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Cloning, expression, purification and preliminary X-ray crystallographic studies of yeast Hsp40 Sis1 complexed with Hsp70 Ssa1 C-terminal lid domain

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Heat-shock protein 70 (Hsp70) plays essential roles in a number of cellular processes such as protein folding, assembly and translocation. Heat-shock protein 40 (Hsp40) transiently interacts with Hsp70 and facilitates Hsp70 functions in these processes within cells. Hsp40 recognizes and binds non-native polypeptide and delivers it to Hsp70. Hsp40 can then stimulate the ATPase activity of Hsp70 to refold the polypeptide. To investigate the molecular mechanism by which Hsp40 interacts with Hsp70 to transport the non-native polypeptide, *Saccharomyces cerevisiae* Hsp40 Sis1 C-terminal peptide-binding fragment complexed with Hsp70 Ssa1 C-terminal lid domain has been produced and crystallized. The complex crystals diffract to 3.3 Å and belong to the space group $P4_12_12$ or $P4_32_12$, with unit-cell parameters a = 112.17, c = 171.31 Å. Structure determination by the MAD method is under way.

1. Introduction

Molecular chaperones, by definition, are a large group of proteins which can recognize, bind and stabilize non-native polypeptides and facilitate protein folding (Gething & Sambrook, 1992; Craig *et al.*, 1994). Heat-shock proteins 70 (Hsp70s) constitute a major molecular chaperone family and play essential roles in cell physiology. Hsp70 pairs with a specific Hsp40 to form transient protein complexes in order to promote essential cellular functions such as protein folding, assembly, translocation and degradation of incorrectly folded polypeptides within the cells (Craig *et al.*, 1994; Hartl, 1996; Bukau & Horwich, 1998; Georgopoulos, 1992; Flynn *et al.*, 1991; Ungermann *et al.*, 1994; Rothman, 1989). Hsp70 contains an N-terminal ATPase domain and a C-terminal peptide-binding domain (Hartl, 1996; Bukau & Horwich, 1998; Zhu *et al.*, 1996; Flaherty *et al.*, 1990). In the crystal structure of *Escherichia coli* Hsp70 DnaK peptide-binding domain complexed with the peptide substrate, two domains were identified: a β-domain and an α-domain (Zhu *et al.*, 1996). The β-domain was composed of two layers of antiparallel β-sheets and formed a peptide-binding groove, while the C-terminal α-domain consisted of four α-helices and constituted a 'lid' to cover the groove (Zhu *et al.*, 1996).

All members of Hsp40 proteins have an N-terminal J domain which regulates the ATPase activity of Hsp70. The Hsp40 family has two subtypes: type I and type II Hsp40. Type I Hsp40 proteins have a zinc-finger motif adjacent to the J domain while type II Hsp40 proteins do not. Both types of Hsp40 proteins contain a C-terminal peptide-binding fragment which may recognize and bind hydrophobic side chains of denatured polypeptides (Sha *et al.*, 2000; Lu & Cyr, 1998). The J domain within Hsp40 can interact with the Hsp70 ATPase domain and stimulate the ATPase activity of Hsp70 (Rothman, 1989; Gething & Sambrook, 1992; Hohfeld *et al.*, 1995). It is suggested that Hsp40 may bind non-native polypeptide first and then deliver the non-native polypeptide to Hsp70 for refolding (Hohfeld *et al.*, 1995; Langer *et al.*, 1992; Sha *et al.*, 2000). Once Hsp70 enters its ADP state, the Hsp40–Hsp70 complex may dissociate. At the molecular level, it is not well understood how Hsp40 interacts with Hsp70 and facilitates the protein refolding by Hsp70.

