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Crystallization and preliminary X-ray diffraction analysis of the red fluorescent protein eqFP611

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A novel red fluorescent protein, eqFP611, from the sea anemone *Entacmaea quadricolor* has been cloned in *Escherichia coli*. With excitation and emission maxima at 559 and 611 nm, this protein shows the most red-shifted emission and the largest Stokes shift of all non-modified proteins in the green fluorescent protein (GFP) family. The protein fluoresces over a wide pH range (4–10) with high quantum yield (0.45). Its photophysical properties make eqFP611 an excellent marker protein for *in vivo* labeling in eukaryotic systems as was shown by expression in a mammalian cell culture. eqFP611 has been crystallized in space group $P6_522$, with unit-cell parameters $a = b = 77.26$, $c = 329.49$ Å. The unit cell contains 12 asymmetric units, with two monomers in each. A molecular-replacement solution has been obtained using the 48.4% homologous red fluorescent protein from *Discosoma* coral (DsRed).

1. Introduction

The green fluorescent protein from *Aequorea victoria* (avGFP) has become very popular in life-science research as protein label, marker of gene expression and reporter of environmental conditions in living cells (Prasher *et al.*, 1992; Chalfie *et al.*, 1994; Tsien, 1998; Ormo *et al.*, 1996; Yang *et al.*, 1996). A large number of variants of avGFP with optimized properties have been created by site-directed and random mutagenesis. Efforts to create stable red fluorescent variants, however, have not met with success. Red fluorescent proteins would be highly desirable because of the reduced cellular autofluorescence in the red spectral range and for use in multicolor labeling or fluorescence resonance energy-transfer (FRET) experiments. Recently, avGFP homologs were discovered in non-bioluminescent Anthozoa species (Wiedenmann, 1997; Wiedenmann *et al.*, 1999, 2000, 2002; Matz *et al.*, 1999; Fradkov *et al.*, 2000). Some of the Anthozoa fluorescent proteins (FPs) show spectral properties that deviate greatly from avGFP, including red-emitting variants.

In a continuation of our previous work (Wiedenmann *et al.*, 1999, 2000), naturally occurring fluorescent proteins were screened to identify candidates with outstanding properties for potential applications as marker proteins. Here, we describe the expression, purification, crystallization and preliminary X-ray diffraction analysis of the red fluorescent protein eqFP611 from the sea anemone *Entacmaea quadricolor* (Wiedenmann *et al.*, 2002). This protein consists of 231 amino acids and has a molecular weight of ∼26 kDa. With an excitation maximum at 559 nm and an emission maximum at 611 nm, it shows the most red-shifted emission and the largest Stokes shift (52 nm) of all non-modified proteins in the green fluorescent protein family. Moreover, its fast and essentially complete maturation and reduced oligomerization tendency makes eqFP611 a viable alternative to red fluorescent protein (DsRed) in a variety of applications (Wiedenmann *et al.*, 2002).

2. Materials and methods

2.1. Expression and purification of recombinant eqFP611

A cDNA library of *E. quadricolor* was constructed as described elsewhere (Wiedenmann *et al.*, 2000). One clone coding for the red fluorescent protein eqFP611 was chosen for further characterization (Wiedenmann *et al.*, 2002). Bacteria (*Escherichia coli* BL21 DE3) were transformed with the eqFP611-coding plasmid (pQE32, Qiagen, Hilden, Germany) and grown on non-inducing agar plates in the presence of 50 mg l^{-1} ampicillin. This plasmid introduces the coding sequence for a 6×His tag to the 5′-terminus of the insert. As soon as the colonies developed fluorescence because of background expression, five weakly fluorescent colonies were used to set up a starter culture (50 ml of LB medium). After overnight growth at 303 K, 1.5 l of 2YT medium was inoculated with the starter culture and grown at 277 K under moderate shaking (120 rev min^{-1}) until the cells developed a pinkish color. Bacteria were harvested by centrifugation. The cells

were resuspended in 50 m*M* sodium phosphate pH 7, 300 m*M* sodium chloride and disrupted by sonication. Cell debris was removed by centrifugation at 50 000*g*. The clear lysate was loaded onto Talon metal-affinity resin (BD Biosciences Clontech), washed with loading buffer and eluted by adding 300 m*M* imidazole to the buffer. The eluate was dialyzed against 50 m*M* sodium phosphate pH 7.0, 300 m*M* NaCl and concentrated. Protein concentration was determined by absorption spectroscopy in the visible range (ε_{559} = 78 000 cm^{-1} M^{-1}).

2.2. Crystallization

Crystals of eqFP611 were grown using the hanging-drop vapor-diffusion method. The Hampton Research Crystal Screen kit I was used to screen for crystallization under a large number of conditions. Each drop was prepared by mixing 5 µl of protein (at a concentration of 5 mg ml^{-1}) and 5 µl of reservoir solution. The well contained 1 ml of buffer solution and 2 µl of β-mercaptoethanol. Initial crystallization occurred at 293 K using condition 20: 25% polyethylene glycol (PEG) 4000, 0.1 *M* sodium acetate pH 4.6, 0.2 *M* ammonium sulfate. The best results were obtained by increasing the drop size to 40 µl and decreasing the protein concentration to 2 mg ml^{-1}. Variations in buffer composition did not improve the results. Buffer solution and protein in 50 m*M* sodium phosphate buffer pH 7.0, 300 m*M* NaCl, 1% β-mercaptoethanol were premixed at a ratio of 1:7. Precipitate was removed by centrifugation. Subsequently, the solution was filtered (0.2 µm pore size) before hanging the drops. Bipyramidal crystals on a hexagonal base with typical dimensions of 0.15 × 0.05 × 0.05 mm, shown in Fig. 1, grew in about one week from mostly amorphous precipitation.

