Expression, purification, crystallization and preliminary X-ray crystallographic analysis of the SH3 domain of human AHI1

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The SH3 domain of human AHI1 was cloned and expressed in *Escherichia coli*. The protein was purified by affinity and size-exclusion chromatography and was crystallized using the sitting-drop vapour-diffusion method at 293 K. A complete data set was collected to 2.5 Å resolution at 110 K. The crystal belonged to space group $P4_12_12$, with unit-cell parameters $a = 67.377$, $b = 67.377$, $c = 98.549$ Å.

1. Introduction

Joubert syndrome (JS) is an autosomal recessive disorder marked by agenesis of the cerebellar vermis, ataxia, hypotonia, oculomotor apraxia, neonatal breathing abnormalities and mental retardation (Dixon-Salazar *et al.*, 2004; Parisi *et al.*, 2007; Sheng *et al.*, 2008). Mutations in the Abelson helper integration site 1 gene (AHI1 gene), which was originally identified in mouse as a common helper provirus integration site for murine leukaemias and lymphomas, have been shown to cause Joubert syndrome (Utsch *et al.*, 2006; Valente *et al.*, 2006; Kroes *et al.*, 2008; Sheng *et al.*, 2008). The human AHI1 gene encodes a 1196 amino-acid protein (AHI1 or jouberin), which is comprised of an N-terminal coiled-coil domain, seven WD40 repeats, an SH3 domain and several potential SH3-binding sites (Jiang *et al.*, 2002; Sheng *et al.*, 2008).

SH3 domains play critical roles in a wide variety of biological processes, including regulation of enzymes by intramolecular interactions, increasing the local concentration or altering the subcellular localization of components of signalling pathways and mediating the assembly of large multiprotein complexes (Mayer, 2001). Most mutations of the AHI1 gene in JS are nonsense or frame-shift mutations, which result in N-terminally truncated AHI1 or loss of WD40 repeats and the SH3 domain (Dixon-Salazar *et al.*, 2004; Ferland *et al.*, 2004). However, the molecular basis of how the loss-of-function mutations in AHI1 cause Joubert syndrome remains to be elucidated.

Recently, AHI1 has been reported to interact with huntingtin-associated protein 1 (Hap1) to regulate cerebellar and brain-stem development in mice (Sheng *et al.*, 2008). This research shed new light on the investigations of Joubert syndrome. Further insight could be provided if an atomic structure of HI1 were available. As an initial effort towards this goal, we have chosen the SH3 domain as our research object. This domain is rather unique since it contains the key conserved residues but shares little amino-acid sequence identity with the 'classical' SH3 domains of Abl, Fyn and Lck (Fig. 1; Musacchio *et al.*, 1994; Carrera *et al.*, 1995). Here, we report the successful purification, crystallization and preliminary X-ray analysis of the human AHI1 SH3 domain (hAHI1 SH3).
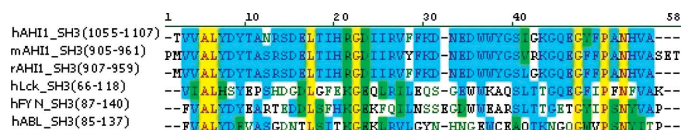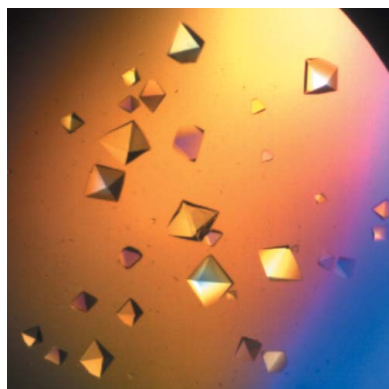
Figure 1
Sequence alignment of SH3 domains from AHI1, Lck, Fyn and Abl.

doi:10.1107/S174430910900774X   **361**
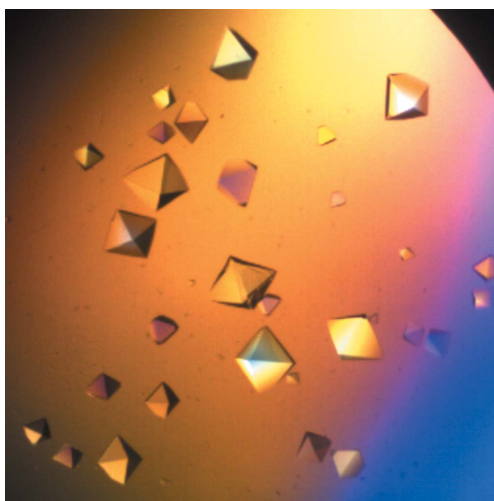
2. Materials and methods

