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Crystallization and preliminary X-ray crystallographic analysis of the 30 kDa membrane-binding domain of protein 4.1 from human erythrocytes

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The 30 kDa membrane-binding domain of protein 4.1 from human erythrocytes has been expressed in *Escherichia coli* and crystallized in a form suitable for X-ray crystallographic study. Crystals were grown using a salting-in technique. Crystals have a tetragonal plate shape and belong to the *C*2 space group, with unit-cell parameters $a = 163.9$, $b = 106.5$, $c = 93.5$ Å, $\beta = 95.5^\circ$. The crystals diffract to 2.8 Å resolution.

1. Introduction

Protein 4.1 is a cytoskeletal protein found in a multitude of eukaryotic cells. In human red blood cells, 80 kDa protein 4.1R is a major component of the spectrin-based membrane cytoskeletal complex which physically supports the cell membrane. This protein binds to spectrin and actin, and anchors the cytoskeleton to the plasma membrane through its interaction with membrane proteins such as glycophorin C/D (Marfatia *et al.*, 1994; Hemming *et al.*, 1994) and band 3 (Pasternack *et al.*, 1985; An *et al.*, 1996). In addition, protein 4.1R binds to calmodulin, indicating its calcium-dependent role in regulating these interactions (Anderson & Morrow, 1987; Tanaka *et al.*, 1991; Lombardo & Low, 1994). Defects in this protein result in an unstable cytoskeleton and an abnormally shaped red blood cell, manifested clinically as elliptocytosis leading to anemia (Conboy *et al.*, 1991).

We have expressed the N-terminal 30 kDa domain (core domain) of the 80 kDa protein 4.1R. This cysteine-rich domain contains binding sites for glycophorin C/D, band 3, p55 (Marfatia *et al.*, 1994, 1995) and calmodulin. We have obtained crystals of the core domain that are suitable for X-ray crystallographic structure determination. The detailed structure of this domain can be expected to provide a basis for understanding its dynamic interactions with membrane proteins and its role in regulating cell shape.

2. Materials and methods

2.1. Purification

The 30 kDa domain of protein 4.1R was expressed in *E. coli* as a fusion protein with glutathione S-transferase. This fusion protein was purified using glutathione affinity chromatography. The exogenous glutathione S-transferase was cleaved from the fusion protein using PreScission protease (Pharmacia). The 30 kDa domain was purified to homogeneity using DEAE ion-exchange chromatography.

2.2. Crystallization

Crystals of protein 4.1R core domain were obtained by the sitting-drop technique utilizing a salting-in vapor-diffusion procedure. In the crystallization wells, the protein, which was concentrated to 20 mg ml^{-1} in 20 m*M* Tris buffer (pH 7.5) containing 0.4 *M* NaCl, was mixed with crystallization solution containing 10% polyethylene glycol (PEG) 3000, 0.4 *M* NaCl and 0.1 *M* sodium citrate buffer (pH 5.5) in a 2:1 volume ratio; the crystallization reservoir was 0.1 *M* NaCl solution. Stacked crystals were obtained in 2–4 weeks. These crystals were harvested and broken into small crystal fragments which were used as macroseeds for new crystallization trials. Using cryo-loops, macroseeds with good morphology were transferred into a washing solution consisting of half-strength mother liquor. The seed crystals were then placed in a new crystallization drop. Single crystals grew from macroseeds in

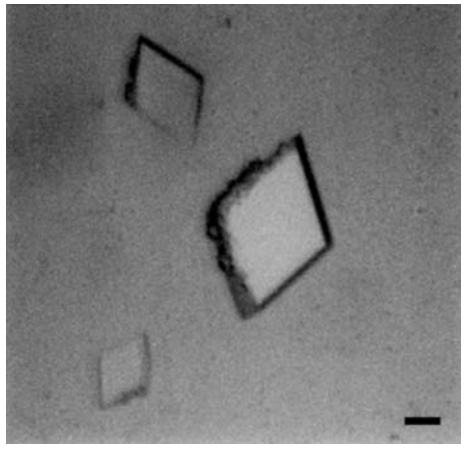

Figure 1
Crystals of the 30 kDa core domain of protein 4.1R. Scale bar indicates 0.1 mm.

