Acta Crystallographica Section F
Structural Biology and Crystallization Communications
ISSN 1744-3091

Crystallization and preliminary X-ray diffraction analysis of a novel wild-type blue fluorescent protein from *Vibrio vulnificus* CKM-1

Chin-Feng Liu,[a]‡ Chao-Hung Kao,[b]‡ Yen-Chao Su[c] and Yeh Chen[b]*

[a]Department of Biochemical Science and Technology, College of Life Science, National Taiwan University, Taipei 106, Taiwan, [b]Department of Biotechnology, Hungkuang University, Taichung 443, Taiwan, and [c]Department of Food Science, Nutrition and Nutraceutical Biotechnology, Shih Chien University, Taipei 106, Taiwan

‡ These authors made equal contributions.

Correspondence e-mail: chyeah6599@sunrise.hk.edu.tw

Received 15 December 2011
Accepted 13 February 2012

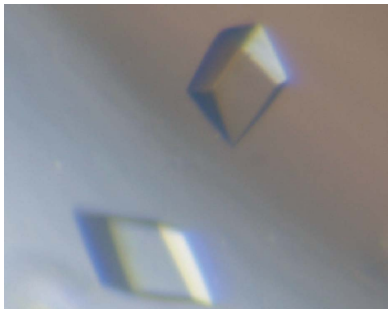
The use of green fluorescent protein (GFP) for non-invasive *in vivo* imaging is limited to aerobic systems, as chromophore formation requires oxygen. However, a novel NADPH-dependent blue fluorescent protein from *Vibrio vulnificus* CKM-1 (BFPvv) that emits blue fluorescence in both aerobic and anaerobic systems has recently been discovered. Wild-type BFPvv was overexpressed in *Escherichia coli*, purified and crystallized using the sitting-drop vapour-diffusion method. The resulting BFPvv crystals diffracted to a resolution of 1.9 Å and belonged to space group *P*3, with unit-cell parameters $a = b = 96.62$, $c = 214.511$ Å. Assuming the presence of eight molecules in the unit cell, the solvent content was estimated to be ∼56.16%.

1. Introduction

Fluorescent proteins are widely used to study protein localization and dynamics in living cells (Heim & Tsien, 1996; Tsien, 1998). Members of the green fluorescent protein (GFP) family, first discovered in the jellyfish *Aequorea victoria*, have been extensively studied owing to their unique biochemistry and widespread use as *in vivo* markers (Shimomura, 2005).

Interestingly, a novel NADPH-dependent blue fluorescent protein from *Vibrio vulnificus* CKM-1 (BFPvv) belonging to the short-chain dehydrogenase/reductase (SDR) family has been identified which does not display the typical β-can structure of GFP and its homologues (Su *et al.*, 2001). *Escherichia coli* transformants expressing the cloned *bfg*V gene exhibited strong blue fluorescence emission around 440 nm following excitation at 352 nm (Su *et al.*, 2001; Chang *et al.*, 2004). In addition, evaluation of the substrate specificity of wild-type (WT) BFPvv indicates that BFPvv favours aldehydes over ketones (Polizzi *et al.*, 2007). Additionally, the chromophore of GFP and its variants is formed *via* oxygen-dependent cyclization steps, which may limit their application under obligate anaerobic conditions. Therefore, BFPvv, an NADPH-based fluorescent protein, may represent an alternative reporter molecule for use in both aerobic and anaerobic systems.

Mutants of BFPvv, including E40K, V76A, L77I, V83M, S124C, G176S and E179K, have previously been generated by directed evolution. In particular, Kao *et al.* (2011) determined the structure of a mutant form of BFPvv and demonstrated that the mutation of Gly176 to Ser or Ala enhanced fluorescence. However, the structure of WT BFPvv has not yet been determined, making it difficult to compare structure and function between WT BFPvv and its variants. In the present study, we describe the cloning, purification, crystallization and initial X-ray analysis of WT BFPvv in order to obtain its tertiary structure and to further understand how chromophore formation occurs *via* a different oxygen-independent mechanism.