2.1. Cloning

The human AHI1 SH3-domain expression vector SH3-pET-28(a) was constructed as follows. The cDNA sequence encoding the mature SH3 domain (amino-acid residues 1047–1116) was amplified from full-length human AHI1 cDNA via PCR. The forward (5′-ATAA-**GGATCC**CATCAGGTAGATACAGCACCAAC-3′) and reverse (5′-ACCT**GTCGAC**TTAAGGCAGTTCTTGATACAGTG-3′) primers used for PCR amplification were designed to introduce a BamHI site at the N-terminus and a SalI site with a stop codon at the C-terminus. The PCR product was purified using a Gel Extraction Kit (Qiagen, Germany), digested with BamHI and SalI and ligated into the pET-28(a) vector. The sequence of the SH3 domain of the AHI1 gene was confirmed by DNA sequencing (Invitrogen, USA).

2.2. Expression and purification

Escherichia coli BL21 (DE3) SH3-pET-28(a) transformants were inoculated into 100 ml LB medium containing 50 µg ml^{-1} ampicillin and incubated with shaking overnight at 310 K and 220 rev min^{-1}. 20 ml of this culture was added to 2 l fresh culture medium and the culture was grown in a shaker (180 rev min^{-1}) at 310 K. When the absorbance at 600 nm reached around 0.7, 200 µl 1 M IPTG was added. For production of the SH3 domain, the culture was incubated for a further 18 h at 289 K. After centrifugation, the bacterial pellets were stored frozen.

The bacterial pellets were resuspended in 40 ml precooled lysis buffer (20 mM Tris–HCl pH 8.0, 300 mM NaCl, 1 mM EDTA, 10% glycerol, 20 mM imidazole) containing 1 mM PMSF. The suspension was sonicated 200 times for 5 s each on ice and centrifuged at 20 000g for 30 min at 277 K. After the cell extracts had been incubated with 3 mg Ni–NTA agarose resin (Novagen) for 3 h at 277 K, the resin was loaded into a column, washed with 15 ml washing buffer (20 mM Tris–HCl pH 8.0, 300 mM NaCl, 1 mM EDTA, 10% glycerol, 40 mM imidazole) and the target protein was eluted with 10 ml elution buffer (20 mM Tris–HCl pH 8.0, 300 mM NaCl, 1 mM EDTA, 10% glycerol, 200 mM imidazole). The fractions obtained were analyzed by 15% SDS–PAGE and the eluted fraction was dialyzed against 20 mM Tris–HCl pH 8.0, 50 mM NaCl to remove imidazole. After filtration with a 0.22 µm filter (Millipore), the protein solution was concentrated to 10 mg ml^{-1} in a 10K concentrator (Millipore) at 3000g. The protein concentration was estimated using Quant-iT Protein Assay Kits (Invitrogen, USA).

The concentrated protein was loaded onto a preparative Superdex 75 size-exclusion column (GE Healthcare) equilibrated with 20 mM Tris–HCl pH 8.0, 50 mM NaCl and run at 1.0 ml min^{-1} on an ÄKTA purification system (GE Healthcare). The protein purity was checked by 15% SDS–PAGE and fractions containing purified SH3 domain were concentrated to 10 mg ml^{-1} in a 10K concentrator at 3000g.
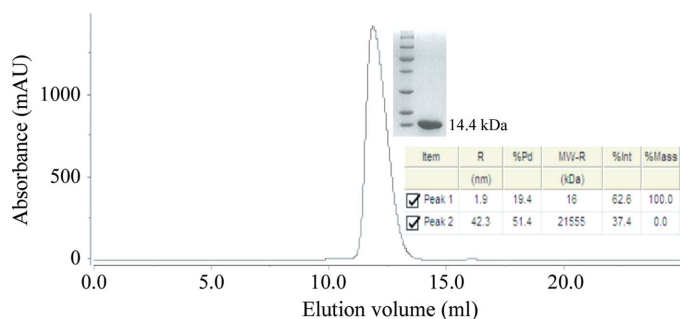
Dynamic light-scattering (DLS) analysis was carried out using a DynaPro-MSXTC molecular-sizing instrument (Protein Solutions). The concentrated protein was diluted to 2 mg ml^{-1} and centrifuged at 15 000g for 15 min at 277 K to remove particulate materials. The solution properties of the purified protein were monitored and data were acquired from 50 scattering measurements at 277 K. Five sets of data were analyzed using the DYNAMICS software package (Protein Solutions Inc.) and were averaged.

