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Crystallization and preliminary X-ray crystallographic analysis of SP1, a novel chaperone-like protein

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SP1 (108 amino acids) is a boiling-stable stress-responsive protein. It has no significant sequence homology to other stress-related proteins or to small heat-shock proteins (sHsps). SP1 activity is ATP-independent, similar to other small heat-shock proteins. Based on these features, it is expected that the structure–function relationship of SP1 will be unique. In this work, the crystallization and preliminary crystallographic data of native SP1 and its selenomethionine derivative are described. Recombinant SP1 and its selenomethionine derivative were expressed in *Escherichia coli* and used for crystallization experiments. SP1 crystals were grown from 0.1 *M* HEPES pH 7.5, 20% PEG 3K, 0.2 *M* NaCl. One to four single crystals appeared in each droplet within a few days and grew to dimensions of about $0.5 \times 0.5 \times 0.8$ mm after about two weeks. Diffraction studies of these crystals at low temperature indicated that they belong to space group *I*422, with unit-cell parameters $a = 89$, $b = 89$, $c = 187$ Å. Efforts to crystallize the selenomethionine derivative of SP1 are in progress.

1. Introduction

SP1 is a stress-responsive protein that has been isolated from aspen plants (*Populus tremula*). SP1 has no significant sequence homology to other stress-related proteins (Pelah *et al.*, 1995; Pelah, Wang *et al.*, 1997; Pelah, Shoseyov *et al.*, 1997; Wang *et al.*, 2002). The cDNA encodes a 12.4 kDa polypeptide that is generally hydrophilic, with a hydrophobic C-terminus. SP1 has no cysteines or potential *N*-glycosylation sites. Amino-acid analysis and gel-filtration HPLC studies of SP1 have revealed it to be a homo-oligomeric protein composed of 12 subunits tightly bound to each other even under extreme conditions (*e.g.* heating at 353 K in the presence of high concentrations of SDS). Chemical cross-linking in combination with MALDI–TOF and preliminary EM indicated that the SP1 oligomer is organized as two stacked hexamer rings and that SP1 possesses chaperone-like activity.

It has been shown that the principal heat-shock proteins with chaperone activity (*i.e.* those involved in the proper folding of nascent polypeptides and in helping damaged proteins to regain their biological active conformation) belong to five conserved groups: Hsp100, Hsp90, Hsp70, Hsp60 and small heat-shock proteins (sHsps; Hartl, 1996). Small heat-shock proteins prevent protein aggregation and thus contribute to the balance between cell survival and cell death. The activity of most small Hsps as well as SP1 activity is ATP independent.

Only a few structural studies on Hsps have been carried out, as small Hsps tend to form oligomeric structures with a variable number of subunits and are thus difficult to crystallize. Two crystal structures of sHsp have recently been reported: the crystal structure of sHsp from *Methanococcus jannaschii* (MW 16.5 kDa; Kim *et al.*, 1998) and the eukaryotic sHsp from wheat (MW 16.9 kDa; Montfort *et al.*, 2001). Both sHsps form large oligomeric complexes. The X-ray structure of sHsp16.9 from wheat showed that it is a dodecamer that consists of two disks, each made up of six monomers. However, SP1 (108 amino acids) has no significant sequence homology with sHsp16.9 (160 amino acids) or other heat-shock proteins.

In the following, we present the crystallization procedure and preliminary crystallographic data of SP1 as well as the expression of a selenomethionine derivative of SP1 (Sel-SP1). Efforts to crystallize Sel-SP1 are in progress.

