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Purification, crystallization and preliminary X-ray analysis of the plectin actin-binding domain

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Plectin is an abundantly expressed cytoskeletal crosslinking protein of enormous size (>500 kDa) and multiple functions. It represents one of the many members of a large family of actin-binding proteins. The actin-binding domain of mouse plectin was expressed in *Escherichia coli* and purified to homogeneity. Crystals of the actin-binding domain of plectin were prepared by the hanging-drop method. They belong to space group $P2_1$, with unit-cell parameters a = 55.92, b = 108.92, c = 63.75 Å, β = 115.25°. Data from a single crystal were collected to 2.0 Å resolution at room temperature using synchrotron radiation at EMBL, Hamburg. The asymmetric unit contains two molecules of the protein, which corresponds to V_M = 3.06 Å^3 Da^{-1} and a solvent content of 60%. The structure was solved by the molecular-replacement method. In addition, the preparation of selenomethionine-derivative crystals is described.

1. Introduction

Plectin and its isoforms are versatile cytolinker proteins of very large size (molecular mass over 500 kDa) which are expressed in a wide variety of mammalian tissues and cell types. Biochemical data indicate that plectin plays an important role in cytoskeleton network organization and regulation, with consequences for viscoelastic properties of the cytoplasm and the mechanical integrity and resistance of cells and tissues. Plectin self-associates and interacts with subcomponents of all three major cytoskeletal filament networks (intermediate filaments, microfilaments and microtubules), the subplasma membrane-protein skeleton and a variety of plasma membrane–cytoskeleton junctional complexes. It was discovered recently that plectin also plays a role in signal transduction and regulation of cellular plasticity and morphogenesis (for reviews, see Wiche, 1998; Steinböck & Wiche, 1999). Defects in plectin genes cause autosomal recessive or dominant hereditary diseases, characterized by severe skin blistering with or without muscular dystrophy (for reviews, see Rouan *et al.*, 2000; Bauer *et al.*, 2001).

Plectin has been well characterized biochemically and genetically. Electron microscopy has revealed that the protein has a dumbbell-like structure comprising a central rod domain (approximately 2000 Å long) flanked by N- and C-terminal globular domains (Foisner & Wiche, 1987). Each of these domains contains several subdomains to which binding sites for various interaction partners have been mapped. So far, about 30 different proteins (vimentin, cytokeratins, desmin, actin, fodrin, desmoplakin, integrin β4 and others) have been found to directly interact with plectin. Plectin's actin-binding domain (ABD) is located in proximity to its N-terminus. It consists of two subdomains called calponin homology (CH) 1 and 2 domains. Actin-binding domains of similar type were found in a large family of actin-binding proteins, among them β-spectrin, dystrophin, utrophin and fimbrin (for a review, see Stradal *et al.*, 1998). Plectin counteracts actin stress-fibre formation and thus positively controls cellular processes requiring actin-filament dynamics. Recombinant versions of plectin's ABD have been shown to bind to actin with a stoichiometry of 1:1 and K_d = 0.32 μM (Andrä *et al.*, 1998; Fontao *et al.*, 2001). The aim of this work was to prepare crystals and collect data for structure determination of the plectin ABD in order to better understand plectin functions involving the interaction of this domain with other proteins.

2. Protein purification

The plectin fragment containing the ABD extended from amino-acid residue 182 to residue 417 (Fuchs *et al.*, 1999; accession No. AF 188008). The cDNA encoding this fragment contained an additional 21 nucleotides (encoding the sequence GSHMEFD) at its 5′

end and six nucleotides (EF) at its 3′ end owing to the cloning strategy used. The recombinant protein that was crystallized consisted of 245 amino-acid residues in total. For protein expression we used pBN120 (Nikolic *et al.*, 1996), a modified version of expression vector pET15a.

Escherichia coli BL21 (DE3) was grown in 800 ml of TB medium containing 100 µg ml^{-1} ampicillin and 1% glucose at 210 K. At OD_{600} = 0.5, expression of ABD was induced by adding 1 m*M* IPTG and 1% lactose. The cells were harvested 3 h post-induction by centrifugation and resuspended in 20 ml of lysis buffer (50 m*M* Tris–HCl pH 9.0, 1% Triton X-100 and 1 m*M* EDTA). Cells were sonicated by applying 3 × 5 min pulses at a 50% cycle and 80% energy (Bandelin Electronic).

After centrifugation (10 min at 20 000*g*) the supernatant was applied to a 7.5 ml HisBindResin (Novagen) column equilibrated with 50 m*M* Tris–HCl pH 9.0 and 20% glycerol. After washing, protein was eluted with buffer containing 50 m*M* Tris–HCl pH 9.0, 20% glycerol, 2 m*M* EDTA and 250 m*M* histidine; $(His)_6$ peptide was removed from the recombinant fusion protein by treatment with thrombin protease at 210 K in elution buffer. The purified protein without $(His)_6$ was dialyzed against $(NH_4)HCO_3$ pH 9.0, concentrated using an Amicon cell (10 YM membrane) and lyophilized. Homogeneity of the protein was assessed by 15% SDS–PAGE and Coomassie Blue staining (Fig. 1).

