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Crystallization of Ranasmurfin, a blue-coloured protein from *Polypedates leucomystax*

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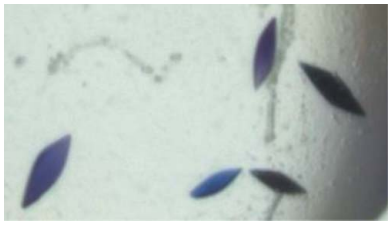

Ranasmurfin, a previously uncharacterized ~13 kDa blue protein found in the nests of the frog *Polypedates leucomystax*, has been purified and crystallized. The crystals are an intense blue colour and diffract to 1.51 Å with $P2_1$ symmetry and unit-cell parameters a = 40.9, b = 59.9, c = 45.0 Å, β = 93.3°. Self-rotation function analysis indicates the presence of a dimer in the asymmetric unit. Biochemical data suggest that the blue colour of the protein is related to dimer formation. Sequence data for the protein are incomplete, but thus far have identified no model for molecular replacement. A fluorescence scan shows a peak at 9.676 keV, indicating that the protein binds zinc and suggesting a route for structure solution.

1. Introduction

Ranasmurfin (Pl-RSF-1) is a previously undescribed blue protein isolated from the foam nests of the tropical frog *Polypedates leucomystax*. Many species of frog found in tropical or subtropical regions deposit their fertilized eggs in foams that presumably function to protect the developing embryos and to act as temporary shelters for the mature larvae from dehydration, predation and other environmental challenges. These foam nests are proving to be a rich source of proteins with unusual primary structures and a range of interesting surfactant and possible antimicrobial properties (Cooper *et al.*, 2005). The current work stems from field observations in Malaysia (AC, MWK, RTYC) and biophysical and biochemical analysis in Glasgow.

P. leucomystax (Java whipping frog) is a common rhacophorid tree frog found abundantly throughout Malaysia and adjacent regions of South East Asia. During mating, the female releases eggs and a secretion that is whipped up by the male (which simultaneously fertilizes the eggs) into a foamy mass, usually attached to vegetation or other surfaces overhanging water. After hatching, the developing tadpoles subsequently emerge from the foam and escape directly into the water below the nest. These foam nests are usually pale pink/orange or colourless when first formed, but develop blue/green coloration with time. Analysis of foam-nest material by SDS–PAGE shows that this pigmentation co-migrates with a ~26 kDa protein, clearly identifiable as a blue/green band in the electrophoresis gel even under denaturing conditions and without staining. The protein is readily purified from the mixture by gel-filtration chromatography. Partial amino-acid sequencing of this protein by high-resolution mass spectrometry (>50% contiguous sequence so far) shows no similarity to any protein sequences yet reported. The function of this protein is currently unknown, but possible functions include a sunscreen to protect the (unpigmented) eggs and developing embryos from potentially harmful solar irradiation, a sink for reactive oxygen species, antimicrobial activity and/or simple camouflage.

2. Experimental methods

2.1. Purification

P. leucomystax nests were collected from well populated sites along the east coast of Peninsula Malaysia with an appropriate permit from the Malaysian Department of Wildlife and National Parks and

Economic Planning Unit (EPU). Foam material was immediately separated from eggs by hand and stored frozen until use. Ranasmurfin is the dominant protein in the nest and was isolated from this mixture by gel-filtration chromatography (Superdex-75) after dispersal of the foam into water by gentle sonication. The protein runs as two bands, one a ~13 kDa monomer which is weakly coloured and the second a much stronger blue band corresponding to a ~26 kDa dimer. The blue fraction was concentrated by centrifugal concentration prior to crystallization trials.

2.2. Crystallization

Protein concentrations were estimated by Bradford assay (Bradford, 1976) and the optimal values (~30 mg ml^{-1}) for initial crystallization screens were determined from pre-crystallization tests (Hampton Research). The protein was buffered in 25 m*M* Tris pH 7. The sitting-drop vapour-diffusion method was used to screen 384 independent conditions (0.1 µl protein + 0.1 µl precipitant with 100 µl reservoir) at 293 K using a nanodrop crystallization robot (Cartesian HoneyBee) as part of the Hamilton-Thermo Rhombix system. Deep blue protein crystals (Fig. 1) were obtained in several different PEG-containing conditions and the best, as judged morphologically, grew overnight to dimensions of 140 × 40 × 40 µm from Hampton PEG/Ion Screen tube 31 (20% PEG 3350, 0.2 *M* Li SO_4). Larger crystals (400 × 80 × 80 µm) were grown from manually set up drops (1 + 1 µl) for easier manipulation and subsequent data collection.