2. Materials and methods

2.1. Cloning, expression and purification

BFPvv was cloned into a T7 promoter-driven expression system (pET-21b vector; Invitrogen), allowing the expression of His_6-tagged

recombinant BFPvv protein (rBFPvv) in *E. coli* BL21 (DE3) cells. The cells were cultured and shaken using an orbital shaking incubator as batches in 1 l flasks containing Luria–Bertani medium at 293 K and 200 rev min^{-1} for 20 h after IPTG induction. Isolation and purification took place when the protein yield reached 30 mg per litre of medium. The cells were harvested by centrifugation and the cell pellet was resuspended in buffer *A* (50 m*M* Tris–HCl pH 8.0, 500 m*M* NaCl, 5 m*M* imidazole). Subsequently, the cells were disrupted by sonication and the crude lysate was centrifuged at 15 000 rev min^{-1} for 90 min at 277 K. The clarified supernatant was applied to Ni–NTA His-bind resin pre-equilibrated with binding buffer. Impurities were removed with Ni–NTA wash buffer (50 m*M* Tris–HCl, 500 m*M* NaCl, 10 m*M* imidazole pH 8.0) and the bound BFPvv was eluted with a 0–200 m*M* linear gradient of imidazole. Fractions containing BFPvv were pooled, concentrated by ultrafiltration using an Amicon Ultra-15 10K Centrifugal Filter Device (Millipore; 10 kDa cutoff) and loaded onto a HiLoad 16/60 Superdex 200 size-exclusion column (GE Healthcare) equilibrated with gel-filtration buffer (50 m*M* Tris–HCl pH 8.0, 100 m*M* NaCl, 5% glycerol, 2 m*M* TCEP). The fractions containing His_6-tagged rBFPvv were pooled and concentrated to 7 mg ml^{-1} for crystallization screening (Fig. 1).

2.2. Crystallization

Crystallization screening was performed with commercial screening kits from Hampton Research (Index, PEG/Ion, PEGRx 1 and 2, Crystal Screen and Crystal Screen 2), Emerald BioSystems (Wizard I–IV) and Molecular Dimensions (PGA Screen and Clear Strategy Screens I and II) using the sitting-drop vapour-diffusion method in 24-well VDX plates (Hampton Research). 1 µl protein solution (7 mg ml^{-1} in gel-filtration buffer) and 1 µl reservoir solution were mixed and equilibrated against 400 µl reservoir solution at 277 K. Initial crystals were grown in a precipitant consisting of 25% PEG 4000, 0.1 *M* MES monohydrate pH 6.0, 6% Tacsimate pH 6.0 (condition No. 29 of PEGRx 2 from Hampton Research). Refinement to determine the optimal crystallization conditions then took place using manual screens varying the pH and the precipitant concentration in a systematic manner. Crystals suitable for diffraction experiments were grown by mixing 1 µl protein solution with 1 µl reagent solution and reached maximum dimensions of 0.25 × 0.25 × 2.0 mm after 30 d (Fig. 2).

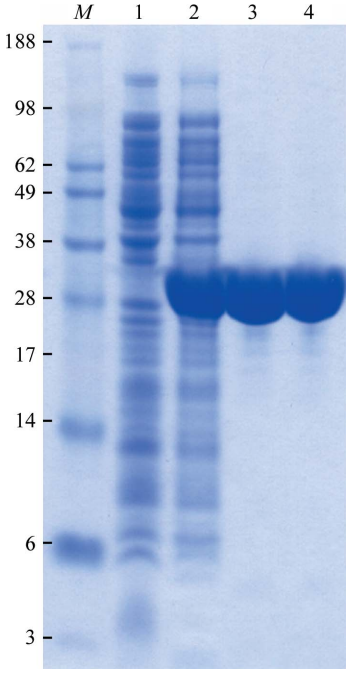


Figure 1
SDS–PAGE of purified rBFPvv. Lane *M*, molecular-weight markers (labelled in kDa); lane 1, whole cell lysate before IPTG induction; lane 2, whole cell lysate after IPTG induction; lane 3, rBFPvv purified with an Ni column; lane 4, rBFPvv purified by gel filtration.

