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Crystallization and preliminary X-ray analysis of a monomeric mutant of Azami-Green (mAG), an *Aequorea victoria* green fluorescent protein-like green-emitting fluorescent protein from the stony coral *Galaxea fascicularis*

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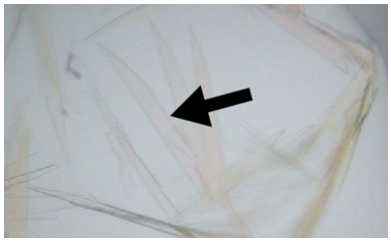


Monomeric Azami-Green (mAG) from the stony coral *Galaxea fascicularis* is the first monomeric green-emitting fluorescent protein that is not a derivative of *Aequorea victoria* green fluorescent protein (avGFP). mAG and avGFP are 27% identical in amino-acid sequence. Diffraction-quality crystals of recombinant mAG were obtained by the sitting-drop vapour-diffusion method using PEG 3350 as the precipitant. The mAG crystal diffracted X-rays to 2.20 Å resolution on beamline AR-NW12A at the Photon Factory (Tsukuba, Japan). The crystal belonged to space group $P1$, with unit-cell parameters a = 41.78, b = 51.72, c = 52.89 Å, α = 90.96, β = 103.41, γ = 101.79°. The Matthews coefficient (V_M = 2.10 Å^3 Da^{-1}) indicated that the crystal contained two mAG molecules per asymmetric unit.

1. Introduction

Green fluorescent proteins (GFPs), which were first isolated from the bioluminescent jellyfish *Aequorea victoria* (avGFP; Shimomura *et al.*, 1962), are crucial tools in molecular and cellular biology as translational reporters, fusion tags and biosensors (Tsien, 1998). avGFP adopts a β-can fold (Yang *et al.*, 1996; Ormö *et al.*, 1996), which consists of an 11-stranded β-barrel and a coaxial central α-helix holding a chromophore. Recent studies have enabled the structure-based mutagenesis of avGFP and other anthozoan fluorescent proteins in order to develop derivatives with desirable properties, *e.g.* enhanced green emission (Heim & Tsien, 1996), red-shifted fluorescence (Mishin *et al.*, 2008) and photo-inducible green-to-red conversion (Tsutsui *et al.*, 2005). All the anthozoan avGFP-like proteins characterized to date form obligatory oligomers (Zhang *et al.*, 2002) which are not suitable for fluorescence resonance energy transfer (FRET) experiments (reviewed by Müller-Taubenberger & Anderson, 2007; Shaner *et al.*, 2007). Karasawa *et al.* (2003) cloned the gene for a tetrameric green-emitting fluorescent protein, Azami-Green (AG), which has 27% sequence identity to avGFP, from the stony coral *Galaxea fascicularis* and established its monomeric mutant, mAG, by introducing three point mutations: Y123T, Y188A and F190K (Fig. 1). mAG is the first monomeric green-emitting fluorescent protein that is not a derivative of avGFP. The crystal structure of mAG would thus contribute to the design and development of new mAG mutants with faster maturation, brighter fluorescence, improved photostability, new colours and other desirable properties. Here, we describe the overproduction, purification, crystallization and preliminary X-ray diffraction analysis of mAG.

2. Materials and methods

2.1. Overproduction and purification of mAG

The gene for mAG from *G. fascicularis* (gi:52839541) was amplified by PCR from pmAG1-S1 (Amalgaam). The PCR primers used were 5′-TCCAGGGTGCT**CATATG**AGTGTGATTAAACCAGAGATG-AA-3′ (including an *Nde*I site; shown in bold) and 5′-GCTCGAAT-TC**GGATCC**TTATTACTTGGCCTGACTCGGCAGCATAGAA-3′