2. Experimental

2.1. Expression and purification of SP1 and selenomethionine SP1

Escherichia coli strain 834 (DE3) (methionine auxotroph) containing pET-29a-SP1 plasmid was grown overnight at 310 K in LB medium containing 30 μg ml^{-1} kanamycin. Cells from overnight culture were then trans-

ferred to M10 methionine-containing medium (Mechaly *et al.*, 2000) and incubated for an additional 4 h until the culture reached an OD of 0.9. The cells were harvested by centrifugation and washed twice in M10 selenomethionine-containing medium. Following resuspension in the same medium containing 10% glycerol, the resuspended cells were divided into 55 µl aliquots that were used to inoculate 50 ml M10 selenomethionine-containing medium. The culture was incubated at 310 K until the OD reached 1.2. SP1 expression was induced using 1 m*M* IPTG for 4 h. After induction, cells were collected by centrifugation and stored at 253 K. Recombinant SP1 was produced in the same way, except that the host was BL21 (DE3) and the medium was TB.

The cell pellet was resuspended in lysis buffer containing 20 m*M* Tris–HCl pH 8, 0.1% Triton 100. DNase I and lysozyme were added to reach final concentrations of 5 and 10 µg ml^{-1}, respectively. Lysis was carried out at 310 K for 45 min, followed by centrifugation (15 000*g*, 15 min, 277 K).

Proteinase K (50 µg ml^{-1}) was added to the supernatant and incubated at 310 K for 45 min. Proteolysis was terminated by adding 1 m*M* of PSMF at room temperature for 30 min and was followed by boiling for 10 min. The aggregates were removed by centrifugation (15 000*g*, 15 min, 283 K). The supernatant containing SP1 protein was washed with TBS buffer (20 m*M* Tris–HCl pH 7.5, 137 m*M* NaCl) and concentrated using a Centricon (30 kDa cutoff) to a final concentration of 10 mg ml^{-1}. 1 µg of recombinant SP1 and selenomethionine SP1 were loaded onto 12% SDS–PAGE and the SP1 protein bands were excised for MALDI–TOF analysis (Technion Protein Center, Technion, Haifa, Israel).

2.2. Crystallization

Prior to crystallization experiments, $CaCl_2$ was added to the protein solution to reach a final concentration of 20 m*M*. Crystallization experiments were set up using the hanging-drop vapour-diffusion method with siliconized cover slips and Linbro 24-well tissue-culture plates. In these experiments, droplets ranging in size from 10 to 20 µl were equilibrated with 1.0 ml of reservoir solution at 293 K. One to four crystals appeared within 3 d and grew to their maximal dimensions within two weeks.

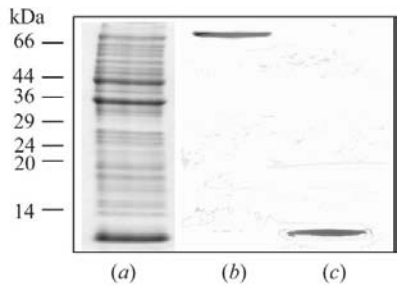

Figure 1
SDS–PAGE analysis of (*a*) crude extract sample, (*b*) isolated SP1 protein, (*c*) isolated SP1 protein boiled in the presence of SDS.

2.3. Data collection

A native data set was collected at the Wolfson Center for Structural Biology at the Hebrew University of Jerusalem. Diffraction data from SP1 crystals were collected at low temperature (100 K) using an R-AXIS IV electronic area detector and a Rigaku RU-200 HB generator. Determination of unit-cell parameters, crystal orientation and integration of reflection intensities were performed using *DENZO* and *SCALEPACK* (Otwinowski & Minor, 1997).

3. Results

Fig. 1 shows SDS–PAGE analysis of the expression and purification of SP1 selenomethionine derivative. Fig. 1(*a*) shows a total protein extract of *E. coli* expressing SP1 selenomethionine derivative. Fig. 1(*b*) shows SP1 without boiling, while Fig. 1(*c*) shows SP1 after boiling in the presence of SDS. SP1 without boiling remained as a complex, whereas upon boiling it dissociated into its monomeric form. The SP1 selenomethionine derivative and native SP1 showed comparable activity in preventing horseradish peroxidase (HRP) inactivation (PCT No. 60/272,771). Preliminary MALDI-TOF–MS experiments on SP1 and selenomethionine SP1 indicated that both proteins exist in their oligomeric form (work submitted for publication).

