The 2.0-Å Crystal Structure of eqFP611, a Far Red Fluorescent Protein from the Sea Anemone *Entacmaea quadricolor*

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We have crystallized and subsequently determined to 2.0-Å resolution the crystal structure of eqFP611, a far red fluorescent protein from the sea anemone *Entacmaea quadricolor*. The structure of the protomer, which adopts a β-can topology, is similar to that of the related monomeric green fluorescent protein (GFP). The quaternary structure of eqFP611, a tetramer exhibiting 222 symmetry, is similar to that observed for the more closely related red fluorescent protein DsRed and the chromoprotein Rtsm5. The unique chromophore sequence (Met63-Tyr64-Gly65) of eqFP611, adopts a coplanar and trans conformation within the interior of the β-can fold. Accordingly, the eqFP611 chromophore adopts a significantly different conformation in comparison to the chromophore conformation observed in GFP, DsRed, and Rtsm5. The coplanar chromophore conformation and its immediate environment provide a structural basis for the far red, highly fluorescent nature of eqFP611. The eqFP611 structure extends our knowledge on the range of conformations a chromophore can adopt within closely related members of the green fluorescent protein family.

The green fluorescent protein (GFP)1 from *Aequorea victoria* has generated widespread interest as a biotechnological tool, acting as a visual reporter for events in living cells. With a view to increasing potential uses for GFP, considerable effort has been invested to evolve new variants of GFP (1). For example, GFP variants and GFP homologs have been reported that together cover almost the entire visible range of emission wavelengths (420–630 nm) (1, 2, 3) while other variants have been optimized or are suitable as biosensors for pH (4), redox potential (5), or Ca2+ ions (6).

In addition to using protein engineering approaches, GFP-like proteins have been isolated from alternative natural sources, exhibiting spectroscopic properties that could be of value as reporters. For example, the range of colors available has been extended with the discovery of naturally occurring red-emitting homologues such as DaRed isolated from the coral morphian *Discosoma sp* (2) and eqFP611 from the sea anemone *Entacmaea quadricolor* (8). Applications using DaRed as a fluorescent marker have been limited however, because of a number of undesirable properties including its oligomeric nature and slow chromophore maturation. Nevertheless, extensive random mutagenesis of DaRed has produced variants with much improved properties (9, 10).

Other fluorescent proteins have been isolated such as the *Kaede* protein with striking photoresponsive behavior (11). In addition, a range of weakly and non-fluorescent chromoproteins, including the pocilloporin Rtsm5 from the reef building coral *Montipora efflorescens* (12) and the GFP homologue asFP595 from *Anemonia sulcata* (13) have been described. Some of these proteins have important biological roles (14). For example, the vividly colored yet often poorly fluorescent pocilloporins possess multiple photoprotective functions, helping to protect the photosystems of their resident microalgae from high-amplitude light fluctuations that can lead to severe photoinhibition (15, 16).

Aside from biotechnology-based considerations, of fundamental interest is the structural basis for the similarities and differences of the spectral properties these fluorescent proteins and chromoproteins exhibit. One common feature is the presence of an extended, conjugated π-system comprising a cyclic tripeptide chromophore (Ser-Tyr-Gly in GFP, Glu-Tyr-Gly in DaRed and Rtsm5) buried within the distinctive β-can topology. Differences in spectral properties arise from the unique environment in which the chromophore resides together with modifications to the π-resonance system. For example, in comparison to GFP, the red-shifted spectral properties of DaRed have been attributed to chemical modifications of the GFP-like chromophore that extend the coplanar π-resonance system (17, 18). However, despite possessing the same chromophore sequence as DaRed, Rtsm5 adopts a markedly different conformation to that observed in DaRed, such that the phenoxy ring of the chromophore in is a trans and non-coplanar conformation (12).

The majority of fluorescent proteins with emission maxima significantly beyond 600 nm have been found to be weakly fluorescent. A notable exception is the highly fluorescent protein eqFP611 recently isolated from the sea anemone *E. quadri-
ricolor that when excited, emits light maximally at 611 nm (8).

In order to increase our understanding of the structural basis for protein fluorescence at the far red end of the color scale, we have determined the crystal structure of the highly fluorescent protein, eqFP611 to 2.0-Å resolution. The crystal structure of eqFP611 provides a detailed view of the chromophore and its environment, thereby providing a platform for the design of variants of eqFP611 with improved spectral and oligomeric properties, as well as clues for altering similar properties of other chromoproteins, such as Rm5.

