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Crystallization and preliminary X-ray crystallographic studies of the PDZ domain of Shank1 from *Rattus norvegicus*

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Shank proteins are a new family of scaffold proteins interacting with various membrane and cytoplasmic proteins. Shank contains multiple protein–protein interaction sites, including ankyrin repeats, an SH3 domain, a PDZ domain, a long proline-rich region and an SAM domain. The PDZ domain of Shank binds to the C-terminus of guanylate kinase-associated protein (GKAP). The PDZ domain of Shank1 from *Rattus norvegicus* and its complex with the C-terminal octapeptide of GKAP were crystallized at 294 K using polyethylene glycol 20 000 and 6000 as precipitants. Diffraction data sets from a peptide-free crystal and a complex crystal were collected to 1.8 and 3.2 Å resolution, respectively, using synchrotron radiation. The peptide-free crystal belongs to space group $P2_1$, with unit-cell parameters a = 42.0, b = 50.3, c = 51.8 Å, β = 106.3°. The complex crystal belongs to space group $P2_12_12_1$, with unit-cell parameters a = 89.4, b = 97.5, c = 108.3 Å.

1. Introduction

Shank proteins, a new family of scaffold proteins localized in the postsynaptic density, bind to various membrane and cytoplasmic proteins in excitatory synapses (Naisbitt *et al.*, 1999). It has been suggested that Shank connects *N*-methyl-D-aspartate receptor–PSD-95 complexes to regulation of the actin cytoskeleton playing a critical role in the organization of cytoskeletal/signalling complexes at the excitatory synapses (Naisbitt *et al.*, 1999). The Shank family has three known members, Shank1, Shank2 and Shank3, containing multiple sites for alternative splicing and each showing a distinct tissue distribution (Sheng & Kim, 2000). Shank proteins, which have a marked heterogeneity in molecular mass (about 200 kDa), contain several protein–protein interaction domains, including ankyrin repeats, an SH3 (Src homology 3) domain, a PDZ domain, a long proline-rich region and a SAM (sterile α motif) domain. The proline-rich region commonly acts as binding sites for SH3, EVH1 (Ena/VSAP homology 1) and WW domains, mediating multiple sets of protein interaction. SAM domains of Shank can bind to each other in a homomeric and heteromeric fashion, enabling the oligomerization of Shank and its interacting proteins. PDZ domains are molecular-recognition elements that mediate protein–protein interactions (Sheng & Kim, 2000). It was originally discovered as a common motif present in three structurally related proteins: PSD-95 (postsynaptic density protein, 95 kDa), Dlg (*discs-large* protein) and ZO-1 (zonula occludens-1) (Garner *et al.*, 2000).

PDZ domains are globular domains containing about 80–100 amino acids. The PDZ domain of Shank, which belongs to the class I PDZ domains, recognizes the C-terminal sequence *X*-T/S-*X*-L; a variety of integral membrane proteins fit this consensus. It is well characterized that the Shank PDZ domain binds to the C-terminus of guanylate kinase-associated protein (GKAP), which interacts with the guanylate kinase domain of PSD-95 (Sheng & Sala, 2001). These specific interactions may play a role in the synaptic targeting and cytoskeletal attachment of the receptors by linking them physically and functionally to the appropriate intracellular signalling pathways (Naisbitt *et al.*, 1999). Here, to elucidate the structural mechanism of the protein–protein interaction of Shank PDZ domain with the C-terminus of GKAP, we subcloned the PDZ domain in a bacterial expression vector and synthesized the C-terminal octapeptide (IPEAQTRL) of GKAP for X-ray crystallographic studies. As the first step toward its structure determination, we report the overexpression, purification and crystallization of the PDZ domain and its complex with the octapeptide as well as preliminary X-ray characterization.

2. Materials and methods

2.1. Expression and purification

The DNA for the Shank PDZ domain (amino acids 584–691) was subcloned in the *Bam*HI site of the expression vector pGEX4T-1 (Pharmacia Biotech) and transformed into *E. coli* strain BL21(DE3). The

bacterial cells were induced by 1 m*M* isopropyl *β*-D-thiogalactopyranoside at 310 K for 2 h and harvested by centrifugation at 4500*g* for 20 min. Cells were resuspended in the lysis buffer (1× PBS, phosphate-buffered saline) and lysed using a French press; the lysate was centrifuged at 16 000*g* for 30 min. Glutathione-Sepharose 4B affinity resin (Peptron) was loaded onto a gravity-flow column (Bio-Rad) and pre-equilibrated with a lysis buffer (2 ml of bed volume per litre of culture). The supernatant was added to the column and the matrix was washed with ten bed volumes of the lysis buffer. The glutathione-S-transferase fusion protein was eluted by addition of 50 m*M* Tris–HCl pH 8.0, 10 m*M* glutathione. The PDZ domain was cleaved by thrombin and further purified by gel filtration on a Superdex 75 column (Pharmacia Biotech) pre-equilibrated with 50 m*M* Tris–HCl pH 7.4. The fractions containing the PDZ domain were collected and concentrated by ultrafiltration (Amicon Centricon 3). The C-terminal octapeptide (IPEAQTRL) of the GKAP was chemically synthesized (Anygen).

