# crystallization papers

# Crystallization and preliminary crystallographic characterization of an SH3 domain from the IB1 scaffold protein

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IB1 is a mammalian scaffold protein that interacts with components of the c-Jun N-terminal kinase (JNK) signal-transduction pathway mainly *via* its protein–protein interaction domains. Crystallization of the key Src homology 3 (SH3) domain of IB1 has been achieved. Crystallization experiments with unmodified protein and deliberately oxidized protein have led to different crystal forms. X-ray data have been collected to 3.0 Å resolution from a crystal form with rectangular prism morphology. These crystals are orthorhombic ($P2_12_12_1$), with unit-cell parameters $a = 45.9$, $b = 57.0$, $c = 145.5$ Å. These are the first crystallographic data on a scaffold molecule such as IB1 to be reported.

## 1. Introduction

Scaffold proteins can be defined as proteins with no intrinsic enzymatic activity that associate with two or more partners to enhance the efficiency and possibly the specificity of cellular signalling pathways (Hall & Lefkowitz, 2002). One class of scaffold proteins is the type that organizes the three-component mitogen-activated protein kinase (MAPK) signalling pathway. This pathway is found in all eukaryotic cells (Widmann *et al.*, 1999). One member of the class is the islet brain 1 (IB1) protein, a mammalian scaffold protein that is mainly expressed in pancreatic islets and in the brain (Bonny *et al.*, 1998). IB1 binds to the MLK3, MKK7 and JNK kinases and sequential activation of these enzymes eventually results in the activation of specific transcription factors (Whitmarsh *et al.*, 1998; Yasuda *et al.*, 1999). A mechanism for the assembly and regulation of the dynamic IB1-JNK module has recently been proposed (Nihalani *et al.*, 2003).

IB1 is known to play a major role in human diseases. An S59N mutation is believed to be associated with type 2 diabetes (Waeber *et al.*, 2000). Furthermore, it is known that IB1 reduces cytokine-induced apoptosis of insulin-secreting cells (Bonny *et al.*, 2000; Haefliger *et al.*, 2003). IB1 is also linked with Alzheimer's disease, as it interacts specifically with the amyloid precursor protein (APP; Matsuda *et al.*, 2001).

IB1 contains several protein–protein interaction domains. These include a JNK-binding domain (JBD), a phosphotyrosine-interaction domain (PID) and a polyproline-binding SH3 domain (Negri *et al.*, 2000). The latter is functionally important and is also responsible for homo-dimerization of IB1 (Bonny *et al.*, unpublished data). Thus, this domain has received particular attention as a therapeutical target and a detailed structural description of its spatial organization could be of value in structure-based design of potential drugs.

In this paper, we describe the crystallization of the 71-amino-acid SH3 domain (IB1-SH3) and the results of preliminary X-ray crystallographic characterization.

## 2. Materials and methods

### 2.1. Expression and purification

The ib1-sh3 cassette was subcloned as a GST-fusion construct in a pGEX-4T vector (Bonny *et al.*, 1998). The plasmid was transformed into chemical competent cells, Rosetta BL21 (DE3) (Novagen), and grown in LB medium containing chloramphenicol and ampicillin (34 and 100 µg ml$^{-1}$, respectively). After 4.5 h expression, the protein was induced by adding isopropyl-$\beta$-D-thiogalactopyranoside to a final concentration of 1 m$M$. The cells were incubated for a further 2.5 h at 310 K.

The expression of selenomethionine-substituted protein from the same construct followed a previously published procedure (Van Duyne *et al.*, 1993). All subsequent steps were the same for the wild-type and seleno-methionine-substituted proteins.

The cells were harvested by centrifugation and stored on ice before disruption in starting buffer [140 m$M$ NaCl, 2.7 m$M$ KCl, 10 m$M$ Na$_2$HPO$_4$, 1.8 m$M$ KH$_2$PO$_4$, 0.50 m$M$ phenyl-methylsulfonyl fluoride (PMSF), 4 µg ml$^{-1}$ DNAse pH 7.3] containing 0.3% Igepal CA-630. Most of the cell debris was removed by centrifugation (40 000$g$, 15 min) and the remainder was removed by passing the supernatant through a 0.22 µm microfilter (Millex-GV, Millipore).