(including a *Bam*HI site; shown in bold). 30 cycles of PCR were performed using PrimeSTAR HS (Takara Bio) with a melting phase at 371 K for 30 s, an annealing phase at 328 K for 30 s and a polymerization phase at 345 K for 60 s. The PCR product and the vector plasmid, a modified pET-28a(+) (Novagen) with a TEV protease-cleavage site substituted for the thrombin-cleavage site, were digested by *Nde*I and *Bam*HI and ligated with Ligation High (Toyobo). DNA-sequence analyses of the mAG-encoding regions in pmAG1-S1 (Amalgaam) and pET-28a(+)-based expression plasmids showed that the mAG proteins encoded in both plasmids had four amino-acid substitutions, R149E, A160R, D182E and V191I, in addition to the three reported monomeric mutations: Y123T, Y188A and F190K (gi:52839541; Fig. 1). N-terminally (6×His)-tagged mAG was expressed in *Escherichi coli* strain KRX (Promega) grown in 2 l LB medium at 310 K. Protein expression was induced by the addition of L-(+)-rhamnose to a final concentration of 0.1%(*w*/*v*) when the OD_{600} reached ∼0.8 and the culture was grown for a further 6 h at 310 K. The harvested cells were resuspended in 40 ml 50 m*M* sodium phosphate pH 8.0 and 300 m*M* NaCl (buffer *A*) supplemented with 10 m*M* imidazole and one tablet of Complete EDTA-free protease-inhibitor cocktail (Roche) and were then disrupted by sonication. The lysate was centrifuged at 40 000*g* at 277 K for 60 min and the resulting supernatant was loaded onto an Ni Sepharose 6 Fast Flow (2.5 ml gel bed volume; GE Healthcare) column equilibrated with buffer *A* containing 10 m*M* imidazole. The column was washed with 25 ml buffer *A* containing 10 m*M* imidazole and then with 15 ml buffer *A* containing 20 m*M* imidazole to remove nonspecifically bound proteins. Finally, N-terminally (6×His)-tagged mAG was eluted with 10 ml buffer *A* containing 250 m*M* imidazole. The eluate was dialyzed against 50 m*M* Tris–HCl pH 8.0, 150 m*M* NaCl and 0.5 m*M* EDTA at 277 K overnight and the dialysate was supplemented with DTT to a final concentration of 10 m*M*. For removal of the N-terminal (6×His) tag from mAG, the protein solution was supplemented with (6×His)-tagged TEV protease (10 000 U ml^{-1}, prepared in our laboratory) to a final concentration of 9 U enzyme per milligram of substrate protein and incubated at room temperature for 3 d. The protein solution was then applied onto a HisTrap HP 1 ml (GE Healthcare) column pre-equilibrated with 10 m*M* Tris–HCl pH 8.0 to remove uncleaved fusion protein, (6×His)-tagged TEV protease and released (6×His) tag. The flowthrough was applied onto a HiLoad 26/60 Superdex 75 pg (GE Healthcare) column equilibrated with 10 m*M* Tris–HCl pH 8.0. The purified protein was concentrated to 5 mg ml^{-1} using a 20 ml Vivaspin concentrator (10 kDa cutoff; Sartorius).

2.2. Crystallization, data collection and preliminary X-ray analysis

Initial screening for crystallization conditions was performed using commercially available kits (Crystal Screen HT and Index HT from Hampton Research, and Wizard Screens I and II from Emerald BioSystems) at 293 K by the sitting-drop vapour-diffusion method using 96-well Intelli-Plates (Art Robbins). Index HT condition F6 [0.1 *M* bis-tris pH 5.5, 25%(*w*/*v*) PEG 3350 and 0.2 *M* ammonium sulfate] and Crystal Screen HT condition B3 [0.1 *M* sodium cacodylate pH 6.5, 30%(*w*/*v*) PEG 8000 and 0.2 *M* ammonium sulfate] gave needle-like crystals. To obtain crystals of better quality, these two conditions were subjected to two-dimensional grid optimizations by the sitting-drop vapour-diffusion method using 24-well Cryschem Plates (Hampton Reasearch). A crystallization drop was prepared by mixing 1 µl protein solution and 1 µl reservoir solution; the drop was then equilibrated against 500 µl reservoir solution. Index HT condition F6 was optimized using a two-dimensional grid of four precipitant concentrations [19, 22, 25 and 28%(*w*/*v*) PEG 3350] and six buffer pH values (5.5, 5.9, 6.3, 6.7, 7.1 and 7.5), while Crystal Screen HT condition B3 was optimized in a similar manner but using three different buffers, sodium citrate (pH 4.5, 4.9, 5.3, 5.7, 6.1 and 6.5),

