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The production, purification and crystallization of a pocilloporin pigment from a reef-forming coral

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Reef-building corals contain fluorescent pigments termed pocilloporins that function by regulating the light environment of coral and acting as a photoprotectant in excessive sunlight. These pocilloporins are related to the monomeric green fluorescent protein and the tetrameric DsRed fluorescent proteins, which have widespread use as biotechnological tools. An intensely blue-coloured pocilloporin, termed Rtms5, was expressed in *Escherichia coli*, purified and crystallized. Rtms5 was shown to be tetrameric, with deep blue crystals that diffract to 2.2 Å resolution and belong to space group $I4_122$. The colour of this pocilloporin was observed to be sensitive to pH and a yellow (pH 3.5) and a red form (pH 4.5) of Rtms5 were also crystallized. These crystals belong to space group $P4_222$ and diffract to 2.4 Å resolution or better.

1. Introduction

The vivid and diverse colours of reef-building corals are a result of host-based pigmentation. The pink and blue pigments of two families of corals have previously been partially characterized and described as pocilloporins (Dove *et al.*, 1995). The pocilloporins exhibit a broad range of spectral and fluorescent behaviours (Dove & Hoegh-Guldberg, 1999; Dove *et al.*, 2001). The pocilloporins are related to the green fluorescent protein from *Aequorea victoria* (GFP) and a red fluorescent protein from *Discosoma* coral (DsRed), whose crystal structures have been determined. Their intrinsic fluorescent properties arise from an extended conjugated π-system comprising a cyclic tripeptide chromophore (Gln-Tyr-Gly in DsRed; Ser-Tyr-Gly in GFP) that is buried within the β-can fold of these structures (Ormo *et al.*, 1996; Yang *et al.*, 1996; Wall *et al.*, 2000; Yarbrough *et al.*, 2001). These proteins are useful widespread biotechnological tools and, to further their biotechnological applications, variants of these proteins have been generated with altered spectral and oligomeric properties (Tsien, 1998), whilst other variants have been optimized or are suitable as biosensors for pH (Miesenbock, 1999), redox potential (Ostergaard *et al.*, 2001) or Ca^{2+} (Miyawaki *et al.*, 1999).

To address the biophysical and structural basis of coral pigmentation as well as the potential biotechnological use of the pocilloporins and other closely related chromoproteins (Lukyanov *et al.*, 2000; Gurskaya *et al.*, 2001), we have initiated structural studies on a pocilloporin, Rtms5, from the reef-building coral *Montipora efflorescens*. Rtms5 is deep blue in colour, yet is weakly fluorescent (quantum yield <0.0001). However, it has been reported that other chromoproteins with sequence homology to Rtms5 can be interconverted to a fluorescent protein by single amino-acid substitutions (Lukyanov *et al.*, 2000).

In this report, we have cloned, sequenced, expressed, crystallized and undertaken preliminary diffraction analysis on Rtms5. We show that Rtms5 is tetrameric and that iodide ions were an essential crystallization agent in order to obtain crystals. The blue crystals belong to space group $I4_122$ and diffract to 2.2 Å resolution. The colour of this pocilloporin was observed to be sensitive to pH and we have additionally crystallized a yellow (pH 3.5) and a red form (pH 4.5) of Rtms5. These crystals belong to space group $P4_222$ and diffract to 2.4 Å resolution or better.

2. Materials and methods

2.1. Protein expression and purification

A DNA cassette encoding Rtms5 was retrieved from a cDNA (Dove *et al.*, 2001) using the oligonucleotide primers 5′-gaagatctaaaacaatgagtgtgatcgctacacaaatg/5′-tatcaaatcgccggcgtcaggcgaccacaggtttg and cloned into the *Bam*HI/*Not*I site of pRSETBN. pRSETBN is a derivative of pRSETB (Invitrogen) modified to contain a *Not*I in the multiple cloning site.