3. X-ray data collection

The crystals were characterized on BM14 at the European Synchrotron Radiation Facility (ESRF) and found to diffract to high resolution (Fig. 2). Crystals were cryoprotected by soaking in mother liquor doped with 25% glycerol and were cryocooled in a loop at 100 K. A 1.51 Å resolution data set of 150 non-overlapping images was collected as 30 s 1° oscillations using a MAR 225 CCD. The crystal-to-detector distance was 140 mm and all data were integrated and reduced using *HKL*-2000 (Otwinowski & Minor, 1997). The crystals are primitive monoclinic, with unit-cell parameters a = 40.9, b = 59.9, c = 45.0 Å, $\alpha = \gamma = 90$, $\beta = 93.3°$. Analyses of over 20 axial reflections demonstrated the presence of a screw axis, confirming the space group as $P2_1$. Data-collection statistics are presented in Table 1. A self-rotation map calculated with data from 12 to 3.5 Å shows a peak at $\omega = 112$, $\psi = 270$, $\kappa = 180°$ at a height of 31% of the origin peak (Collaborative Computational Project, Number 4, 1994). This suggests there is noncrystallographic twofold rotation axis within the asymmetric unit.

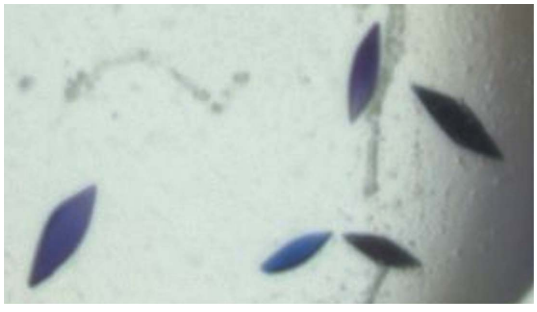

Figure 1
Crystals of Pl-RSF-1 grown in a 0.1 + 0.1 µl sitting drop from Hampton PEG/Ion Screen tube 31.

Table 1
Pl-RSF-1 data-collection statistics.

Values in parentheses refer to the highest resolution shell.

Wavelength (Å)	0.98
Resolution (Å)	50.00–1.51 (1.56–1.51)
Space group	$P2_1$
Temperature (K)	100
Detector	MAR 225 CCD
Unit-cell parameters (Å, °)	a = 40.9, b = 59.9, c = 45.0 $\alpha = \gamma = 90$, $\beta = 93.3$
Solvent content (%)	51
Unique reflections	179029
$I/\sigma(I)$	29.1 (2.3)
Average redundancy	5.6 (3.5)
Data completeness (%)	92.7 (64.8)
$R_{\rm merge}$† (%)	5.3 (43.9)

† $R_{\rm merge} = \sum\sum I(h)_i - \langle I(h)\rangle / \sum I(h)_i$, where $I(h)$ is the measured diffraction intensity and the summation includes all observations.

4. Results and discussion

Primary structure information from mass spectrometry is incomplete (60 residues) and the interpretation is complicated by many chemically modified amino acids. A reliable search for a molecular-replacement model is therefore difficult. Searches with the partial sequence data available do not reveal any match to any known protein or structure. This reinforces the notion that the Pl-RSF-1 may be a novel protein. As the protein must currently be purified from natural sources, biosynthetic selenomethionine incorporation is impossible. Based on the strong blue colour of the protein, it was anticipated that the protein may bind a metal ion such as copper or nickel, either of which would allow MAD phasing of the structure. Fluorescence scans at the appropriate energies provided no evidence for either metal. Surprisingly, the scans revealed a strong signal for zinc (9.676 keV), which is not normally found in blue complexes. Zinc can of course be used for MAD phasing (Fig. 3) and should allow us to solve the structure. The unit-cell parameters are consistent with the presence of two molecules in the asymmetric unit