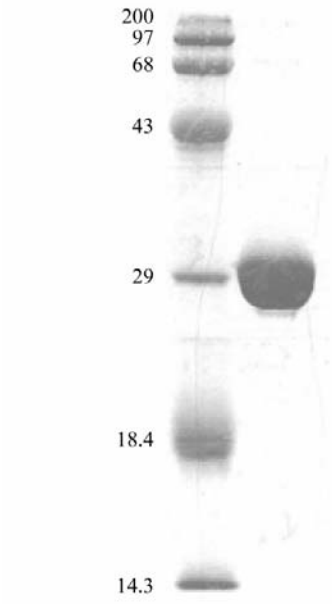

Figure 1
Coomassie-stained SDS–PAGE of plectin ABD. The left lane contains molecular-weight markers (kDa).

3. Preparation of selenomethionine derivative

In parallel with the native form, a selenomethionine derivative of the plectin ABD was prepared and crystallized for multi-wavelength anomalous diffraction (MAD). Selenomethionine-containing protein was produced in *E. coli* strain BL21 (DE3) by inhibition of the methionine pathway (Van Duyne *et al.*, 1993). An aliquot (1.8 ml) of a frozen stock solution of cells was added to 1 l of minimal medium M9 supplemented with 0.4% glucose and 100 µg ml^{-1} ampicillin. When cells had grown to OD_{600} = 0.6, protein expression was induced with 1 m*M* IPTG, 0.8% lactose and nitrogen base for yeast (Difco) and the culture was grown for another 20 h. Lysine, phenylalanine and threonine (to a concentration of 100 mg l^{-1}), isoleucine, leucine and valine (50 mg l^{-1}) and L-selenomethionine (50 mg l^{-1}) were added 15 min before induction. Purification of the selenomethionyl ABD was similar to that of the native protein, except that the washing buffer was enriched to 100 m*M* NaCl and 2 m*M* DTT was added to all solutions to prevent oxidation of the selenomethionyl protein. The efficiency of the selenomethionine-labelling procedure was scrutinized by MALDI mass spectrometry. A shift of +351.65 Da was found for the intact labelled protein, corresponding well to the predicted additional mass of 328 Da (seven methionine residues per chain). ABD containing sulfur methionine was undetectable in the mass spectrum (Fig. 2).

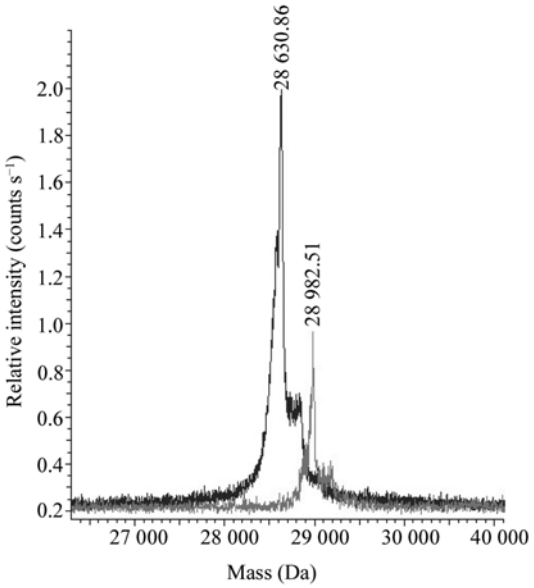

Figure 2
The matrix-assisted laser desorption (MALDI) mass-spectrometric analysis of selenomethionyl plectin ABD. Expected molecular masses of 28 655 (native protein) and 28 983 Da (selenomethionine derivative) are in a good agreement with observed values of 28 631 and 28 983 Da, respectively.

4. Crystallization

Crystals of the plectin ABD were grown by the hanging-drop diffusion method at room temperature. Initial crystallization conditions were found using the screening method of Jancarik & Kim (1991). Microcrystals appeared under several conditions, which were further optimized. The best crystals were grown under the following conditions: lyophilized protein was dissolved in 0.1 *M* NH_4HCO_3 pH 9.0 or Tris–HCl pH 9.0 to a concentration of 2 mg ml^{-1}. The precipitant solution contained 10%(*w*/*v*) PEG 8000 and 0.1 *M* Tris–HCl pH 8.5–9.0. Drops (4 µl) containing protein and precipitant solution in a 1:1 ratio were equilibrated against 0.9 ml of precipitant solution. Under these conditions, protein formed a precipitate from which relatively large crystals grew after a few days. Crystals were fragile and susceptible to radiation damage. They diffracted to 2.2–2.6 Å. Enrichment of precipitant solution by dioxane to a concentration of 2% improved the diffraction quality of the crystals. The dimensions of the crystal used for data collection were about 0.8 × 0.4 × 0.4 mm (Fig. 3).