Table 1
Data-collection statistics for the rBFPvv crystal.

Values in parentheses are for the highest resolution shell.

X-ray wavelength (Å)	0.97622
Space group	*P*3
Unit-cell parameters (Å)	a = 96.62, b = 96.62, c = 214.511
Resolution (Å)	30.0–1.90 (1.97–1.90)
No. of measured reflections	738320 (72340)
No. of unique reflections	176161 (17644)
Multiplicity	4.2 (4.1)
R_{merge} (%)	6.1 (38.7)
Data completeness (%)	100 (100)
$\langle I/\sigma(I)\rangle$	22.28 (3.65)

2.3. Data collection

Crystals of rBFPvv were cooled in liquid nitrogen using ethylene glycol as a cryoprotectant. The cryoprotectant solution was prepared by diluting the reservoir solution with ethylene glycol to obtain a final concentration of 25%(*v*/*v*) ethylene glycol. After soaking for 5 min in the cryoprotectant solution, the crystals were flash-cooled by plunging them into liquid nitrogen. X-ray diffraction data were collected on BL13C1 at the National Synchrotron Radiation Research Center (NSRRC), Taiwan. A native data set was obtained and the data were indexed and integrated using the *HKL*-2000 processing software (Otwinowski & Minor, 1997). An X-ray diffraction data image collected at the NSRRC is shown in Fig. 3.

3. Results and discussion

The *bfp* gene consists of 717 bp that encode 239 amino-acid residues. The purified rBFPvv protein produced a single band of approximately 26 kDa following SDS–PAGE, which is in good agreement with the calculated value of 26.3 kDa. In addition, the protein purity

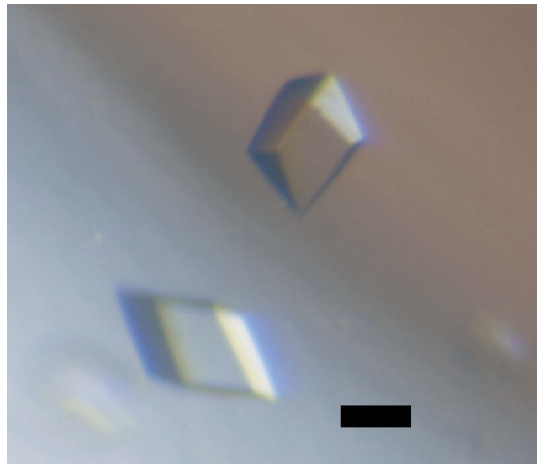


Figure 2
Diamond-shaped crystals of rBFPvv from *V. vulnificus* grown by the sitting-drop vapour-diffusion method. Crystals suitable for X-ray diffraction had dimensions of 0.2 × 0.2 × 0.25 mm. The scale bar represents 100 µm.