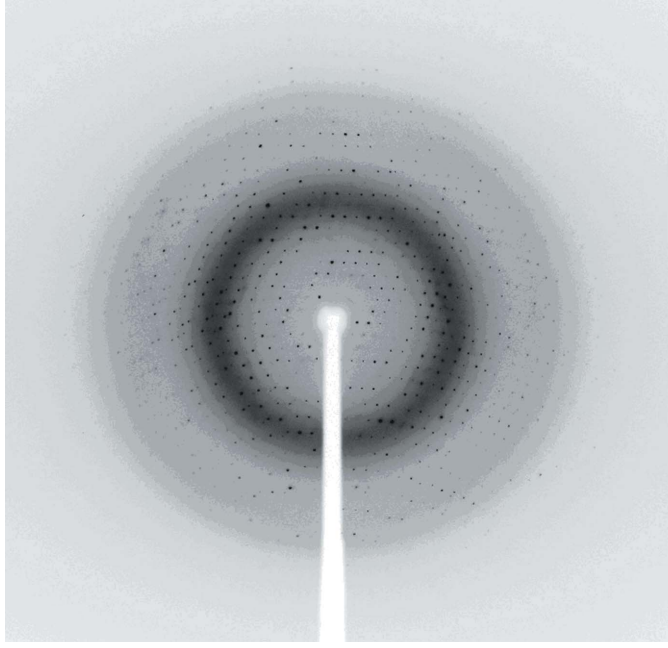

Figure 2
High-resolution diffraction pattern of Pl-RSF-1 recorded at BM14 at the ESRF using a MAR 225 CCD. The edge of the plate is 1.4 Å.

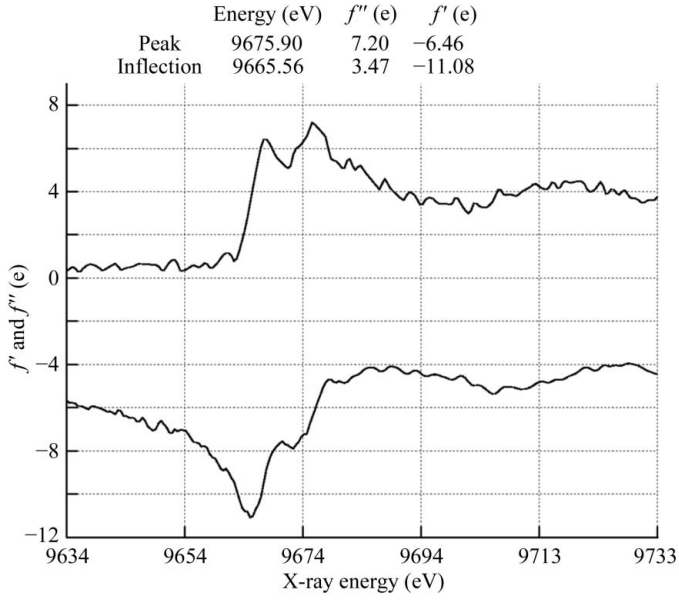

Figure 3
Zinc fluorescence measured from a crystal of Pl-RSF-1. The peak is at 9675.9 eV, compared with the theoretical value of 9658.6 eV.

($V_M = 2.51$ Å^3 Da^{-1}) and a solvent content of 51%. The existence of a dimer would be consistent with SDS–PAGE analysis of the protein, which revealed an entity of ∼26 kDa, and the noncrystallographic twofold rotation axis detected in the asymmetric unit. Prolonged heating of the protein at 373 K does not affect the appearance of the dimer band in the gel, suggesting a very tight association between the dimer partners. Given the absence of colour in the monomer-sized band, it seems reasonable to suggest the colour of the protein requires dimer formation. The monomer band has essentially identical tryptic fragmentation mass spectra, confirming it is definitely Pl-RSF-1. Pl-RSF-1 is clearly a highly unusual protein, whose natural colour may involve a chromogen of novel composition that may or may not relate to the presence of a zinc ion. Structure elucidation of Pl-RSF-1 will provide important clues towards an understanding of the chemistry involved in the creation of the chromophore. A high-resolution structure will greatly assist in identifying the sequence of the protein and thus in cloning it.

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