Sis1 is an essential type II Hsp40 protein in *S. cerevisiae* (Luke *et al.*, 1991). Sis1 can bind to ribosomes and is required for the normal initiation of translation (Zhong & Arndt, 1993). The yeast Hsp70 homologues Ssb1 and Ssb2 have been shown to be components of the ribosome–nascent chain complex (Nelson *et al.*, 1992; Pfund *et al.*, 1998); it is therefore possible that Sis1 cooperates with Ssb1 and Ssb2 during the translation process *in vivo*. Coupling with Ssb1, Sis1 is also involved in modulation of intracellular protein degradation in yeast (Ohba, 1997). Sis1 can also stimulate the ATPase activity of Ssa1 and refold denatured protein along with Ssa1 *in vitro* (Lu & Cyr, 1998). Ssa1 is also one of the yeast Hsp70 homologs. The crystal structure of Sis1 C-terminal peptide-binding fragment has been determined recently in our group (Sha *et*

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al., 2000). The Sis1 C-terminal fragment forms a homodimer in the crystal and in solution. Two hydrophobic depressions on the dimer surface were identified as the non-native polypeptide binding sites. A huge cleft generated by Sis1 dimer formation can accommodate the C-terminal peptide-binding domain of Hsp70 (Sha *et al.*, 2000; Zhu *et al.*, 1996). Based on the crystal structure, we have proposed a 'docking model' to illustrate the mechanism by which the type II Hsp40 Sis1 interacts with Hsp70 and delivers the non-native polypeptides to Hsp70 (Sha *et al.*, 2000).

The Sis1 C-terminal peptide-binding fragment can physically interact with yeast Hsp70 Ssa1 C-terminal lid domain and form a stable complex (Qian & Sha, 2001). To further investigate how the Sis1 C-terminal fragment interacts with Hsp70, we plan to carry out structural studies on the complex of the Sis1 C-terminal fragment and the yeast Hsp70 Ssa1 C-terminal lid domain.

2. Experiments

2.1. Cloning, expression and purification of Sis1 C-terminal peptide-binding domain and Ssa1 lid domain

The cloning, expression and purification of Sis1 C-terminal peptide-binding domain has been described previously (Sha & Cyr, 1999). The calculated molecular weight is 20 454 Da for the monomer and 40 908 Da for the homodimer of Sis1 C-terminal peptide-binding domain. Based on a sequence alignment between *S. cerevisiae* Ssa1 and *E. coli* Hsp70 DnaK, the 'lid' domain of Ssa1 was determined to be within amino-acid residues 524–642 of Ssa1 (Zhu *et al.*, 1996). The calculated molecular weight for Ssa1 lid domain is 12 921 Da. The Ssa1 lid domain (residues 524–642) was cloned into vector pet15b (Novagen). The PCR products were digested by restriction endonucleases *Nde*I and *Bam*HI as indicated in the instructions (New England). The inserts were then ligated into the digested pet15b vector by T4 ligase. The nucleotide sequence was confirmed by DNA sequencing. The plasmid encoding the Ssa1 lid domain was finally transformed into *E. coli* strain BL21(DE3) for protein expression.

