Asn170, Glu172, Tyr200, and Ser202 lie within the buried surface between the two subunits, whereas the same EYFP residues are solvent-accessible (Fig. 2B). These differences leave Venus with 5% more buried surface area relative to EYFP. In addition, the average intersubunit distance between atoms located within
the buried surface of the Venus dimer is somewhat shorter than that of EYFP (Table III). These concurring results indicate that Venus has a larger dimerization interface than EYFP.

Wachter et al. (10, 18) have shown that the fluorescence intensity of YFP is inversely related to the concentration of halide ions in solution. This group identified a halide-binding site proximal to the EYFP-H148Q chromophore, which suppresses chromophore fluorescence upon ion binding. The halide-binding cavity is adjacent to the backbone carbonyl groups of Tyr145, Asn149, and Arg168 (Fig. 4B). Two mutations (T203Y and V68L) located at the buried halide ion-binding cavity of EYFP (Fig. 4B) have been implicated in halide sensitivity (18). The halide ion sensitivity of YFPs has led to their use as halide ion sensors (7). The β-bulge residues Tyr145, Asn149, and Arg168 participate in dimer contacts in both Venus and wtGFP, while the same residues are fully solvent exposed in EYFP (Fig. 2B). These results suggest that the intersubunit interface could influence the chromophore environment, linking the dimerization mechanism to the fluorescence property of GFP and its variants (8).

### Venus-specific Mutations

As stated previously, Venus contains five additional mutation sites relative to EYFP (Table I). Comparison of the corresponding crystal structures helped explain the effects of these mutations on Venus properties. M153T, V163A, and S175G were found to improve the rate of maturation, while mutations F46L and F64L induced a number of local and global structural changes. Here we describe the individual effect of each mutation site relative to EYFP (Table I). Comparison of the corresponding crystal structures helped explain the effects of these mutations on Venus properties. M153T, V163A, and S175G were found to improve the rate of maturation, while mutations F46L and F64L induced a number of local and global structural changes. Here we describe the individual effect of each mutation site relative to EYFP (Table I). Comparison of the corresponding crystal structures helped explain the effects of these mutations on Venus properties. M153T, V163A, and S175G were found to improve the rate of maturation, while mutations F46L and F64L induced a number of local and global structural changes. Here we describe the individual effect of each mutation site relative to EYFP (Table I). Comparison of the corresponding crystal structures helped explain the effects of these mutations on Venus properties. M153T, V163A, and S175G were found to improve the rate of maturation, while mutations F46L and F64L induced a number of local and global structural changes. Here we describe the individual effect of each mutation site relative to EYFP (Table I). Comparison of the corresponding crystal structures helped explain the effects of these mutations on Venus properties. M153T, V163A, and S175G were found to improve the rate of maturation, while mutations F46L and F64L induced a number of local and global structural changes. Here we describe the individual effect of each mutation site relative to EYFP (Table I). Comparison of the corresponding crystal structures helped explain the effects of these mutations on Venus properties. M153T, V163A, and S175G were found to improve the rate of maturation, while mutations F46L and F64L induced a number of local and global structural changes. Here we describe the individual effect of each mutation site relative to EYFP (Table I). Comparison of the corresponding crystal structures helped explain the effects of these mutations on Venus properties. M153T, V163A, and S175G were found to improve the rate of maturation, while mutations F46L and F64L induced a number of local and global structural changes. Here we describe the individual effect of each mutation site relative to EYFP (Table I). Comparison of the corresponding crystal structures helped explain the effects of these mutations on Venus properties. M153T, V163A, and S175G were found to improve the rate of maturation, while mutations F46L and F64L induced a number of local and global structural changes. Here we describe the individual effect of each mutation site relative to EYFP (Table I).
was found to improve maturation at 37 °C (15) by removing steric and energetic constraints, which may hinder folding of the polypeptide chain (Table I). Furthermore, the Venus F46L substitution accelerates the oxidation of the Co-Cβ bond of Tyr56 during chromophore formation (15). Interestingly, major functional changes caused by the F46L mutation resemble those seen in GFP-S65T (6, 21) and DsRed N42H/Q (22), which show an increased rate of oxidation relative to their “precursor” proteins, wtGFP and wild-type DsRed, respectively. In GFP-S65T, the introduction of the threonine side chain at chromophore position 65 repositions the Leu44 and Leu42 side chain. In DsRed-N42H/Q, the more bulky side chain of His42 or Gln42 shifts toward a position similar to Leu42 in Venus, leading to a repositioning of Gln66, an equivalent to residue 65 in Venus and EYFP. In brief, the introduction of F46L causes a series of changes at residues 42, 44, and 65, facilitating an increased rate of maturation. In addition, a change concerning residue 65 (66 in DsRed) in the Venus chromophore may lead to an increased rate of oxidation.