The supernatant was applied to a 5 ml glutathione *S*-transferase (GST) column (Amersham Pharmacia) at room temperature. The column was then washed with 250 ml phosphate-buffered saline (PBS) buffer (140 m$M$ NaCl, 2.7 m$M$ KCl, 10 m$M$ Na$_2$HPO$_4$, 1.8 m$M$ KH$_2$PO$_4$ pH 7.3) and eluted with standard GST-elution buffer (50 m$M$ Tris–HCl, 10 m$M$ reduced glutathione pH 8.0).

The protein was then precipitated by addition of ammonium sulfate to 70% saturation. After resuspension in a minimal volume of PBS buffer, the N-terminal GST tag was removed proteolytically by incubation with thrombin at room temperature. After another 70% ammonium sulfate precipitation, the protein was dissolved in a buffer suitable for gel filtration (5 m$M$ Tris–HCl, 100 m$M$ NaCl, 1 m$M$ dithiothreitol pH 8.0) and subjected to Superdex 200 (Amersham Pharmacia) chromatography.

## 2.2. Crystallization

The protein solution was concentrated directly from gel-filtration fractions to a concentration of 1.4–2.0 mg ml$^{-1}$ using 3 kDa cutoff membrane-filter devices (Microcon, Millipore). Initially, very small rectangular-shaped crystals were obtained after two months using Crystal Screen 1 (Hampton Research). Drops consisting of equal amounts (2 µl) of protein solution and reservoir solution (1.4 $M$ trisodium citrate dihydrate, 0.1 $M$ HEPES pH 7.5) were equilibrated using the hanging-drop vapour-diffusion technique over 200 µl reservoir solution. The very small size of these crystals, 10–20 µm in the largest dimension, presented difficulties in the mounting procedure and prevented data collection.

Numerous optimization experiments were carried out and larger crystals were obtained by addition of hydrogen peroxide to the protein stock solution to a final concentration of 0.3%. The larger crystals were obtained with the same reservoir solution contents as described above but at pH 6.5. Two different crystal forms were at first observed in the drops, one in which the edges are rounded (round form) and another with sharper edges (rectangular prism form; see Fig. 1).

Continued optimization experiments using hydrogen peroxide-treated protein led to a new trapezoidal prism crystal morphology. Trapezoidal prism crystals of oxidized IB1-SH3 selenomethionine protein (OxSeMetSH3) and native crystals were obtained using our standard gel-filtration buffer (excluding DTT) and reservoir solution conditions (pH 7.0).

Recently, crystallization of OxSeMetSH3 has been achieved using either 1.5 $M$ lithium sulfate, 0.1 $M$ HEPES pH 7.5 or 2%($v/v$) polyethylene glycol 400, 2.0 $M$ ammonium sulfate, 0.1 $M$ HEPES pH 7.5 as reservoir solutions. It is notable that these crystals were obtained within the pH range 6.5–7.5, which was also noted as the successful pH conditions in crystallization using sodium citrate.

## 2.3. Crystallographic data

A complete 3.0 Å resolution data set has been collected from a good-sized native crystal of the rectangular prism form. Data (102 frames in total) were collected on an ADSC Q210 detector at beamline ID29, ESRF (Grenoble) using a wavelength of 0.98 Å, an oscillation range of 1° and an exposure time of 10 s per frame. Systematic absences in the data clearly indicate that the crystal belongs to space group $P2_12_12_1$, with unit-cell parameters $a$ = 45.9, $b$ = 57.0, $c$ = 145.5 Å. The estimated solvent content is 56% ($V_M$ = 2.8 Å$^3$ Da$^{-1}$) with four molecules in the asymmetric unit using the Matthews formula (Matthews, 1968). Data-collection statistics are given in Table 1 and a native diffraction image is shown in Fig. 2.

Native crystals of the round crystal form did not diffract at all. The native trapezoidal prism crystals diffracted to around 4 Å, but suffered from inappropriate handling upon cryoprotection. Trapezoidal prism crystals of OxSeMetSH3 have not yet given useable diffraction; the weak diffraction is probably a result of the very small crystal dimensions (10 × 10 × 5 µm). In all cases, crystals were transferred to a cryosolution [1.26 $M$ trisodium citrate, 10%($v/v$) glycerol, 0.09 $M$ HEPES pH 7.5] before flash-freezing in liquid nitrogen. All data processing was performed using *DENZO* and *SCALE-PACK* (Otwinowski, 1993).