10 ml LB medium was inoculated using the transformed *E. coli* stocks. The cells were allowed to grow at 310 K in a shaker for 12 h. 10 ml of LB medium was then used to inoculate 1 l of LB medium. 0.5 ml of 1 *M* IPTG was added to 1 l of medium to induce protein expression when the OD_{600} of the medium reached 0.6. The cells were harvested 3 h after induction. The *E. coli* cells from 1 l of medium were pelleted by centrifugation and resuspended in 100 ml 10 m*M* phosphate buffer pH 7.2, 150 m*M* NaCl. The cell walls and membranes were broken down by sonication. The debris and insoluble materials were pelleted at 15 000 rev min^{-1} using a Beckman JA20 rotor and the supernatant was collected. As the Ssa1 lid domain was histidine tagged, it could be relatively easily purified by a metal-chelating column. The metal-chelating beads (Pharmacia) were charged with 50 m*M* $NiSO_4$ and washed with an extensive amount of 10 m*M* phosphate buffer pH 7.2, 150 m*M* NaCl. The collected supernatant was pumped through the Ni-charged column and the protein-bound beads were washed thoroughly with 10 m*M* phosphate buffer pH 7.2, 0.5 *M* NaCl and 50 m*M* imidazole to remove contaminating protein. The bound Ssa1 lid-domain protein was then eluted with 10 m*M* phosphate buffer pH 7.2, 0.5 *M* NaCl and 200 m*M* imidazole. The eluted protein was dialyzed against 10 m*M* Tris buffer pH 7.2, 50 m*M* NaCl. The typical yield of Ssa1 lid domain from 1 l of medium is ~20 mg. The N-terminal histidine tag of the Ssa1 lid domain was then digested by thrombin treatment. 1 unit of thrombin (Sigma) was utilized per milligram of Ssa1 lid-domain protein. Digestion took place for 12 h at room temperature and was stopped by the addition of 10 µl 0.2 *M* PMSF, a protease inhibitor. The protein was further purified using a Superdex 200 gel-filtration column (Pharmacia) mounted on an AKTA HPLC system (Pharmacia) to remove thrombin and digested peptides. The apparent molecular weight of the Ssa1 lid domain is shown to be ~20 kDa from the elution time of the protein peak, indicating that the Ssa1 lid domain is a monomer in solution.

2.2. Production and purification of the Sis1–Ssa1 complex

Purified Sis1 C-terminal peptide-binding fragment and Ssa1 lid domain were mixed together in an approximately 1:2 molar ratio in Tris buffer pH 7.2, 50 m*M* NaCl to produce the Sis1 C-terminal fragment/Ssa1 lid-domain complex (henceforth, this complex will be referred to as the 'Sis1–Ssa1 complex'). Excess Ssa1 lid domain was added to the mixture to saturate the Sis1 C-terminal fragment in order to simplify the complex purification. The Sis1–Ssa1 complex was purified by Superdex 200 column (Pharmacia) to remove unbound Ssa1 lid domain. The apparent molecular weight of the Sis1–Ssa1 complex appears to be ~60 kDa from the elution time of the protein peak. Because Sis1 forms a dimer in solution, it is possible that one Sis1 dimer may bind to one Ssa1 lid-domain monomer in the complex (Sha & Cyr, 1999; Sha *et al.*, 2000; Qian & Sha, 2001). The SDS–PAGE gel densitometry analysis of the purified complex and the complex crystals (see below) also suggest that one Sis1 C-terminal fragment dimer binds to one Ssa1 lid-domain monomer.

2.3. Crystallization, data collection and processing of the Sis1–Ssa1 complex

The Sis1–Ssa1 complex was concentrated to 20 mg ml^{-1} in 10 m*M* HEPES buffer pH 7.2, 50 m*M* NaCl and subjected to crystallization trials. Large diffraction-quality crystals (0.8 × 0.8 × 0.8 mm) were obtained within 10 d by the hanging-drop vapor-diffusion method using Linbro boxes at room temperature. The well solution consisted of 1 ml 100 m*M* Tris buffer pH 7.0, 42.5% ammonium sulfate. The hanging drops consisted of 2 µl protein solution plus 2 µl well solution. The crystals of Sis1–Ssa1 complex can be stored in 100 m*M* Tris buffer pH 7.0, 60% ammonium sulfate for several months without any damage. To examine whether the crystals are composed of Sis1–Ssa1 complex, the crystals were collected, washed several times and then dissolved in water. The sample was then subjected to SDS–PAGE analysis and mass-spectrum measurement using a MALDI-TOF mass spectrometer. Both the results from the SDS–PAGE gel and the mass spectrum showed the crystal samples to contain Sis1 C-terminal fragment and Ssa1 lid domain. Therefore, we concluded that the protein that we crystallized was the Sis1–Ssa1 complex.