EXPERIMENTAL PROCEDURES

Protein Expression and Purification—Single colonies of Escherichia coli (BL21-DE3) freshly transformed with a vector encoding eqFP611 with an N-terminal His tag (8) were inoculated into LB medium. Cells were incubated overnight with orbital shaking (200 rpm) at 28 °C. 2 ml of the overnight culture were transferred to 500 ml of 2YT medium and after 4 h incubation isopropyl-β-D-galactopyranoside was added to a final concentration of 0.16 mM. Incubation was continued overnight after which the cells were harvested. Recombinant protein was isolated and purified by Ni-nitrilotriacetic acid and gel filtration chromatography, after which the cells were harvested. Recombinant protein was isolated and purified by Ni-nitrilotriacetic acid and gel filtration chromatography at micromolar concentrations and under earlier report that eqFP611 behaves as a tetramer when subjected to eqFP611 (data not shown). Minor amounts of earlier eluting material indicated possible aggregation of the protein. These results confirm an earlier report that eqFP611 behaves as a tetramer when subjected to gel filtration chromatography at micromolar concentrations and under certain conditions of expression can form aggregates (8). Denaturing SDS-PAGE indicated a single polypeptide of M₆ 26,000. Column fractions corresponding to the tetramer were pooled and concentrated with a centrifugal ultrafiltration device (Millipore, MWCO 10,000) to 26 mg/ml.

Crystallization—Crystals of eqFP611 were obtained at 20 °C using the hanging drop vapor diffusion method. Purified eqFP611 protein at 26 mg/ml was mixed with an equal volume of a reservoir solution containing 11.5% polyethylene glycol 8000, 0.1 M sodium acetate pH 4.3 and 0.2 M calcium acetate. Crystals formed after 1–3 days grew to maximal size between 7 and 14 days. The absorption and fluorescence excitation/emission spectra were determined for crystals redissolved at pH 8.0. Spectra were similar for eqFP611 solutions at pH 8.0 (purification buffer) and published values (data not shown, Ref. 8).

Data Collection—The crystals were flash cooled during data collection using 15% PEG 400 as the cryoprotectant. A 2.0-Å data set was collected using a RU-3HBR x-ray source and an R-AXIS IV++ detector by inverse phi geometry. The data were merged and processed with the HKL software package (20). The crystals, with unit cell dimensions a = 78.8 Å, b = 78.8 Å, c = 328 Å belong to space group P6₃22. See Table I for a summary of statistics. Vₘ calculations suggested there were two protomers per asymmetric unit. Inspection of the native Patterson map suggested that the two protomers had a common orientation within the asymmetric unit.

Structure Determination—The crystal structure of eqFP611 was determined using the molecular replacement method with the program AmoRe in the CCP4 suite. A modified protomer of the tetrameric DsRed structure (Protein Data Bank code, 1GXE) was used as the search probe, with all sequence differences mutated to alanine and the chromophore deleted. A clear peak in the rotation function led to the elucidation of two translation function solutions, which packed well within the unit cell and defined a 222 tetramer upon applying the crystallographic symmetry operators.

The progress of refinement was monitored by the Rₚₚ value (% of the data) with neither a sigma, nor a low resolution cut off being applied to the data. The structure was refined using rigid-body fitting followed by the simulated-annealing protocol implemented in CNS (version 1.0) (21). Both rounds of model building using the program O (22). NCS restraints were used throughout refinement, although residues deviating from NCS were released from these restraints. Tighty restrained individual B-factor refinement was employed, and bulk solvent corrections were applied to the data set. Water molecules were included in the model if they were within hydrogen-bonding distance to chromophore. For the DsRed and Rm5 structures, the electron density around the chromophore structure, dihedral restraints that might force the 4-hydroxyphenyl group to be co-planar were removed.

During the refinement and model building of eqFP611, a peak was observed in the Fₚ - F₂ map above the plane of the chromophore, contacting the imidazolinone group, which was initially interpreted as a water molecule. However, further refinement indicated that a water molecule alone was insufficient to account for the observed electron density, whereas an acetate ion clearly was (an ingredient of the crystallization buffer).

The final model, which comprises two protomers (residues 2–226), 285 water molecules, 2 calcium ions, and two acetate ions, has an Rₚₚ of 21.4% and an Rₚₚ of 25.3% for all reflections between 20 and 2.0 Å. See Table I for summary of refinement statistics and model quality. The coordinates and structure factors have been deposited in the PDB database (code: 1UIS).