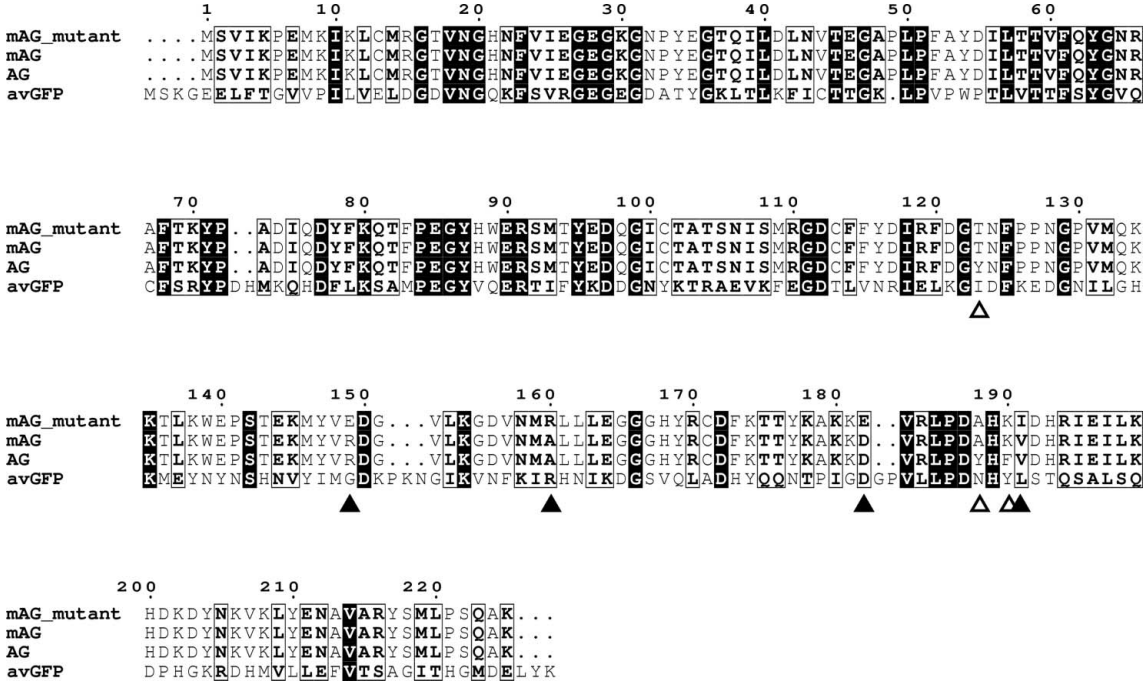


Figure 1
Amino-acid sequence alignment of the mAG mutant that was used in this study, mAG, AG and avGFP. The three point mutations, Y123T, Y188A and F190K, introduced to produce mAG from AG are indicated by white arrowheads. The four amino-acid substitutions, R149E, A160R, D182E and V191I, between mAG and the mAG mutant are indicated by black arrowheads. This figure was generated by *ESPript* (Gouet *et al.*, 1999).

MES (pH 5.2, 5.5, 5.9, 6.3, 6.7 and 7.1) and bis-tris (pH 5.5, 5.9, 6.3, 6.7, 7.1 and 7.5), instead of the originally used sodium cacodylate buffer. Diffraction-quality crystals were obtained in the two-dimensional grid optimization of Index HT condition F6 with the reservoir composition 0.1 *M* bis-tris pH 5.5, 22%(*w*/*v*) PEG 3350 and 0.2 *M* ammonium sulfate. X-ray diffraction data were obtained on beamline AR-NW12A at Photon Factory (Tsukuba, Japan). A crystal was picked up with a mounting loop and frozen in a cold nitrogen-gas stream without the use of any cryoprotectant. The crystal was then annealed by blocking the cold nitrogen-gas stream for 15 s. A diffraction data set consisting of 360 images was collected with a wavelength of 1.0000 Å, a crystal-to-detector distance of 166.2 mm, a rotation angle of 1° and an exposure time of 2 s per image using an ADSC Quantum 210r detector. The data were indexed and scaled using the program package *XDS* (Kabsch, 1993).

3. Results and discussion

N-terminally (6×His)-tagged mAG was expressed in *E. coli* KRX using a pET-28a(+)-based expression plasmid; approximately 5 mg of purified mAG was obtained from 1 l *E. coli* culture. Crystals of mAG were obtained by the sitting-drop vapour-diffusion method and typically grew to final dimensions of 0.3 × 0.04 × 0.01 mm within 2 d at 293 K (Fig. 2). The best crystal diffracted X-rays to a resolution of 2.20 Å on beamline AR-NW12A at Photon Factory (Fig. 3*a*). A 15 s annealing of the crystal was effective in removing the ice rings that had resulted from flash-cooling without any cryoprotectant (Fig. 3*b*). As a result, the completeness was improved from 88.9 to 97.1%, from 85.2 to 97.8%, from 51.6 to 95.3% and from 65.2 to 97.5% in the 4.02–3.72, 3.72–3.48, 2.32–2.26 and 2.26–2.20 Å resolution shells, respectively. The data-collection statistics are summarized in Table 1. The Matthews coefficient (V_M = 2.10 Å^3 Da^{-1}; Matthews, 1968) indicated that the crystal contained two mAG molecules per asymmetric unit, with a solvent content of 41.3%. Structure determination of mAG by molecular replacement is currently under way using the coordinates of two similar proteins as search models: Dronpa (76% sequence identity to mAG; PDB code 2ie2, Wilmann *et al.*, 2006; PDB code 2iov, Stiel *et al.*, 2007; PDB codes 2gx0 and 2gx2, Nam *et al.*, 2007; PDB code 2pox, Andresen *et al.*, 2007; PDB codes 2z6x, 2z6y, 2z6z and 2z1o, Mizuno *et al.*, 2008), a photochromic green fluorescent protein whose fluorescence can be reversibly turned on and off on demand by exposure to different wavelengths of light (Ando *et al.*, 2004), and Dendra2 (76% sequence identity to mAG; PDB code 2vzx; Adam *et al.*, 2009), a fluorescent protein that undergoes irreversible green-to-red photoconversion upon exposure to purple–blue light (Gurskaya *et al.*, 2006).