The complex crystals were then taken to a synchrotron X-ray source for data collection at the BioCars beamline BMC14 at the APS. The crystals are very sensitive to X-rays and had to be frozen in order to collect a full data set from a single crystal. The crystal was transferred into a cryoprotectant containing 100 m*M* Tris buffer pH 7.0, 60% ammonium sulfate and 20% glycerol for 5 min before being immediately frozen at 100 K in a nitrogen-gas stream. The Sis1–Ssa1 complex crystals diffracted to 3.3 Å using synchrotron X-ray sources at a wavelength of 1.00 Å. The crystal-to-detector distance was 300 mm and the oscillation angle was 1.0°. 150 images were collected and processed using *DENZO* and *SCALEPACK* (Minor, 1993; Otwinowski, 1993). The crystals belong to space

group $P4_12_12$ or $P4_32_12$, with unit-cell parameters a = 112.17, c = 171.31 Å. The data were about 91.4% complete to 3.3 Å and R_{sym} was 0.057; the redundancy for the data is 5.5. The overall $I/\sigma(I)$ is 31.2. The statistics of the data set are shown in Table 1. The V_M value is 2.30 $Å^3 Da^{-1}$ for two complex molecules per asymmetric unit. The mosaicity value for the best crystals after freezing is 0.352. The total number of unique reflections is 15 723.

We initially planned to determine the crystal structure of the Sis1–Ssa1 complex by the molecular-replacement method. However, we failed to find a solution for the Sis1 molecule using various programs. Our next approach is to utilize the MAD method to solve the complex structure. Selenomethionyl Sis1 C-terminal fragment and Ssa1 lid domain have been produced following the protocols described previously (Sha & Cyr, 1999). The production, purification and crystallization of the Se-Met Sis1–Ssa1 complex were carried out using the same conditions as for the wild-type Sis1–Ssa1 protein complex. The selenomethionyl complex crystals diffract as well as the native complex crystals using a synchrotron X-ray source. The crystal structure determination of Sis1–Ssa1 complex by MAD method is under way.

Table 1
The statistics of the native data set for Sis1–Ssa1 complex from APS BioCars beamline BMC14.

Resolution shells (Å)	$I/\sigma(I)$	R_{sym}	Completeness (%)
100.0–6.00	36.5	0.030	97.9
6.00–4.76	38.7	0.051	100.0
4.76–4.16	34.5	0.074	100.0
4.16–3.78	19.8	0.161	100.0
3.78–3.51	7.77	0.331	92.4
3.51–3.30	4.10	0.434	65.6

3. Discussion

We have crystallized the protein complex of the yeast Hsp40 Sis1 C-terminal peptide-binding fragment and the yeast Hsp70 Ssa1 C-terminal lid domain. The complex crystals belong to the space group $P4_12_12$ or $P4_32_12$, with unit-cell parameters a = 112.17, c = 171.31 Å, and diffract to 3.3 Å at a synchrotron X-ray source.

It is surprising that we failed to determine the complex crystal structure by the molecular-replacement method. This result may suggest that the Sis1 C-terminal peptide-binding fragment undergoes certain conformational changes when Hsp40 Sis1 interacts with Hsp70 Ssa1. Sis1 C-terminal peptide-binding fragment has an elongated molecular shape and contains two domains; it is therefore possible that positional changes between the two domains may occur in order for Sis1 to bind to Hsp70 Ssa1 (Sha *et al.*, 2000).

Little work has been performed on the structural study of Hsp40–Hsp70 complexes. The crystal structure of yeast Hsp40 Sis1 complexed with Hsp70 Ssa1 will have a major impact on understanding the mechanism by which Hsp40 delivers non-native peptide to Hsp70. It would be of great interest to see what conformational changes may occur when Hsp40 interacts with Hsp70 and how the conformational changes may facilitate the transfer of non-native peptides from Hsp40 to Hsp70.

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