RESULTS

The crystal structure of eqFP611 has been determined to 2.0-Å resolution (Fig. 1, Fig. 2, Table I). Following superposition of the two protomers within the asymmetric unit, the r.m.s. deviation is 0.22 Å for all Cα atoms; accordingly, unless otherwise stated, structural analyses will be confined to one protomer. The eqFP611 tetramer, which displays 222 symmetry, is generated via crystallographic symmetry (Fig. 1). Unless explicitly stated structural comparisons will be restricted to Rm5 and DsRed, homologues that fluoresce in the red range.

The eqFP611 protomer has a very similar fold to that de-
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Table I

Data collection and refinement statistics

| Parameter                               | Value |
|-----------------------------------------|-------|
| Temperature                             | 100K  |
| Space Group                             | P622  |
| Cell dimensions (Å) (a,b,c)             | 77.9, 77.9, 327.5 |
| Resolution (Å)                          | 2.0   |
| Total no. of observations               | 134722|
| No. of unique observations              | 37625 |
| Multiplicity                            | 3.6   |
| Data completeness (%)                   | 91.2 (79.7) |
| No. data > 2σt                         | 76.9 (38.3) |
| Rmerge (%)                              | 21.0 (2.9) |
| Rfree (%)                               | 4.6 (23.0) |

Refinement statistics

| Parameter                               | Value |
|-----------------------------------------|-------|
| Non-hydrogen atoms                     |       |
| Protein                                 | 3548  |
| Chromophore                             | 46    |
| Calcium                                 | 2     |
| Acetate                                 | 8     |
| Water                                   | 285   |
| Resolution (Å)                          | 50.2  |
| Rfactor (%)                             | 21.4  |
| Rfree (%)                               | 25.3  |
| r.m.s. deviations from ideality         |       |
| Bond lengths (Å)                        | 0.007 |
| Bond angles (*)                        | 1.37  |
| Improperas (*)                          | 0.81  |
| Dihedrals (*)                           | 26.03 |
| Ramachandran plot                       |       |
| Most favored                            | 91.0  |
| And allowed region (%)                 | 9.0   |
| B-factors (Å²)                          |       |
| Average main chain                     | 37.0  |
| Average side chain                     | 39.5  |
| Average water molecule                  | 42.3  |
| Average chromophore                    | 31.6  |
| Calcium                                 | 41.9  |
| Acetate                                 | 39.0  |
| r.m.s. deviation bonded Bs             | 1.70  |

scribed for GFP, DsRed, and Rtms5, namely a 11 stranded β-barrel (β-can) in which a central helix, that is shielded from bulk solvent, runs co-axial with the axes of the β-barrel and represents the secondary structural element that covalently connects the chromophore to the protein.

Compared with other members of the GFP family whose structures are known, the overall structure of eqFP611 is most similar to that of DsRed (49.1% sequence identity, 214 equivalent Ca atoms having an r.m.s deviation of 0.69 Å) and Rtms5 (47.6% sequence identity, 211 equivalent Ca atoms having an r.m.s deviation of 0.69 Å) while showing less similarity to GFP (23.0% sequence identity, 204 equivalent Ca atoms having an r.m.s deviation of 1.24 Å) (Fig. 3). The largest differences reside within the N and C termini, and the loop regions, the most notable of which is a surface loop in eqFP611 (residues 181–188), which contains a three residue insert with respect to Rtm5 and DsRed (Fig. 3). This eqFP611 loop appears to be mobile as judged by high B-factors (50–70 Å²). Similar to DsRed and Rtm5, eqFP611 has a C-terminal tail that makes inter-subunit contacts at the AB interface. A number of sequence and structural differences were observed to cluster around the chromophore (positions 39, 41, 59, 60, 62, 67, 106, 108, 143, 158, 160, 197; Fig. 3), which have a significant impact on the conformation and spectral properties of the chromophore.

The eqFP611 chromophore structure can be likened to that of DsRed and Rtm5, as it possesses a 5-[(4-hydroxyphenyl)methylen]-imidazolinone group (Fig. 4). The Mes³⁺ Ca originally in the sp² hybrid configuration is observed to be planar and sp³ hybridized. This result is consistent with the formation of a double bond between Ca and N at position 63, namely acylimine formation. A similar observation was seen for the equivalent position (Glu⁶⁶) in DsRed and Rtm5 (12, 17, 18), but is absent in GFP. This additional double bond extends the π-bonding system of the eqFP611 chromophore and is consistent with the red-shifted emission of the matured protein.

It is clear from the electron density that the eqFP611 chromophore adopts a coplanar conformation (Figs. 2 and 4). The overall temperature factor for the chromophore is 31.6 Å², similar to that of the neighboring side chains, suggesting that there is limited mobility of the chromophore, which is consistent with the large number of interactions the chromophore participates in: namely 9 hydrogen bonds, 2 water-mediated hydrogen bonds and a myriad of van der Waals interactions (see Table II). There are notable differences in the chromophore...
conformation between eqFP611, Rtms5 and DsRed (Fig. 4), which must relate to differences in the amino acids that interact with the respective chromophores (Figs. 5 and 6).

