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Preliminary X-ray crystallographic studies of yeast Hsp40 Ydj1 complexed with its peptide substrate

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The mechanism by which Hsp40 functions as a molecular chaperone, recognizing and binding non-native polypeptides, is not understood. To investigate the mechanism, the yeast type I Hsp40 protein Ydj1 C-terminal peptide-binding fragment has been crystallized complexed with its peptide substrate. The crystals diffract to 2.70 Å using a synchrotron X-ray source and belong to space group $P3_221$ or $P3_121$, with unit-cell parameters $a = b = 55.21$, $c = 161.87$ Å, $\alpha = \beta = 90$, $\gamma = 120°$. There is one Ydj1 peptide-binding fragment and one peptide substrate in the asymmetric unit, which corresponds to a solvent content of approximately 42%. Structure determination by MAD methods is under way.

1. Introduction

Molecular chaperones are a large group of proteins that can recognize, bind and stabilize non-native polypeptides and facilitate protein folding (Gething & Sambrook, 1992; Craig *et al.*, 1994). Members of the Hsp70 and Hsp40 families of molecular chaperones function in specific pairs that form a transient complex that promotes the folding, assembly and transport of proteins within cells (Hartl, 1996; Bukau & Horwich, 1998; Hartl & Hayer-Hartl, 2002). 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 its peptide substrate two domains were identified: a β-domain and an α-domain (Zhu *et al.*, 1996). The β-domain consisted of two layers of antiparallel β-sheets and formed a peptide-binding groove, while the α-domain consisted of four α-helices and constituted a lid that covered the groove.

Hsp40 proteins act as molecular chaperones that bind and deliver non-native polypeptides to Hsp70. All members of the Hsp40 family contain an N-terminal J domain, which regulates the ATPase activity of Hsp70 (Langer *et al.*, 1992; Misselwitz *et al.*, 1998). Both type I and type II Hsp40 proteins contain a conserved C-terminal peptide-binding fragment and form homodimers in solution. The type I Hsp40 proteins, such as *E. coli* DnaJ, human Hdj2 and yeast Ydj1, have zinc-finger-like regions between the N-terminal J domain and the conserved C-terminus (Szabo *et al.*, 1996; Banecki & Zylicz, 1996; Lu & Cyr, 1998; Goffin & Georgopoulos, 1998). Type II Hsp40 proteins, such as human Hdj1 and yeast Sis1 (Luke *et al.*, 1991; Zhong & Arndt, 1993), lack zinc-finger-like motifs. It has been suggested that Hsp40 may bind non-native polypeptides first and then deliver the non-native polypeptides to Hsp70 for subsequent refolding (Hohfeld *et al.*, 1995; Langer *et al.*, 1992; Sha *et al.*, 2000). At the molecular level, it is not fully understood how Hsp40 binds non-native polypeptides and facilitates folding with Hsp70.

Ydj1, a type I Hsp40 from *Saccharomyces cerevisiae*, play critical roles in yeast growth. Knockout of Ydj1 leads to a severe growth defect at elevated temperatures. Ydj1 has two zinc-finger-like motifs adjacent to the conserved C-terminal domain. It has been proposed that the zinc-finger-like motifs and the conserved C-terminal fragment are both involved in peptide binding (Szabo *et al.*, 1996; Lu & Cyr, 1998).

Sis1 is an essential type II Hsp40 protein in *S. cerevisiae* (Luke *et al.*, 1991). The crystal structure of the Sis1 C-terminal peptide-binding fragment has been determined by our group (Sha *et al.*, 2000). The two Sis1 C-terminal fragment monomers are associated through a short C-terminal dimerization motif. Two hydrophobic depressions on the dimer surface were identified as putative non-native polypeptide-binding sites.

To investigate the structural basis of the chaperone action of type I Hsp40 proteins, we have cloned and expressed the Ydj1 C-terminal peptide-binding fragment and crystallized it in complex with the peptide substrate. The complex structure may demonstrate the mechanisms by which Hsp40 inter-

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acts with its peptide substrate and facilitates protein folding.

2. Experimental and discussion

2.1. Cloning, expression and purification of Ydj1

The Ydj1 peptide-binding fragment has been determined to be within amino-acid residues 102–384 of the full-length Ydj1 by limited proteolysis (Lu & Cyr, 1998). The DNA fragment encoding Ydj1 (102–384) was amplified from Ydj1 cDNA by PCR using the 5′ primer GGAATTCCATA-TGGGCGCACAAAGACCAAGAGGTC-CCCAAAGAGG and the 3′ primer CCC-AAGCTTTCATTCTAACTTCTTCAAGT-TTTCTTCTGATGTG. The PCR products were digested using the restriction endonucleases *Nde*I and *Hin*dIII (New England BioLabs). The digested inserts were then ligated into the digested pet28b vector by T4 ligase.

To eliminate possible intramolecular flexibility within the Ydj1 dimer, we intended to construct a monomeric form of the Ydj1 peptide-binding fragment. The crystal structure of the type II Hsp40 Sis1 peptide-binding fragment dimer indicated that Tyr336 plays an important role in its dimerization through hydrophobic interactions (Sha *et al.*, 2000). A sequence alignment between the type I Hsp40 Ydj1 and the type II Hsp40 Sis1 predicts that Phe335 of Ydj1 may function as the counterpart of Tyr336 of Sis1 and contribute to Ydj1 dimerization. To generate a monomeric form of Ydj1, we constructed a mutant Ydj1 F335D (102–384) that covers amino-acid residues 102–384 of the full-length Ydj1 molecule with the mis-sense mutation Phe to Asp at position 335 to change its hydrophobic nature. The site mutation Phe335Asp was performed by use of the QuikChange Site-directed Mutagenesis Kit (Stratagene). The nucleotide sequence was confirmed by DNA sequencing. The plasmid was transformed into *E. coli* strain BL21(DE3) for protein expression.

