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Crystallization and preliminary crystallographic analysis of the Rho-binding domain of bovine Rho-kinase

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Rho-kinase binds to a small GTPase Rho in a GTP-dependent manner and regulates many cytoskeletal events in the cell. The minimum region of bovine Rho-kinase sufficient for Rho-binding was expressed as a fusion protein with glutathione S-transferase. After removal of the glutathione S-transferase, thin plate crystals were obtained. The selenomethionine-substituted protein was introduced and crystallized, as was the native protein. The crystals of the Rho-binding domain of Rho-kinase belong to the space group $C2$, with unit-cell parameters a = 148.0 (2), b = 26.1 (1), c = 39.6 (1) Å, β = 90.3 (1)°. The crystals diffract to a resolution beyond 1.5 Å.

1. Introduction

Rho-kinase is a serine/threonine kinase, the catalytic activity of which is enhanced by GTP-bound Rho GTPase; Rho-kinase was originally isolated from bovine brain tissue (Matsui *et al.*, 1996). This kinase was shown to phosphorylate myosin light chain, the myosin-binding subunit of myosine phosphatase and adducin (Amano *et al.*, 1996; Kimura *et al.*, 1996, 1998). These phosphoryl-driven regulations are directly related to cytokinesis, cell motility and smooth muscle contraction (Amano *et al.*, 1997; Chihara *et al.*, 1997). The Rho-kinase is identical to ROK from rat brain (Leung *et al.*, 1995) and ROCK from human megakaryocytic leukaemia cells (Ishizaki *et al.*, 1996). A recent report has suggested that ROCK phosphorylates LIM-kinase to induce the reorganization of the actin cytoskeleton (Maekawa *et al.*, 1999). In this manner, Rho-kinase plays an important role in cytoskeletal regulation at Rho-dependent pathways (reviewed by Machesky & Hall, 1996; Van Aelst & D'Souza-Schorey, 1997; Fukata *et al.*, 1999).

Of several Rho GTPase target proteins, only the structure of protein kinase N (PKN), complexed with RhoA, has recently been determined and was identified as the anti-parallel coiled-coil structure in the Rho-binding domain of PKN (Maesaki *et al.*, 1999). The binding modes of PKN–Rho GTPase and Rho-kinase–Rho GTPase are thought to be different (Fujisawa *et al.*, 1998). However, amino-acid sequence analysis suggests that the conformation of the Rho-binding domain of Rho-kinase is similar to the coiled-coil motif of the Rho-binding domain of PKN. In order to obtain more definitive information about Rho-binding motifs, we crystallized the minimum region of bovine Rho-kinase sufficient for Rho-binding and analyzed its crystallographic features.

2. Expression and purification

The minimum Rho-binding domain (RhoBD) of bovine Rho-kinase has been determined previously; it consists of 69 amino acids (residues 979–1047; Leung *et al.*, 1995; Fujisawa *et al.*, 1996). This region was cloned into plasmid pGEX-2T (Pharmacia). RhoBD was expressed in the *Escherichia coli* expression strain DH5α as a fusion protein with glutathione S-transferase (GST); this GST fusion protein was purified using a column of glutathione Sepharose (Pharmacia). Removal of GST from GST-RhoBD was achieved within the column using 2 units ml^{-1} thrombin (Nippon Ham). RhoBD was eluted with a buffer containing 20 mM HEPES pH 7.0 and 50 mM KCl. This eluate was applied to HiTrap S and HiTrap Q (Pharmacia) and the eluates from each column were collected and concentrated to 6 mg ml^{-1} in the same buffer using Centricon-3 (Millipore). The Rho-binding activity was confirmed by an overlay assay using ^{35}S-labelled RhoA complexed with guanosine 5′-3-*O*-(thio)-triphosphate (GTPγS) (unpublished data). The N-terminal sequence analysis (M492, Applied Biosystems) of the resulting sample showed that the additional glycine and serine residues at the N-terminus originate from the site of cleavage by thrombin. The protein was also verified with matrix-assisted laser desorption/ionization time-of-flight mass spectroscopy (MALDI-TOF MS; PerSeptive), which gave a peak of 7996 Da, corresponding to the calculated value of 7997 Da for 71 amino acids.

3. Crystallization

Crystals were obtained at 277 K by the hanging-drop vapour-diffusion method from a solution containing 2.7 mg ml^{-1} RhoBD, 55 m*M* HEPES pH 7.0, 23 m*M* KCl, 11% polyethylene glycol (PEG) 1000, 14% ethylene glycol and 22 m*M* *n*-octanoylsucrose (Hampton Research) equilibrated against 100 m*M* HEPES pH 7.0 containing 25% PEG 1000 and 30% ethylene glycol. Clusters of crystals appeared in a few days and were successively micro-seeded and macro-seeded, which gave single crystals suitable for diffraction analysis. Typically, crystals grew to dimensions of 0.7 × 0.2 × 0.05 mm in a week (Fig. 1).

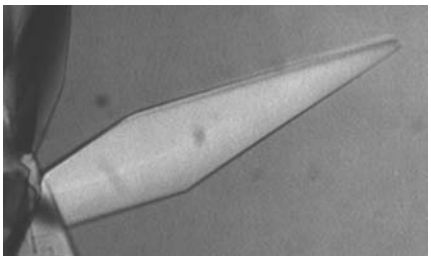

Figure 1
A native crystal of the Rho-binding domain of bovine Rho-kinase. The approximate dimensions of this crystal are 0.7 × 0.2 × 0.05 mm.