The tripeptide sequence of the chromophore in eqFP611 is Met-Tyr-Gly, whereas that in DsRed and Rtms5 is Gln-Tyr-Gly, and in GFP is Ser-Tyr-Gly. Methionine and glutamine have long side chains of approximately similar length; the Met side chain of the eqFP611 chromophore was observed to adopt an extended conformation that superposed well with the equivalent glutamine residues of the DsRed and Rtms5 structures (Fig. 4). Nevertheless, methionine has a hydrophobic side chain, and correspondingly forms van der Waals contacts exclusively within a deep pocket that is surrounded by the side chains of Glu215, Gln213, and Leu199 (Fig. 6). This pocket is largely conserved in the Rtms5 and DsRed structures, although the polar Gln chromophore residue additionally participates in hydrogen bonding interactions (Fig. 6).

The imidazolinone moiety is wedged between the turns of the central helix, making a number of hydrogen bonds and van der Waals contacts with the neighboring side chains. Of the residues in eqFP611, Rtms5 and DsRed that contact the chromophore, only Glu215, Glu145, Arg92, and Trp90 are strictly conserved. Three of these residues (Glu215, Arg92, and Trp90) mediate interactions with the imidazolinone moiety, the orientation of which is largely conserved between the differing structures (with respect to eqFP611, the chromophores of DsRed and Rtms5 are rotated 20° and 10° about the center of the imidazolinone ring respectively). The glycal moiety, whose conformation is also conserved between the DsRed and Rtms5 structures (Fig. 5), forms van der Waals contacts with Ser66, Lys67, and Trp90, hydrogen-bonding contacts with Trp90 and Gln106 as well as water-mediated hydrogen bonds to Gln106 and Thr108.

The hydroxyphenyl group of the eqFP611 chromophore, which is coplanar with the imidazolinone ring, is in the trans configuration, sandwiched between His197 and Phe174, making van der Waals contacts with Lys67, Arg92, Asn143, Ser158, Met160, and Glu215 (Fig. 4). The imidazolinone ring of His197, which dominates the interactions with the hydroxyphenyl group, is coplanar with respect to this moiety, whereas Phe174 adopts a perpendicular orientation with respect to the chromophore. The hydroxyphenyl group hydrogen bonds onto Asn143 and Ser158. The Ser158 residue in eqFP611 packs against the side chain of Phe174, a residue that is conserved in both DsRed and Rtms5, however, in these proteins, the side chain is rotated away from the chromophore (Fig. 4, 6a and 6b).

A cavity proximal to the eqFP611 chromophore was observed that was large enough to accommodate an acetate ion. In Rtms5 and DsRed a water molecule at this equivalent, but smaller site, hydrogen bonds onto the chromophore N2 atom.

### Table II

| Protein contacts | Nature of interaction |
|-----------------|-----------------------|
| Methionine moiety |                      |
| C               | Ser66, Phe62          |
| C               | Glu215, Gln213        |
| C               | Met41                 |
| Imidazolinone moiety |                  |
| C               | Thr60                 |
| N               | Thr60                 |
| N               | Glu215                |
| N               | Thr60                 |
| C              | Ser66                 |
| C              | Thr60                 |
| O               | Lys67, Arg92          |
| 4-Hydroxyphenyl-methylene moiety |     |
| C               | Glu215                |
| C               | His197                |
| C               | Lys67, His197, Arg92  |
| C               | Asn143, Met160, His197|
| C               | Ser158, Asn143, Phe174, His197|
| C               | Ser158                |
| Glycal moiety |                      |
| C               | Ser66, Trp90          |
| C               | Glu215, Lys67, Trp90  |
| C               | Trp90                 |
| C               | Glu106                |
| C               | Gly106                |
| C               | Thr106                |

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There appears to be a degree of compensatory interplay between the residues at positions 197 and 67 in the respective structures, which impacts on the chromophore environment (Fig. 6). For example, in DsRed, position 67 (Lys70) swings toward the smaller side chain of Ser 197 and additionally forms salt bridges with Glu148 and Glu215. Whereas, in eqFP611, the bulkier His 197 appears to push Lys 67 away such that it only forms a salt bridge with Glu 145, and concomitantly creates a cavity that is large enough to accommodate an acetate ion. In Rtms5, position 67 is occupied by an Ile, however the guanidinium group of Arg197 compensates as it is observed to occupy a similar position to that of DsRed Lys70N (Fig. 6. b and c).