2.3. Crystallization

In crystallization trials, His-tagged SH3 was used at a concentration of 10 mg ml^{-1} in a buffer consisting of 20 mM Tris–HCl pH 8.0 and 50 mM NaCl. Commercial crystallization kits (Hampton Research Crystal Screens I and II from Hampton Research, USA) were screened using the sitting-drop vapour-diffusion method. All drops consisted of 1 µl protein solution and 1 µl reservoir solution and were equilibrated against 0.1 ml reservoir solution at a temperature of 293 K. Small crystals appeared within one week in several conditions. Crystallization conditions were then manually optimized with home-made solutions. 2.5 µl protein solution was mixed with 2.5 µl reservoir solution consisting of 0.2 M ammonium sulfate, 0.1 M sodium acetate trihydrate pH 4.6, 30%(w/v) polyethylene glycol monomethyl ether 2000 and equilibrated over 800 µl reservoir solution, leading to large crystals (Fig. 2).

2.4. X-ray diffraction

Crystals were transferred to a series of mother-liquor solutions containing 5, 10, 15 and 20%(v/v) glycerol as cryoprotectant. The looped crystals were then placed in a cold nitrogen stream maintained at 110 K. X-ray data were collected on a Gemini R Ultra with a 135 mm diagonal Ruby CCD detector (Oxford Diffraction, England). The crystal-to-detector distance was 55 mm. A total of 103 frames were collected with 240 s exposure per frame. The data were indexed, integrated and scaled using the CrysAcispro program package (Oxford Diffraction, England) and the CCP4 program suite (Collaborative Computational Project, Number 4, 1994).

Figure 2
Crystals of recombinant hAHI1 SH3 domain obtained using the sitting-drop method.

Figure 3
Size-exclusion chromatography and dynamic light-scattering (DLS) analysis of the SH3 domain.

Table 1
Data-collection statistics.

Values in parentheses are for the highest resolution shell.

Wavelength (Å)	1.5418
Temperature (K)	110
Crystal-to-detector distance (mm)	55
Unit-cell parameters (Å)	$a = b = 67.377$, $c = 98.549$
Space group	$P4_12_12$
Resolution range (Å)	19.9–2.50 (2.59–2.50)
Observed reflections	112374
Unique reflections	8299 (1922)
Redundancy	6.7 (7.4)
Completeness (%)	99.4 (98)
$I/\sigma(I)$	8.56 (2.09)
R_{merge} (%)	12.6 (36.2)

3. Results

The expression of His-tagged SH3 domain of hAHI1 in BL21 (DE3) cells resulted in soluble protein. Recombinant protein was purified in a two-step procedure using affinity and size-exclusion chromatography to give a final yield of 30 mg highly purified protein per litre of culture medium. The molecular weight of the monomeric form was estimated to be 14.4 kDa by SDS–PAGE. The protein eluted from the Superdex 75 size-exclusion chromatography column with an apparent molecular weight of 14.4 kDa, corresponding to the molecular weight of the monomer. DLS analysis revealed a monomodal distribution, with a polydispersity value of 19.4% and an estimated molecular weight of 16 kDa, which is in good agreement with the results of the SDS–PAGE analysis (Fig. 3).

The sample was 95% pure as estimated by SDS–PAGE. High-throughput screening of initial conditions resulted in several leads. After extensive optimization of the crystallization conditions, crystals suitable for diffraction experiments were obtained in 0.2 M ammonium sulfate, 0.1 M sodium acetate trihydrate pH 4.6, 30%(w/v) polyethylene glycol monomethyl ether 2000.

The crystal diffracted to 2.5 Å resolution and belonged to the tetragonal space group $P4_12_12$, with unit-cell parameters $a = 67.377$, $b = 67.377$, $c = 98.549$ Å, $\alpha = \beta = \gamma = 90°$. Data-collection statistics are reported in Table 1. With one monomer in the asymmetric unit, the Matthews volume (V_M; Matthews, 1968) was calculated to be 1.94 Å^3 Da^{-1} and the estimated solvent content was 36.69%. Further structure determination using the molecular-replacement method is in progress.

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