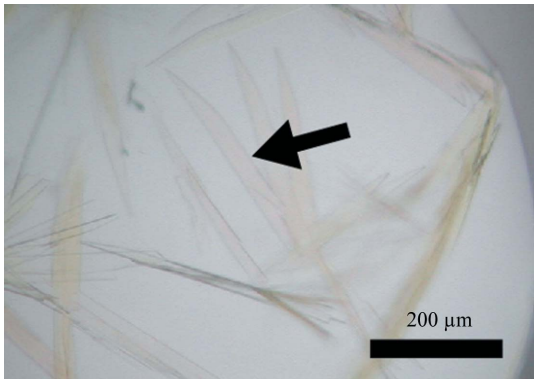


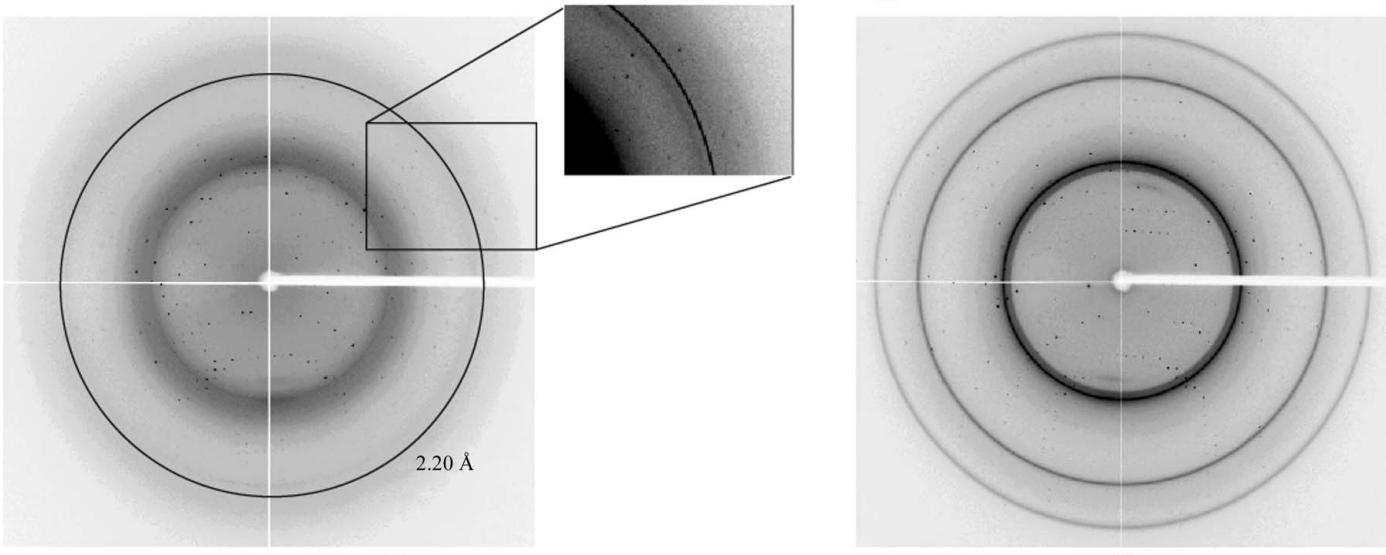
Figure 2
A diffraction-quality crystal of mAG (indicated by an arrow) together with thin crystals.

Table 1
Summary of data-collection statistics.

Values in parentheses are for the highest resolution shell.