**DISCUSSION**

We have determined the 2.0-Å crystal structure of eqFP611 (Fig. 1 and Table I) and compared it to the structures of DsRed and Rtms5. A key finding of this work is the novel conformation of the eqFP611 chromophore. In comparing the chromophore conformation of eqFP611 with the highly fluorescent DsRed and the weakly fluorescent Rtms5, the most noticeable difference is to be seen in the positioning of the 4-hydroxyphenyl group (Figs. 4–6). In eqFP611, this aromatic ring is in a trans coplanar conformation; in DsRed the aromatic ring is in a cis coplanar conformation, whereas in Rtms5 the aromatic ring is in a trans conformation, and rotated 43° out of plane with the heterocycle. In comparison to DsRed, the 4-hydroxyphenyl group is rotated 180° about the Cα-Cβ bond (Figs. 4–6). Given that Rtms5 is weakly fluorescent, whereas DsRed and eqFP611 are highly fluorescent, the conformations of the respective chromophores in these structures suggest that coplanarity of the chromophore is required for a high fluorescence quantum...
yield. The fluorescence quantum yields of DsRed, eqFP611 and Rtms5 are 0.70, 0.45, and <0.001, respectively (7, 8, 12).

A number of amino acids appear to contribute toward the observed trans coplanar conformation of the eqFP611 chromophore. Firstly, the parallel ring-stacking interaction with His197 appears to play a predominant role, whereas in Rtms5 the guanidinium group of Arg197, appears to partly dictate the orientation of the hydroxyphenyl ring (Fig. 6). Secondly, two interactions of the eqFP611 phenoxo group with residues on β7b and β8 strands appear to be important for determining the orientation of the hydroxyphenyl ring (Figs. 3 and 6). In eqFP611, the OH group hydrogen bonds with Ser158 (β8 strand). The equivalent residue in Rtms5 is Asn161, which also hydrogen bonds with the phenoxy group. The presence of a larger residue in Rtms5 additionally appears to favor the rotation of the hydroxyphenyl group out of the plane of the imidazolone ring. On strand β7b, residue Aan143 of eqFP611 hydrogen bonds with the phenoxy group. The equivalent residues in Rtms5 and DsRed are His146 and Ser146, respectively. Ser146 in DsRed hydrogen bonds with the phenoxy group in a similar fashion to Ser158 in eqFP611, whereas His146 in Rtms5 stacks against the hydroxyphenyl moiety. In Rtms5, this appears to allow the hydroxyphenyl group to rotate out of the plane of the rest of the chromophore.

The presence of an acylimine within the eqFP611 chromophore extends the π-bonding system and, as observed in DsRed and Rtms5, is consistent with the red-shifted emission of the matured protein. Compared with DsRed the fluorescence emission maximum of eqFP611 is red-shifted by almost 30 nm (8), while the weak emission of Rtms5 (12) is red-shifted by more than 50 nm.

Differences in the chromophore conformations may contribute to, but are probably not the only reason for, these spectral differences. Significant contributions to red emission appear to arise from the individual chromophore environments in eqFP611, DsRed and Rtms5 respectively, as the contacts the chromophores make with their surrounding residues differ significantly. One candidate for the observed red-shift in the fluorescence spectrum of eqFP611 is the π-stack of the chromophore with His197. The aromatic ring of the His197 is highly polarizable and would help stabilize the excited state of the chromophore. It has been proposed that π-stacking interactions of the eqFP611 phenoxy group with residues on strand β7b, residue Aan143 of eqFP611 hydrogen bonds with Ser158, the OH group hydrogen bonds with Ser158 (7b, residue Asn 143 of eqFP611 hydroxyphenyl moiety. Interestingly, several mutations present in the highly engineered monomeric DsRed mutant are already present in eqFP611, arguing for weaker protomer interaction in eqFP611 (8).

Since GFP was first cloned new variants have been engineered and GFP homologs isolated that have intriguing and potentially useful properties. One phenomenon, termed kindling, has been demonstrated for the non-fluorescent protein asFP595 isolated from A. sulcalta. This protein becomes fluorescent (kindles) when irradiated with intense green light and has been developed for precise photolabeling in vivo (26). A model of the kindling mechanism has been proposed in which the key event in the transition from the non-fluorescent to fluorescent form of the protein was a trans to cis isomerization of the chromophore (7). The data presented here together with further mutagenesis and structural analysis of related proteins such as Rtms5 and eqFP611 may help develop a detailed understanding of this phenomenon.

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