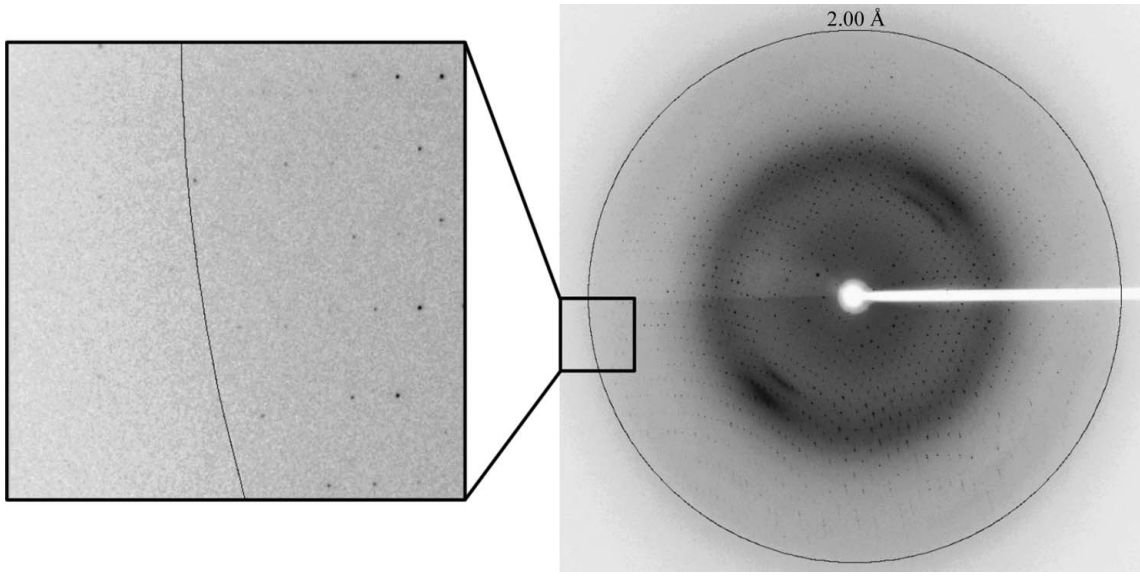


Figure 3
Diffraction pattern of rBFPvv collected on beamline 13C1 at NSRRC from a crystal flash-cooled in 25% ethylene glycol. The black circle indicates a resolution of 2.0 Å.

was determined by scanning densitometry of Coomassie Blue-stained protein on a 12% polyacrylamide gel. As shown in Fig. 1, rBFPvv demonstrated >95% purity.

An rBFPvv crystal was obtained using the sitting-drop vapour-diffusion method in a buffer comprising 6% Tacsimate pH 6.0, 0.1 *M* MES monohydrate pH 6.0, 25% PEG 4000. After 30 d, a single crystal suitable for X-ray diffraction was obtained with dimensions of 0.25 × 0.25 × 2.0 mm. Crystallographic data revealed significant diffraction to a resolution of 1.9 Å and data processing indicated that the crystal belonged to space group *P*3. Data-collection statistics are presented in Table 1. Calculation of the Matthews coefficient suggested that there are eight subunits in the asymmetric unit, with a V_M value of 2.8 Å^3 Da^{-1} and a solvent content of 56.16% (Matthews, 1968). Molecular replacement was performed with *Phaser* (McCoy *et al.*, 2005) using BFPvvD8 (PDB entry 3p19; Kao *et al.*, 2011) as the search model. Refinement and manual refitting of the initial model are now in progress. These results may provide structural information on WT BFPvv in order to compare the structures of WT and mutant BFPvv.

We thank the National Synchrotron Radiation Research Center (NSRRC, Taiwan) for assistance during data collection. This work was supported by grants from the National Science Council (NSC99-2313-B-241-001 and NSC100-2313-B-241-006 to YC).

References

Chang, C. C., Chuang, Y. C., Chen, Y. C. & Chang, M. C. (2004). *Biochem. Biophys. Res. Commun.* **319**, 207–213.
Heim, R. & Tsien, R. Y. (1996). *Curr. Biol.* **6**, 178–182.
Kao, T.-H., Chen, Y., Pai, C.-H., Chang, M.-C. & Wang, A. H.-J. (2011). *J. Struct. Biol.* **174**, 485–493.
Matthews, B. W. (1968). *J. Mol. Biol.* **33**, 491–497.
McCoy, A. J., Grosse-Kunstleve, R. W., Storoni, L. C. & Read, R. J. (2005). *Acta Cryst.* D**61**, 458–464.
Otwinowski, Z. & Minor, W. (1997). *Methods Enzymol.* **276**, 307–326.
Polizzi, K. M., Moore, D. A. & Bommarius, A. S. (2007). *Chem. Commun.*, pp. 1843–1845.
Shimomura, O. (2005). *J. Microsc.* **217**, 1–15.
Su, J.-H., Chuang, Y.-C., Tsai, Y.-C. & Chang, M.-C. (2001). *Biochem. Biophys. Res. Commun.* **287**, 359–365.
Tsien, R. Y. (1998). *Annu. Rev. Biochem.* **67**, 509–544.