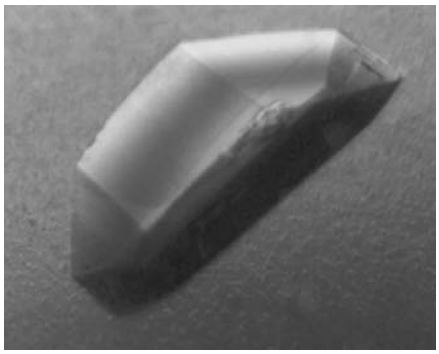

Figure 3
Crystal of plectin ABD (0.8 × 0.4 × 0.4 mm).

Selenomethionine-derivative crystals grew under similar conditions to native plectin ABD crystals, except that the protein solution was enriched by DTT to a concentration of 10 m*M* to prevent oxidation of the protein. 4–6%(*w*/*v*) PEG 4000 was used as a precipitant. Dimensions of the crystals were 0.4 × 0.2 × 0.2 mm two weeks after micro-seeding.

Table 1
Data-collection statistics.

Values in parentheses refer to the last resolution shell.

Wavelength (Å)	1.100
Resolution range (Å)	20.0–1.95 (1.97–1.95)
Space group	$P2_1$
Unit-cell parameters	
a (Å)	55.31
b (Å)	108.92
c (Å)	63.75
β (°)	115.25
Molecules in asymmetric unit	2
Mosaicity (°)	0.3
Unique reflections	47802 (1187)
Completeness (%)	96.2 (71.7)
$R(I)_{\rm merge}$† (%)	6.0 (40.0)
Redundancy	3.5 (2.2)
$I/\sigma(I)$	22.2 (2.1)

† $R(I)_{\rm merge} = \sum_h |I_h - \langle I\rangle| / \sum_h I_h$, where I_h is an individual intensity measurement and $\langle I\rangle$ is the average intensity for this reflection with summation over all data.

5. Data collection and preliminary X-ray diffraction analysis

Crystals of the recombinant plectin ABD were mounted in glass capillaries and data were collected at room temperature to 2.0 Å resolution on the EMBL X-31 beamline at the DORIS storage ring, DESY, Hamburg with a MAR 345 image-plate scanner. The wavelength used in data collection was 1.10 Å, the crystal-to-detector distance was 260 mm and the crystal rotation angle per image was 1.0°. Data were processed using *DENZO* and *SCALEPACK* packages (Otwinowski & Minor, 1997). Crystals belong to space group $P2_1$, with unit-cell parameters $a = 55.31$, $b = 108.92$, $c = 63.75$ Å, $\beta = 115.25°$. Data-collection statistics are given in Table 1.

The unit-cell volume (351 190 Å^3), the molecular mass of the protein (28 655 Da) and the space group gave a crystal packing density $V_{\rm M}$ of 3.06 Å^3 Da^{-1}, with a solvent content of 60% for two protein molecules in the asymmetric unit (Matthews, 1968). Alternatively, three protein molecules in the asymmetric unit would correspond to a $V_{\rm M}$ of 2.04 Å^3 Da^{-1} and a solvent content of 40%.

In spite of the fact that the actin-binding protein family is large, in a FASTA search (Pearson & Lipman, 1988) using the amino-acid sequence of the plectin ABD, only the structures of the ABDs of utrophin (PDB code 1qag), dystrophin (1dxx) and T-fimbrin (1aoa) were found. The amino-acid sequence identities of these proteins with plectin ABD are 48, 47 and 23%, respectively. The plectin ABD structure was solved by the molecular-replacement method using *MOLREP* (Vagin & Teplyakov, 1997) with the utrophin ABD (Keep *et al.*, 1999; PDB code 1qag) as the search model. Based on differences in the orientation of the CH1 *versus* CH2 subdomains in the structures of utrophin, dystrophin and fimbrin, one might also expect differences for plectin and therefore the CH1 and CH2 subdomains were used as individual search models. *MOLREP* was used twice with CH1 and twice with CH2 as models. In each subsequent step, the solution from the previous run was fixed. In the four steps, the R factors were 57.6, 52.5, 45.4 and 38.0% and the corresponding correlation coefficients were 24.6, 38.8, 54.3 and 68.1%. Searching for additional protein molecules made the R factor and correlation coefficient worse, thus confirming the presence of two protein molecules in the asymmetric unit. Refinement of the structure is under way.

The diffracting power of selenomethionine-derivative crystals was tested using synchrotron radiation on EMBL beamline X11. The crystals diffracted to 3.0 Å resolution at 100 K. Precipitant solution enriched with glucose to a concentration of 25%(*w*/*v*) was used as a cryoprotectant. As the structure of the plectin ABD had already been solved by molecular replacement, data were not collected from the selenomethionine-derivative crystals.

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