F64L—In EYFP, the V68L substitution reorients both the central helix and the chromophore toward the protein surface (8). In Venus, however, the substitution of phenylalanine with leucine at position 64 counteracts any change in orientation caused by V68L, and the central helix and the chromophore remain roughly in their wild-type positions. Therefore, when compared with EYFP, the Venus chromophore is shifted ~0.5 Å toward the center of the molecule (Figs. 3B and 4A). A similar dislocation of neighboring residues Tyr145, Tyr203, Leu220, and Ghu222 accompanies this slight movement of the central helix. Such structural changes also influence the position of β-barrel strands G, J, and K as well as part of strand H. The shifted β-strands contain residues involved in the dimer interface, such as N144, S147, and A206 (Fig. 4A). Relocation of these residues causes a small decrease in the average diameter of the β-barrel and geometric “flattening” of the β-barrel itself, increasing the surface area buried between the two monomers relative to EYFP. The β-strand movement occurs in close parallel to the movement of the chromophore and the central helix, and hence the distances between the residues surrounding the chromophore remain essentially unchanged in Venus relative to EYFP. Possible hydrogen bonds in EYFP are shown as dashed lines.

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on its location near to the halide-binding site, the V163A mutation may also be involved in eliminating chloride sensitivity in Venus (18) (Fig. 4B).

**S175G**—The S175G mutation is located in a short turn between strands H and I. The removal of the hydroxyl group of serine drastically alters the backbone conformation of the region spanning residues 173–175. Such a change involves breaking the three-centered hydrogen bond found in EYFP between the side chain carboxyl oxygen atom of Asp173 and the amide nitrogens of Gly174 and Ser175. This is caused by −180° rotation of the carboxyl side chain of Asp173, which flips away from the backbone carbonyl of Gly174, resulting in its complete exposure to the solvent (Fig. 4C). The S175G mutation in cycle-3 GFP (5) and Venus (15) has been shown to facilitate the protein folding process in vitro. S175G also enhances the fluorescence intensity of ECFP 2-fold in an in vitro experiment. While the exact structural basis of this increased folding rate is unclear, it should be noted that a localized conformational change outside of the β-barrel (Fig. 4C) significantly impacts the properties of the chromophore within the β-barrel, including maturation.

**Concluding Remarks**—The five mutations that distinguish Venus from EYFP increase the rate of maturation and abolish the sensitivity to halide ions (15). The 2.2-Å x-ray structure of Venus reveals several structural differences when compared with EYFP and wtGFP, thus helping to elucidate the structure-function relationship of this fluorescent protein. Among the five mutations, those known as “folding mutations” (4, 15) seem to facilitate the maturation process by introducing highly flexible, smaller side chains within the loop regions located outside the β-barrel (M153T, V163A, S175G) or near the chromophore (F64L). The F46L mutation promotes a local structural change near the chromophore, which consequently accelerates oxidation, a rate-limiting step in chromophore maturation. In addition, we found that the F64L mutation is responsible for a major structural change inside the β-barrel, leading to an alteration of the intersubunit orientation, ultimately preventing halide ion access to the binding cavity near the chromophore.

Two properties of Venus, however, remain to be explained, including the small red shift of the absorption peak attributed to a neutral chromophore and the increased resistance to low pH when compared with EYFP. This observed small red shift might be attributed to the fact that the aromatic rings of the Venus chromophore and Tyr203 have a more parallel orientation, minimizing the energy interaction. Alternatively, the pH resistance may result from the slightly smaller distance between His14 and the chromophore, thus stabilizing the associated hydrogen bond and promoting the ionic chromophore form. As both properties are pH-dependent, a comparison of the present structure at pH 8.4, with a Venus structure obtained at lower pH is needed to address these questions.

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