Cultures (BL21-DE3) were inoculated to an OD_{650nm} of 0.6 and, after 1 h incubation at 301 K, expression was induced by the addition of isopropyl β-D-thiogalactoside to a final concentration of 0.5 m*M* and was then continued overnight at 301 K. Cells harvested

from 250 ml cultures were lysed by incubation at 277 K in lysis buffer (100 m*M* NaH_2PO_4 pH 8.0, 300 m*M* NaCl, 10 m*M* imidazole) containing 1 mg ml^{-1} lysozyme, followed by sonication to reduce viscosity. Lysates were centrifuged at 10 000*g* and applied to a 2 ml column of Ni–NTA agarose (Qiagen, Hilden, Germany) equilibrated in lysis buffer containing 20 m*M* imidazole. Columns were washed with ten column volumes of lysis buffer and Rtms5 protein was eluted with lysis buffer containing 250 m*M* imidazole. Typically, the eluted protein was approximately 90% pure at this stage. Eluted protein was further purified and buffer-exchanged by chromatography on a calibrated Superdex 200 column (60 × 2.6 cm) equilibrated with lysis buffer without added imidazole. Fractions corresponding to the tetrameric form of Rtms5 were pooled, concentrated and quantified using the BCA Protein Assay Kit (Pierce) following the manufacturer's protocol.

2.2. Crystallization and data collection

Crystallization experiments were performed at room temperature using the hanging-drop vapour-diffusion technique. All data were collected in-house on an R-AXIS IV++ detector with Cu $K\alpha$ X-rays generated by a Rigaku RU-H3RHB rotating-anode generator and focused using Osmic mirrors. X-ray data were collected from the frozen crystals using the inverse-φ geometry. The crystals were flash-frozen prior to data collection using 25% glycerol in the precipitant buffer as the cryoprotectant. To avoid an unacceptable increase in mosaicity upon freezing, the crystals were transferred stepwise (5% increments) into increasing amounts of glycerol over a time period of 2 h. The diffraction data were processed and analyzed using *d*TREK* (Pflugrath, 1999).

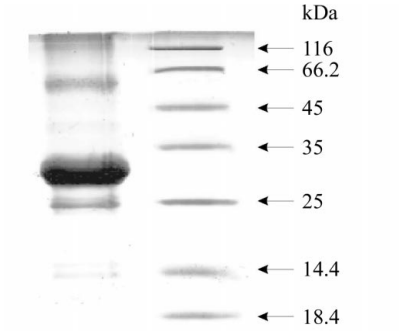

Figure 1
Following Ni–NTA chromatography, 70 µg of Rtms5 protein were subjected to SDS–PAGE under reducing conditions on a 15% acrylamide gel (lane 1). The position of molecular-size markers are indicated (lane 2). Gels were stained using Coomassie Brilliant Blue.

3. Results and discussion

3.1. Expression and purification

A cDNA isolated from the reef-building coral *M. efflorescens* encoding Rtms5 was expressed in BL21 *Escherichia coli*. Suspension cultures were a deep blue colour after inducing expression of Rtms5 for 18 h at 301 K. After cell lysis, the majority of the blue colour was released into the high-speed supernatant, indicating that the majority of the Rtms5 protein was present in a soluble form. Protein was eluted from the Ni–NTA chromatography medium using 250 m*M* imidazole, resulting in >90% purity as estimated by SDS–PAGE (Fig. 1) and silver stains (data not shown). A polypeptide of $M_r \simeq 29$ kDa was observed that matched the predicted size (29.2 kDa) for Rtms5 including the N-terminal hexahistidine tag encoded by pRSET-B. Yields of pure proteins were typically 5 mg per litre of culture. Pocilloporins isolated from different species of reef-building corals can exist as trimers or dimers (Dove *et al.*, 1995). In order to exclude heterogeneity in the preparation, the protein was applied directly to a calibrated S200 size-exclusion column. The intensely blue-coloured protein was readily monitored by eye during chromatography. The majority of the protein eluted from the size-exclusion column with an apparent size (112 kDa) that corresponded closely to the size predicted (117 kDa) for the tetrameric form of Rtms5 (Fig. 2). A small amount of the protein eluted as a shoulder peak corresponding in size to the dimeric form of Rtms5, suggesting that a mixture of tetrameric and dimeric species are present. Given that the dimeric species was blue, it was concluded that the ∼60 kDa band was Rtms5 and not an impurity. Fractions corresponding to the tetramer were pooled and concentrated to 15 mg ml^{-1} ready for crystallization trials. Protein eluted from Ni–NTA columns without further purification by size-exclusion chromatography did not yield crystals.