1–2 weeks as tetragonal plates, with the largest crystals reaching dimensions of 0.3 × 0.3 × 0.1 mm (Fig. 1).

2.3. X-ray data collection

Prior to flash-freezing, crystals were soaked in a cryoprotectant solution of 25% MPD and 2% PEG 3000 in 20 m*M* sodium citrate buffer (pH 5.9) for 30 min. The crystals were mounted in cryo-loops and then plunged into liquid nitrogen. The cryo-loops were placed on the goniometer head and maintained below 123 K during diffraction data collection. Diffraction data for both native and heavy-atom treated crystals were collected using synchrotron light sources at both the Advanced Light Source (ALS), Lawrence Berkeley National Laboratory and the National Synchrotron Light Source (NSLS), Brookhaven National Laboratory. Diffraction data were processed using *DENZO*/*SCALEPACK* (Otwinowski & Minor, 1997).

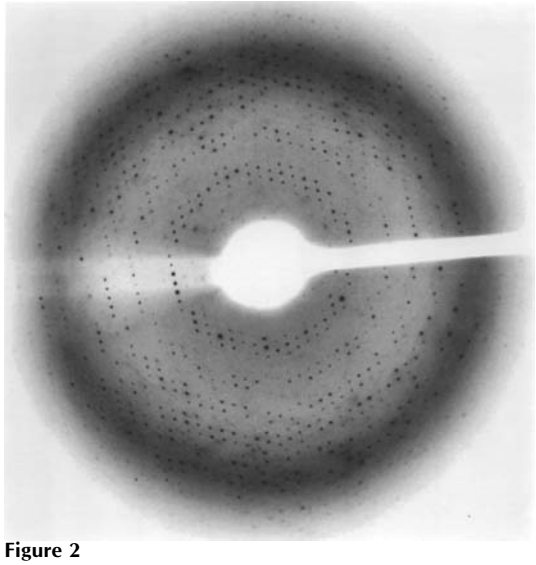

Figure 2
An X-ray diffraction image from a crystal of the protein 4.1R core domain. This diffraction pattern was recorded from a 1° osciallation at NSLS beamline X25 and shows diffraction spots extending to better than 2.8 Å resolution.

3. Results and discussion

Crystallization trials conducted using commercial screening kits failed to produce any crystals. The solubility of the protein 4.1R core domain undergoes a major transition at 0.1–0.3 *M* NaCl, indicating that another crystallization approach involving a salting-in technique might be required. The solubility of the protein is low at low salt concentrations, increases to a maximum at around 0.4–0.5 *M* and decreases again at high salt concentration. Before concentrating the purified protein, the salt concentration of the sample buffer was adjusted to 0.4 *M* in order to optimize the protein solubility. Crystallization trials using a salting-in method were performed with and without the use of additives. In the course of our trials, addition of PEG was found to be necessary to obtain crystals. Furthermore, the concentration of NaCl in the sample drop and reservoir was critical in producing high-quality crystals. The best crystals were obtained with 0.4 *M* NaCl and 3% PEG in the sample drop and 0.1 *M* NaCl in the reservoir. After several days, the volume of the sample drops increased two- or three-fold and crystals started to grow. Stacked multi-crystals were obtained within a month and macroseeding was necessary to produce single crystals, which achieved maximum size in two weeks and started to form multiple stacked crystals after three weeks. Single crystals were harvested within two weeks of macroseeding (Fig. 1). Several cryoprotectants were screened; the best crystal preservation was obtained with a solution containing 25% MPD and 2% PEG 3000. The diffraction data collected under cryo-conditions extend to better than 2.8 Å resolution (Fig. 2). The processed and refined data show that the crystals adopt the monoclinic space group *C*2, with unit-cell parameters a = 163.9, b = 106.5, c = 93.5 Å, β = 95.5°. Complete native data sets and several potential heavy-atom derivative data sets extending up to 2.8 Å resolution have been obtained. Overall $R_{\rm sym}$ factors are less than 5% for the native data set and 5% for derivative data sets. Both anomalous and isomorphous derivative diffraction data will be used to solve the structure.

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