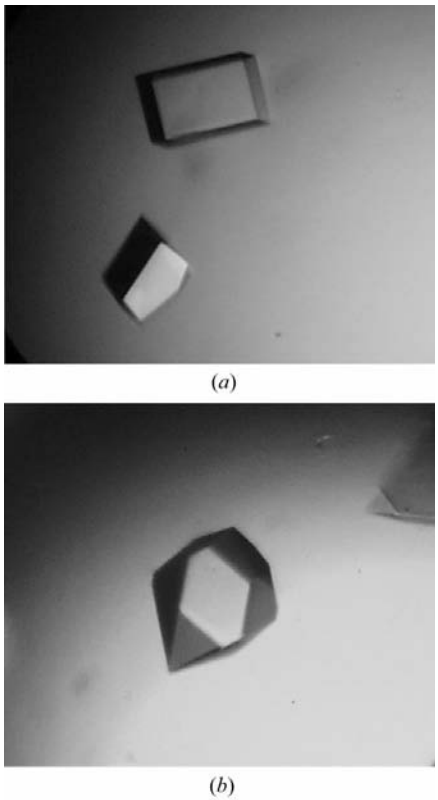

Figure 1
(*a*) Crystals of the Shank1 PDZ domain grown for 1 d using 10% PEG 20 000, 0.2 *M* NaBr, 20% glycerol and 100 m*M* sodium acetate pH 4.5. Its approximate dimensions are 0.1 × 0.15 × 0.25 mm. (*b*) Crystal of Shank1 PDZ complexed with the GKAP C-terminal octapeptide grown for 2 d using 16% PEG 6000 pH 6.5 as a precipitating agent. Its approximate dimensions are 0.2 × 0.2 × 0.2 mm.

2.2. Crystallization and data collection

The PDZ domain was crystallized at room temperature (294 ± 1 K) using the hanging-drop vapour-diffusion method. Crystals were grown on a siliconized cover slip by equilibrating a mixture containing 2 µl of protein solution (12 mg ml^{-1} protein in 50 m*M* Tris–HCl pH 7.4) and an equal volume of well solution [10%(*w*/*v*) PEG 20 000, 0.2 *M* NaBr, 20%(*v*/*v*) glycerol and 100 m*M* sodium acetate pH 4.5) against 0.5 ml of well solution. Crystals appeared from the precipitate after 1 d and grew to dimensions of 0.1 × 0.15 × 0.25 mm (Fig. 1*a*). Crystals of the PDZ–peptide complex were grown by equilibrating a mixture composed of 2 µl of protein solution containing the synthesized peptide (12 mg ml^{-1} protein in 50 m*M* Tris–HCl pH 7.4 plus 2 m*M* peptide) and an equal volume of reservoir solution [16%(*w*/*v*) PEG 6000, 0.2 *M* KCl and 100 m*M* MES–NaOH pH 6.5] against 0.5 ml of reservoir solution. Crystals of the PDZ–peptide complex grew over 2 d to a maximum dimension of 0.2 mm (Fig. 1*b*). Successful flash-freezing was achieved when the crystals were transferred directly to the reservoir solution containing 20% glycerol. Multiwavelength anomalous dispersion (MAD) data were collected from a single peptide-free crystal containing bromine, included in the crystallization conditions, to 1.8 Å resolution at 100 K with an ADSC Quantum 4R CCD detector at beamline BL-18B, Photon Factory, Japan. Diffraction data were collected at the inflection point (λ = 0.9205 Å), the peak (λ = 0.9201 Å) and a high-energy remote wavelength (λ = 0.9180 Å) by scanning the fluorescence signal of bromine. Diffraction data from a complex crystal were collected to 3.2 Å resolution. Single crystals were soaked in cryoprotectant (16% PEG 6000, 0.2 *M* KCl, 100 m*M* MES–NaOH pH 6.5 and 20% glycerol) for 30 s and flash-frozen in liquid nitrogen. Diffraction images were processed using the *MOSFLM* program suite (Leslie, 1994) and the structural factors were scaled and reduced using the *SCALA* program from the *CCP*4 package (Collaborative Computational Project, Number 4, 1994).

2.3. Identification of protein–peptide complex crystal

Complex formation with octapeptide in the crystal was identified by HPLC analysis. The crystals grown in the protein–peptide mixture were collected and washed with reservoir solution. The samples for HPLC analysis were prepared by solubilizing the harvested crystals in deionized water containing 0.1% trifluoracetic acid. Verification of complex formation was performed by comparing the elution profile with those of the protein and the protein–peptide solution used for the crystallization.