3.2. Crystallization

All screens were conducted at room temperature. The initial Crystal Screen Kit (Hampton Research, Laguna Niguel, USA) yielded precipitation, phase separation or clear drops. In addition, a conventional coarse PEG/pH screen yielded phase separation only. Given the observations using the latter screen, it was thought that selected ions may modulate the solubility of Rtms5 further and possibly yield crystals. Accordingly, a PEG/ion screen was established and, although many of the conditions yielded phase separation only, conditions containing iodide resulted in small tetragonal blue crystals. Fine tuning around these initial conditions (20% PEG 3350, 0.2 *M* potassium iodide, unbuffered) yielded large blue crystals (0.4 × 0.3 × 0.3 mm). The final crystallization conditions were as follows: the crystals were grown within 3–5 d by mixing equal volumes of 17 mg ml^{-1} Rtms5 protein with reservoir buffer (10–15% PEG 3350, 0.1 *M* Tris–HCl pH 8.2, 0.2 *M* potassium iodide). The crystals belong to space group $I4_122$, as judged by systematic absences, with unit-cell parameters $a = b = 131$, $c = 151$ Å. The Matthews coefficient suggested there to be either one or two monomer(s) in the asymmetric unit, with the former implying a solvent content of over 70%. There were no significant

Table 1
Data-collection statistics.

The values in parentheses are for the highest resolution bin (approximate interval 0.1 Å).

Temperature (K)	100
X-ray source	RU-3HBR
Detector	R-AXIS IV++
Space group (Å)	$I4_122$
Unit-cell parameters (Å)	$a = b = 131.70$, $c = 150.51$
Resolution (Å)	2.2
Total No. observations	109869
No. unique observations	32494
Multiplicity	3.4
Data completeness (%)	96.1 (98.5)
No. data >2$\sigma(I)$	79.8
$I/\sigma(I)$	6.4 (2.3)
R_{merge}† (%)	7.9 (24.7)

† $R_{merge} = \sum |I_{hkl} - \langle I_{hkl}\rangle| / \sum I_{hkl}$.

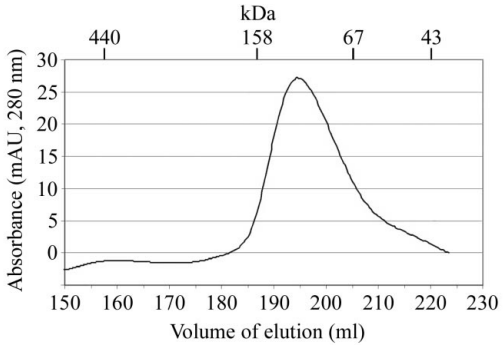

Figure 2
Following Ni–NTA chromatography, Rtms5 protein was subjected to size-exclusion chromatography on a calibrated Superdex 200 column. The major eluting species corresponded in size to an Rtms5 tetramer. The shoulder peak eluted later corresponded in size to an Rtms5 dimer.

pH 3.5 pH 4.5 pH 8.0

Figure 3
Tetragonal crystals of Rtms5 generated at different pH values.

peaks in either a native Patterson map or a self-rotation function, which may suggest a monomer in the asymmetric unit with the biologically active tetramer being generated *via* crystallographic symmetry. For the quality of the data-collection statistics, see Table 1. Preliminary molecular-replacement studies suggested a monomer in the asymmetric unit.

The colour of some pocilloporins is known to be sensitive to pH. Accordingly, we investigated this phenomenon using the pre-existing crystals. Reducing the pH caused the crystals to change colour from red (pH 4.5) to yellow (pH 3.5) and this colour change was reversible. However, these pH-lowered crystals were not suitable for X-ray diffraction analysis because a very large increase in mosaic spread was observed, which significantly reduced the diffraction limit of the crystals. To overcome this, crystallization conditions were screened at low pH (3–4.5) in the presence of potassium iodide. Smaller crystals appeared within one week, either exhibiting a red colour or a yellow colour (Fig. 3). Both these crystals were shown to belong to space group $P4_222$, with unit-cell parameters $a = b = 92.8$, $c = 75.0$ Å, suggesting one monomer per asymmetric unit.

Rtms5 and homologous chromoproteins (Gurskaya *et al.*, 2001) are of significant scientific and biotechnological interest. These proteins represent an important biological source that could be used for developing assays with colour (reflectance) readouts. Rtms5 (data not shown) and homologous proteins (Bulina *et al.*, 2002) can be interconverted to a fluorescent protein with far-red fluorescence. However, little is known about the structural basis for these transitions. With further development, these proteins offer a range of powerful new tools for studying biological function *in vivo* (Ntziachristos *et al.*, 2002). This work will allow us to begin to address the structural basis for chromoprotein pigmentation and the fluorescence interconversions with a view to developing biosensors having novel properties.

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