### Table 1
Data-collection and reduction statistics for IB1-SH3.

Values for the outer resolution shell are given in parentheses.

| Space group | $P2_12_12_1$ |
| --- | --- |
| Unit-cell parameters (Å) | $a$ = 45.9, $b$ = 57.0, $c$ = 145.5 |
| Resolution limits (Å) | 30.0–3.0 (3.11–3.0) |
| Total No. of observations | 32574 |
| No. of unique reflections | 8075 |
| Completeness (%) | 99 (100.0) |
| Average $I/\sigma(I)$ (observed/absent) | |
| $\quad h00$ reflections | 13.1/0.38 |
| $\quad 0k0$ reflections | 4.27/0.37 |
| $\quad 00l$ reflections | 7.20/0.39 |
| Average $I/\sigma(I)$ | 12.1 (2.9) |
| $R_{merge}$ | 0.102 (0.461) |

## 3. Results and discussion

### 3.1. Purification and crystallization

The 9 kDa protein has a strong tendency to aggregate. It travels on a Superdex 200 column (HR 10/30) as a protein of molecular weight around 80 kDa. This aggregation could perhaps explain the long crystallization time observed for IB1-SH3. We have observed that a protein concentration of 1.4–2.0 mg ml$^{-1}$ is optimal for crystallization. Although the protein also exists as a multimer at this concentration, nucleation is probably facilitated compared with crystallization at higher protein concentrations (2.0–3.0 mg ml$^{-1}$). At lower concentrations, the time taken for crystals to appear is reduced from months to two weeks. Protein concentrations higher than 3.5 mg ml$^{-1}$ do not yield crystals at all.

During our optimization experiments, we discovered several factors that influence the crystal morphology. The most important is the pH of the crystallization droplet. We have seen at least four different crystal
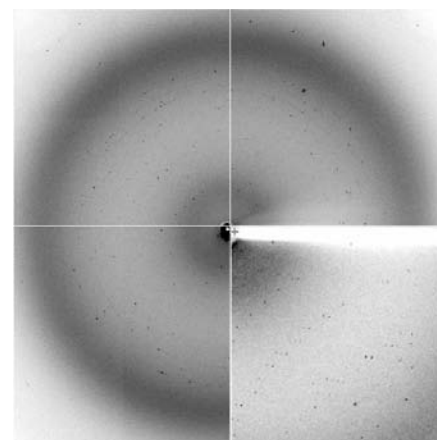


**Figure 1**
Rectangular prism crystal of oxidized IB1-SH3.



**Figure 2**
Native crystal diffraction image with one quadrant of the detector enlarged. The resolution extends to 3.0 Å (exposure details: 1° oscillation range, 102 frames collected with 10 s exposure time per frame).

morphologies from reservoir solutions in the pH range 6.5–7.5. We observe that even a slight change (0.2 pH units) is pivotal for crystal quality. Large crystals of the rectangular prism form were obtained at pH 6.5. The ion strength in the reservoir solution and in the droplet is not crucial, but we produced the largest crystals at a low level of saturation (1.4 $M$ trisodium citrate). The best rectangular prism crystals were obtained using a hydrogen peroxide concentration of 0.3% in the protein stock solution. We have produced only a few crystals using the oxidizing agent at concentrations above 0.3%, but without any noticeable increase in the crystal quality. Streak seeding has been attempted, but has not provided better diffracting crystals.

Deliberate protein oxidation has been mentioned previously as a useful tool in protein crystallization (Kristensen *et al.*, 2002). The actual effect of the addition of this oxidizing agent is still not understood in detail. Formation of intermolecular disulfide bonds can be excluded, as there are no cysteines in the 71-amino-acid sequence of IB1-SH3. However, there are two methionines and at least one appears to be surface-exposed according to IB1-SH3 modelling based on three known SH3 domain structures (1gcq, 1pnj and 1fyn) from the Protein Data Bank using SWISSMODEL (Guex & Peitsch, 1997). During treatment with hydrogen peroxide, one or both methionines could be oxidized to a methionine-*R*,*S*-sulfoxide isomer and this may well result in the creation of an environment that is more favourable for crystal contacts on the introduction of the hydrophilic oxo group. Certainly, other chemical modifications are also possible and the structure determination of the IB1-SH3 domain will shed light on this issue.

## 3.2. Perspectives

Solving the structure by the technique of molecular replacement is not trivial owing to the weak signal arising from the four probable molecules in the asymmetric unit, but solution attempts are in progress. The crystallization conditions of OxSeMetSH3 are also currently being optimized. The fact that crystals of OxSeMetSH3 are obtained with salts other than sodium citrate as precipitating agents is a valuable discovery. With a continued search for new salts that can act as successful precipitants and proper optimization of lithium and ammonium sulfate-based crystallization, MAD phasing is another promising method for structure solution.

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