2.3. Data collection and processing

Crystals were mounted within a cryoloop (Hampton Research) and were flash-cooled using mother liquor with the addition of 25% PEG 200 as cryoprotectant. A 3.2 Å resolution data set was collected at 100 K using synchrotron radiation at a wavelength λ = 1 Å at Elettra (beamline XRD1). The crystals diffracted to 2.5 Å; the extremely long *c* axis (330 Å) resulted in spot overlap at higher resolution, however, which could not be avoided by reorienting the crystal. Therefore, we proceeded with data collection at a crystal-to-detector distance of 250 mm. This resulted in a final resolution of the data of 3.2 Å. The crystal diffraction power could not be fully exploited because of severe overlap of reflections in the region 3.2–2.5 Å. Data collection at higher wavelength (1.9 Å) was also pursued to minimize this problem, but this only marginally reduced the overlap and resulted in greater crystal damage. The best data set was obtained at a wavelength of 1 Å by collecting a total of 340 images with an oscillation angle of 0.3 on a MAR CCD detector. Data were indexed with *DENZO* and reduced and scaled with *SCALEPACK* (Otwinowski & Minor, 1997). Subsequent calculations were performed using the *CCP*4 package (Collaborative Computational Project, Number 4, 1994).

3. Results

Inspection of diffraction patterns and systematic absences and subsequent structure determination by molecular replacement with *AMoRe* (Collaborative Computational Project, Number 4, 1994) allowed the assignment of the eqFP611 crystal to the hexagonal space group $P6_522$, with unit-cell parameters $a = b = 77.26$, $c = 329.49$ Å (unit-cell volume = 1 03 293.4 Å^3). In the past, crystals of *A. victoria* GFP with similar unit-cell parameters have been obtained (Perozzo *et al.*, 1988), but their poor quality prevented a structural determination. The solvent content of the unit cell is 56.3%, with two molecules in the asymmetric unit (V_M = 2.84 Å^3 Da^{-1}). The expected tetrameric assembly is achieved along one of the twofold symmetry axes of the space group. The final data are complete to 96%, with an overall R_{merge} of 0.075 and a crystal mosaicity of 0.35°. Data-collection statistics are summarized in Table 1.

As an initial model for molecular replacement, we used three polyalanine models of different dimers from the homotetrameric DsRed from which the chromophore region had been excised (PDB code 1ggx; Wall *et al.*, 2000). DsRed bears a 48.4% identity to eqFP611. Good agreement for both rotation and translation functions for space group $P6_522$ was achieved using a dimer corresponding to the *AB* assembly of the original model, whereas the other space groups belonging to the hexagonal system did not yield significant solutions. The model was subsequently refined using *REFMAC* (Collaborative Computational Project, Number 4, 1994) with a final R = 0.397 and R_{free} = 0.475; electron-density maps showed consistent density in the chromophore region, main chain and for some bulky side chains. We are currently applying for beam time at suitable lines that would allow data collection at higher resolution. A highly resolved molecular structure is a prerequisite for understanding the functional properties of eqFP611 in detail.

Table 1
Statistical data-collection parameters for eqFP611 crystals as a function of resolution.

A total of 93 232 reflections were measured, for a total of 9930 unique reflections, with an average $\langle I/\sigma(I)\rangle$ = 20.

Resolution (Å)	$I > 3\sigma$† (%)	χ^2‡	R_{merge}§	Completeness (%)
28.0–5.46	94.3	0.375	0.04	99.8
5.46–4.34	93.4	0.332	0.045	99.9
4.34–3.79	84.7	0.588	0.092	100.0
3.79–3.45	77.7	0.825	0.139	100.0
3.45–3.20	82.6	0.887	0.254	79.5
Overall	86.5	0.587	0.074	96.0

† Unique reflections with $I/\sigma > 3$, with measured intensity I and error in intensity σ. ‡ $\chi^2 = \sum_h \sum_i (I_{hi} - \langle I_h\rangle)^2/\sigma_h^2(N-1)$, where N is the number of observations. Here, I_{hi} is the ith observation of the reflection h and $\langle I_h\rangle$ is the mean intensity of reflection h. § $R_{merge} = \sum_h \sum_i |I_{hi} - \langle I_h\rangle| / \sum_h \sum_i I_{hi}$.

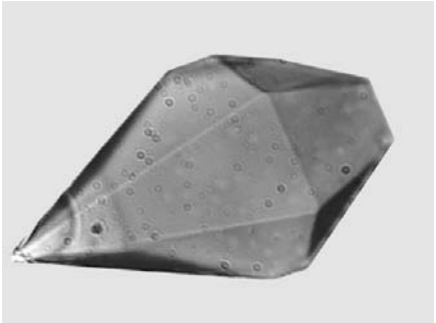

Figure 1
A typical crystal of recombinant eqFP611.

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