3. Results

The MAD data of peptide-free crystal were collected to 1.8 Å resolution. Bromine is a very convenient anomalous scatterer, with a *K* absorption edge at 0.920 Å. For the MAD experiment, bromine was incorporated by substituting the 0.2 *M* NaCl by 0.2 *M* NaBr in the crystallization solution. From this

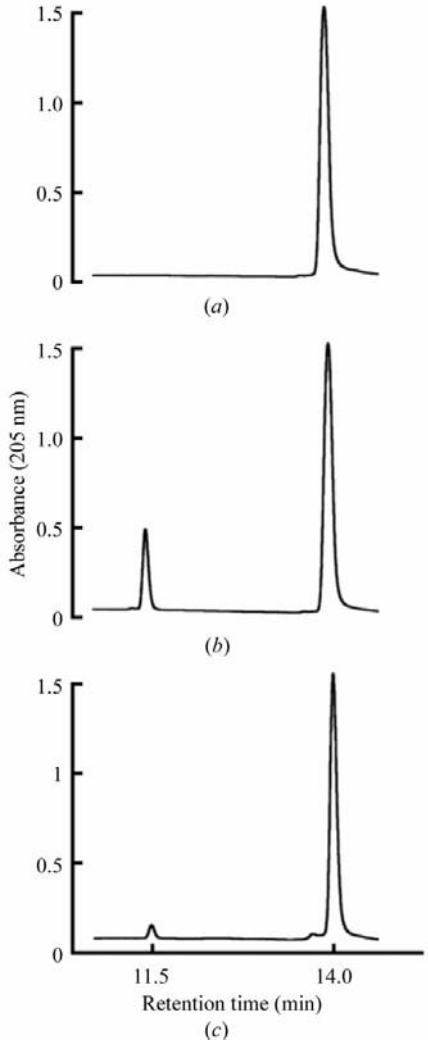

Figure 2
Identification of protein–peptide complex crystal by HPLC analysis. The samples were analyzed using an ODS (octadecyl silica) column with a continuous gradient of 5–95% acetonitrile in deionized water containing 0.1% triflouracetic acid. (*a*) Protein solution used for crystallization, (*b*) protein–peptide mixture solution, (*c*) complex crystal.

Table 1
Summary of data statistics.

Values in parentheses indicate the statistics for the last resolution shell.

	Peptide-free crystal (MAD)			PDZ–peptide complex
X-ray source	BL-18B (PF)			BL-18B (PF)
Wavelength (Å)	0.9205	0.9201	0.9180	1.0000
Resolution range (Å)	27.7–1.8	27.7–1.8	27.7–1.8	47.3–3.2
No. of observations	58394	58363	58396	158199
Unique reflections	19164	19164	19165	16204
Completeness (%)	99.0 (99.0)	99.0 (99.0)	99.0 (99.0)	99.9 (99.9)
Mean $I/\sigma(I)$	13.8 (2.7)	10.1 (2.3)	9.8 (2.1)	6.2 (2.8)
$R_{\rm merge}$† (%)	4.8 (37.7)	4.8 (32.6)	4.9 (33.7)	9.2 (26.0)

† $R_{\rm merge} = \sum_h \sum_i |I(h)_i - \langle I(h)\rangle| / \sum_h \sum_i I(h)_i$, where $I(h)$ is the intensity of reflection h, $\sum_h$ is the sum over all reflections and $\sum_i$ is the sum over i measurements of reflection h.

modified solution crystals appeared as readily as from the standard NaCl-containing crystallization solution. The crystal belongs to space group $P2_1$, with unit-cell parameters $a = 42.0$, $b = 50.3$, $c = 51.8$ Å, $\beta = 106.3^\circ$. The calculated molecular weight of single molecule is 12 174 Da. Assuming two molecules per asymmetric unit, the Matthews coefficient ($V_{\rm M}$) was calculated to be 2.18 Å^3 Da^{-1}, corresponding to a solvent content of 56.4% (Matthews, 1968). The location of four Br atoms in the asymmetric unit and the initial phases were calculated using the program *SOLVE* (Terwilliger & Berendzen, 1997). The phases were further improved using the program *RESOLVE* (Terwilliger, 2000), with a figure of merit of 0.62. The resulting electron-density map clearly showed the two molecules in an asymmetric unit and the quality of the initial map was high enough to build most of the residues. The model building and refinement of the Shank PDZ domain is ongoing.

Complex crystals grew over 2 d to a maximum dimension of 0.2 mm in a protein–peptide mixture with a molar ratio of 1:2 in the crystallization solution. Complex formation with the octapeptide was identified by HPLC analysis (Fig. 2). A data set from a complex crystal was collected to 3.2 Å resolution. The crystal belongs to space group $P2_12_12_1$, with unit-cell parameters $a = 89.4$, $b = 97.5$, $c = 108.3$ Å. Assuming six or eight molecules per asymmetric unit, the Matthews coefficient ($V_{\rm M}$) was calculated to be 3.05 or 2.27 Å^3 Da^{-1}, corresponding to solvent contents of 59.6 or 45.8%, respectively. Table 1 summarizes the statistics of the data collection.

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