10 ml of LB medium containing 30 μg ml^{-1} kanamycin was inoculated using the transformed *E. coli* stock. The cells were allowed to grow at 310 K in a shaker for 12 h and the 10 ml of LB medium was then used to inoculate a further 1 l of LB medium containing 30 μg ml^{-1} kanamycin. 0.5 ml of 1 *M* IPTG was added to the 1 l of medium in order 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 the 1 l of medium were pelleted down by centrifugation and resuspended in 100 ml of 100 m*M* Tris buffer pH 7.9, 0.5 *M* NaCl. The cells were lysed by sonication at 277 K. The debris and insoluble materials were pelleted down at 15 000 rev min^{-1} using a Beckman JA20 rotor. Since the Ydj1 F335D (102–384) was histidine-tagged, it could be relatively easily purified by a metal-chelating column. The supernatant was pumped through an Ni-charged column containing about 10 ml resin. The column was thoroughly washed with 50 m*M* Tris buffer pH 7.9, 0.5 *M* NaCl and 50 m*M* imidazole in order to remove contaminating proteins. The bound protein was then eluted with 50 m*M* Tris buffer pH 7.9, 0.5 *M* NaCl and 200 m*M* imidazole. The eluted protein was dialyzed against 2 l of 10 m*M* HEPES buffer pH 7.2, 150 m*M* NaCl. The typical yield of soluble Ydj1 F335D (102–384) (∼95% pure from SDS–PAGE analysis) from 1 l of culture is ∼15 mg. The N-terminal histidine tag of Ydj1 was then digested by thrombin treatment. 1 unit of thrombin (Sigma) was utilized per milligram of Ydj1 protein. Digestion took place for 12 h at room temperature and was stopped by the addition of 0.2 m*M* PMSF. The protein was further purified on a Superdex 200 gel-filtration column (Pharmacia) mounted on an AKTA HPLC system (Pharmacia) in order to remove thrombin and digested peptides. The apparent molecular weight of the Ydj1 F335D (102–384) was shown to be about 30 kDa based on the protein elution time from the gel-filtration column, indicating that the Ydj1 F335D (102–384) forms a monomer in solution as predicted.

2.2. Crystallization, data collection and processing

A short peptide of sequence GWLYEIS has been identified as the peptide substrate of Ydj1 by using phage peptide display library screening and isothermal titration calorimetry (data not shown). Ydj1 F335D (102–384) and the peptide substrate GWLYEIS were mixed in a 1:2 molar ratio to constitute the protein complex. The complex was concentrated to 15 mg ml^{-1} in 10 m*M* MES buffer pH 6.0, 150 m*M* NaCl and was subjected to crystallization trials. Rod-shaped crystals (0.5 × 0.1 × 0.1 mm) were obtained by the hanging-drop vapour-diffusion method using Linbro plates at 298 K. The well solution consisted of 1 ml 100 m*M* Tris buffer pH 7.0, 3–5% PEG 4K, 15% ethylene glycol. To examine whether the crystals contained Ydj1 and the peptide substrate, several crystals were collected, washed extensively and then dissolved in water. The sample was then subjected to MALDI–TOF mass-spectrometric analysis. The results showed that the crystals only contained Ydj1 peptide-binding fragment and peptide.

Table 1
The statistics of the data set from Ydj1 peptide-substrate complex crystals.

Values in parentheses are for the highest resolution shell.

Unit-cell parameters	
$a = b$ (Å)	55.21
c (Å)	161.87
$\alpha = \beta$ (°)	90
γ (°)	120
Wavelength (Å)	1.284
Resolution (Å)	30–2.7 (2.87–2.7)
Unique reflections	8050 (1085)
Mosaicity	0.581
Completeness (%)	93.6 (80.1)
Average $I/\sigma(I)$	19.9 (7.7)
R_{sym} (%)	4.9 (11.2)

The complex crystals were then taken to BioCars beamline BMD14 at APS for data collection. The crystals were sensitive to X-ray radiation and had to be frozen in order to collect a full data set from a single crystal. The crystal was flash-frozen at 100 K in a nitrogen-gas stream in a cryoprotectant consisting of 100 m*M* Tris buffer pH 7.0, 10% PEG 4K and 20% ethylene glycol.

The Ydj1–peptide substrate complex crystals diffracted X-rays to 2.70 Å using beamline BioCars BMD14. The data were collected using a Quantum 4 CCD detector (ADSC). During data collection, the crystal-to-detector distance was kept at 180 mm. The oscillation angle for the crystal was 1.0°. 50 images were collected and processed using *DENZO* and *SCALEPACK* (Minor, 1993; Otwinowski, 1993). The data set revealed that the crystals belonged to space group $P3_221$ or $P3_121$, with unit-cell parameters $a = b = 55.21$, $c = 161.87$ Å, $\alpha = \beta = 90$, $\gamma = 120°$ (Table 1). Crystal analysis shows that one asymmetric unit contains one Ydj1 peptide-binding fragment complex, corresponding to a solvent content of 42% (V_M = 2.55 Å^3 Da^{-1}; Matthews, 1968). Structure determination of the complex by the MAD method is under way.

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