3.1. Incorporation of selenomethionine into SP1

The sequence analysis revealed that SP1 has three methionine residues: Met1, Met43 and Met54. Two peptide fragments containing methionine were obtained using trypsin treatment, one of which consists of residues 24–44 (molecular weight 2512.23 Da) and the other of residues 45–61 (molecular weight 1926.87 Da). The methionine- and selenomethionine-derivative fragments are presented in Fig. 2. It is estimated that the incorporation rates for the 1926.87 and 2512.23 Da peptides are 90 and 85%, respectively. The N-terminal methionine peptide is a low-mass peptide and was not detected owing to matrix suppression.

3.2. Preliminary crystallographic characterization

Crystallization experiments were carried out by the fast-screening method using Hampton Research Crystal Screen Kit I. The initial screenings resulted in the appearance of small crystals in some of the solutions (solutions 4, 8, 14, 23 and 48). Based on these results, we further refined the crystallization conditions in order to reach a final solution containing 0.1 *M* HEPES, pH 7.5, 20% PEG 3K, 0.2 *M* NaCl.

A native X-ray diffraction data set (1.0° oscillation images) in the resolution range 50.0–2.5 Å was collected on an R-AXIS IV image-plate detector. The data set consisted

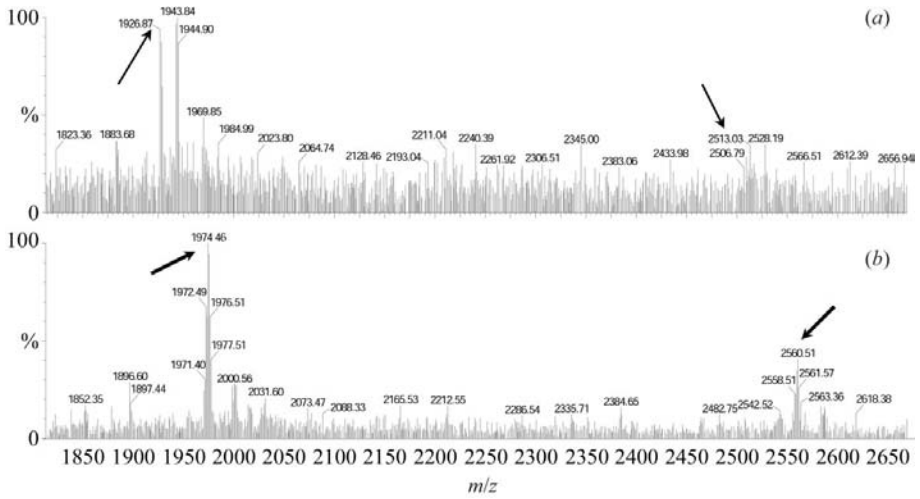

Figure 2
Tryptic digestion fragments of (*a*) recombinant SP1 and (*b*) recombinant selenomethionine SP1. Fragments containing methionine or selenomethionine are indicated by arrows.

of a total of 31 968 reflections, which were merged to obtain 13 153 unique reflections with $I/\sigma(I) > 8.0$. For the last shell, 2.52–2.50 Å, $I/\sigma(I)$ was 4.0. $R_{\rm merge}$ for this complete data set is 0.097 and for the last shell $R_{\rm merge}$ is 0.385. This data set includes more than 96% of the data to 2.5 Å.

SP1 crystals belong to the tetragonal space group $I422$, with unit-cell parameters $a = 89$, $b = 89$, $c = 187$ Å. The Matthews ($V_{\rm M}$) coefficient for these crystals is 2.43 Å^3 Da^{-1} for a trimer as the asymmetric unit.

In summary, we have succeeded in crystallizing native SP1 and in overexpressing its selenomethionine derivative. Efforts to crystallize the selenomethione derivative under conditions similar to those used for native SP1 are under way. These crystals will be used for structure determination of SP1.

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