4. Data collection and processing

Crystals were mounted in a cryo-loop and flash-cooled in a stream of nitrogen gas at 100 K. Intensity data were collected at 100 K using an R-AXIS IV imaging-plate detector with Cu $K\alpha$ X-rays generated by a rotating-anode generator FR (Rigaku). The crystals were found to diffract to a resolution of 2.0 Å and belonged to the space group *C*2, with unit-cell parameters a = 148.1 (2), b = 25.9 (1), c = 39.4 (1) Å, β = 90.1 (1)°. The diffraction data were processed with *DENZO* and *SCALEPAC*K (Otwinowski & Minor, 1997). Each data intensity $I(hkl)$ was evaluated from the imaging plates and transformed to the amplitude of the structure factor $F(hkl)$. Under zero σ-cutoff conditions, we observed 64 203 reflections containing 9933 unique reflections to a resolution of 2.0 Å (the outermost shell was 2.07–2.00 Å and contained 833 unique reflections); a completeness of 94.7% was achieved (81.7% for the outermost shell) and an $R_{\rm merge}$ of 5.8% was observed (22.6% for the outermost shell). The multiplicity of reflections was 3.5 (2.2 for the outermost shell). The RhoBD crystal was estimated to contain two molecules in the asymmetric unit (Z = 8) and to have a V_m value of 2.36 Å^3 Da^{-1} (Matthews, 1968). The solvent content of the crystal was about 48%.

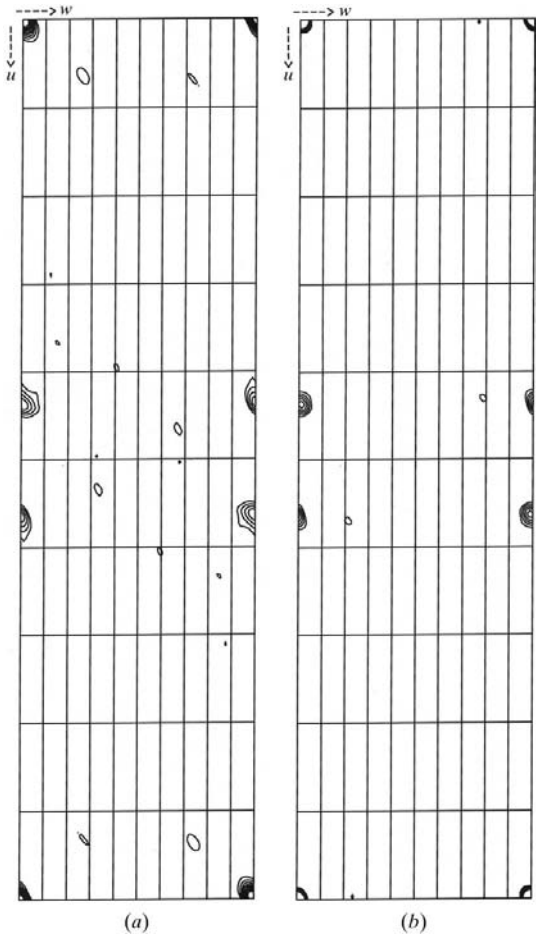

Figure 2
The v = 0 Harker sections of the (*a*) Bijvoet (λ_2) and (*b*) dispersive ($\lambda_1 - \lambda_3$) difference Patterson functions. The resolution range is 10–2.5 Å and the contour level is 3–20σ with 2σ intervals.

As attempts to find heavy-atom derivatives had failed because of their high-order non-isomorphism, we used selenomethionyl RhoBD and its crystals, which were obtained as outlined above. The number of methionines per monomer is one. X-ray intensity data were collected with FUJI large X-ray image-plates (400 × 800 mm) using synchrotron radiation at the beamline BL-18B of the Photon Factory (PF; Tsukuba, Japan; Watanabe *et al.*, 1995). Use of synchrotron radiation allowed collection of high-resolution diffraction data beyond a resolution of 1.5 Å; we calculated phases using four-wavelength anomalous dispersion data (λ_1 = 0.9600, λ_2 = 0.9778, λ_3 = 0.9784, λ_4 = 1.0000 Å). The best crystal had unit-cell parameters a = 148.0 (2), b = 26.1 (1), c = 39.6 (1) Å, β = 90.3 (1)°. Under zero σ-cutoff conditions, we observed 90 227 reflections containing 22 368 unique reflections to a resolution of 1.5 Å (the outermost shell extended from 1.55 to 1.50 Å and contained 1844 unique reflections), with a completeness of 90.6% (75.8% for the outermost shell) and an $R_{\rm merge}$ of 2.6% (14.3% for the outermost shell). The multiplicity of reflections was 3.4 (2.2 for the outermost shell). Both Harker sections of Patterson functions, which were calculated using anomalous differences between Bijvoet pairs in λ_2 and dispersive differences between λ_1 and λ_3, clearly show only one peak originated from Se atom (Fig. 2). Because the expected number of selenomethionines in the asymmetric unit is two, an overlap of two selenomethionine sites in Patterson space must be occurring. Determination of the crystal structure is currently in progress.

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