X-ray source	Photon Factory beamline AR-NW12A
Wavelength (Å)	1.0000
Resolution range (Å)	20.0–2.20 (2.26–2.20)
No. of observed reflections	81625
No. of unique reflections	20674
Data completeness (%)	96.9 (97.5)
R_{merge}†	0.056 (0.315)
$\langle I\rangle/\langle\sigma(I)\rangle$	19.82 (4.85)
Space group	*P*1
Unit-cell parameters (Å, °)	a = 41.78, b = 51.72, c = 52.89, α = 90.96, β = 103.41, γ = 101.79

† $R_{merge} = \sum_{hkl}\sum_i |I_i(hkl) - \langle I(hkl)\rangle| / \sum_{hkl}\sum_i I_i(hkl)$.



(*a*) (*b*)

Figure 3
(*a*) A diffraction image of the mAG crystal after annealing. The ring corresponds to a resolution of 2.20 Å. (*b*) A diffraction image of the mAG crystal before annealing.

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References

Adam, V., Nienhaus, K., Bourgeois, D. & Nienhaus, G. U. (2009). *Biochemistry*, **48**, 4905–4915.
Ando, R., Mizuno, H. & Miyawaki, A. (2004). *Science*, **306**, 1370–1373.
Andresen, M., Stiel, A. C., Trowitzsch, S., Weber, G., Eggeling, C., Wahl, M. C., Hell, S. W. & Jakobs, S. (2007). *Proc. Natl Acad. Sci. USA*, **104**, 13005–13009.
Gouet, P., Courcelle, E., Stuart, D. I. & Métoz, F. (1999). *Bioinformatics*, **15**, 305–308.
Gurskaya, N. G., Verkhusha, V. V., Shcheglov, A. S., Staroverov, D. B., Chepurnykh, T. V., Fradkov, A. F., Lukyanov, S. & Lukyanov, K. A. (2006). *Nature Biotechnol.* **24**, 461–465.
Heim, R. & Tsien, R. Y. (1996). *Curr. Biol.* **6**, 178–182.
Kabsch, W. (1993). *J. Appl. Cryst.* **26**, 795–800.
Karasawa, S., Araki, T., Yamamoto-Hino, M. & Miyawaki, A. J. (2003). *Biol. Chem.* **278**, 34167–34171.
Matthews, B. W. (1968). *J. Mol. Biol.* **33**, 491–497.
Mishin, A. S., Subach, F. V., Yampolsky, I. V., King, W., Lukyanov, K. A. & Verkhusha, V. V. (2008). *Biochemistry*, **47**, 4666–4673.
Mizuno, H., Mal, T. K., Wälchli, M., Kikuchi, A., Fukano, T., Ando, R., Jeyakanthan, J., Taka, J., Shiro, Y., Ikura, M. & Miyawaki, A. (2008). *Proc. Natl Acad. Sci. USA*, **105**, 9227–9232.
Müller-Taubenberger, A. & Anderson, K. I. (2007). *Appl. Microbiol. Biotechnol.* **77**, 1–12.
Nam, K. H., Kwon, O. Y., Sugiyama, K., Lee, W. H., Kim, Y. K., Song, H. K., Kim, E. E., Park, S. Y., Jeon, H., Hwang, K. Y. (2007). *Biochem. Biophys. Res. Commun.* **354**, 962–967.
Ormö, M., Cubitt, A. B., Kallio, K., Gross, L. A., Tsien, R. Y. & Remington, S. J. (1996). *Science*, **273**, 1392–1395.
Shaner, N. C., Patterson, G. H. & Davidson, M. W. (2007). *J. Cell Sci.* **120**, 4247–4260.
Shimomura, O., Johnson, F. H. & Saiga, Y. (1962). *J. Cell. Comput. Physiol.* **59**, 223–239.
Stiel, A. C., Trowitzsch, S., Weber, G., Andresen, M., Eggeling, C., Hell, S. W., Jakobs, S. & Wahl, M. C. (2007). *Biochem. J.* **402**, 35–42.
Tsien, R. Y. (1998). *Annu. Rev. Biochem.* **67**, 509–544.
Tsutsui, H., Karasawa, S., Shimizu, H., Nukina, N. & Miyawaki, A. (2005). *EMBO Rep.* **6**, 233–238.
Wilmann, P. G., Turcic, K., Battad, J. M., Wilcek, M. C., Devenish, R. J., Prescott, M. & Rossjohn, J. (2006). *J. Mol. Biol.* **364**, 213–224.
Yang, F., Moss, L. G. & Phillips, G. N. Jr (1996). *Nature Biotechnol.* **14**, 1246–1251.
Zhang, J., Campbell, R. E., Ting, A. Y. & Tsien, R. Y. (2002). *Nature Rev. Mol. Cell Biol.* **3**, 906–918.