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X-ray diffraction analysis of a crystal of HscA from *Escherichia coli*

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HscA is a constitutively expressed Hsp70 that interacts with the iron–sulfur cluster assembly protein IscU. Crystals of a truncated form of HscA (52 kDa; residues 17–505) grown in the presence of an IscU-recognition peptide, WELPPVKI, have been obtained by hanging-drop vapor diffusion using ammonium sulfate as the precipitant. A complete native X-ray diffraction data set was collected from a single crystal at 100 K to a resolution of 2.9 Å. The crystal belongs to the orthorhombic space group $P2_12_12_1$, with unit-cell parameters a = 158.35, b = 166.15, c = 168.26 Å, and contains six molecules per asymmetric unit. Phases were determined by molecular replacement using the nucleotide-binding domain from DnaK and the substrate-binding domain from HscA as models. This is the first reported crystallization of an Hsp70 containing both nucleotide- and substrate-binding domains.

1. Introduction

Hsp70 molecular chaperones are ubiquitously distributed and participate in multimer assembly and disassembly as well as protein folding, degradation and trafficking (reviewed in Young *et al.*, 2004; Mayer *et al.*, 2002). The protein-binding and chaperone activity of Hsp70s is regulated by ADP and ATP as well as by interactions with specific auxiliary co-chaperones which modulate nucleotide binding and ATPase activity and help mediate peptide delivery. All members of the Hsp70 family contain two distinct domains, an N-terminal nucleotide-binding domain (NBD; ∼45 kDa) and a C-terminal substrate-binding domain (SBD; ∼25 kDa), and allosteric interactions between the domains act to regulate chaperone activity. Binding of ADP to the NBD favors peptide binding to the SBD, whereas ATP binding favors peptide release, but the molecular mechanism of these heterotropic interactions is not known. High-resolution X-ray crystallographic and NMR studies of the isolated NBD (Zhang & Zuiderweg, 2004; Sriram *et al.*, 1997; Harrison *et al.*, 1997; Flaherty *et al.*, 1990) and of the isolated SBD (Cupp-Vickery *et al.*, 2004; Morshauser *et al.*, 1999; Wang *et al.*, 1998; Zhu *et al.*, 1996) have been determined and these have provided detailed information about nucleotide and peptide binding to the individual domains. However, no X-ray crystal structure containing both the NBD and the SDB has been determined and the nature of the interactions between the two domains is poorly understood.

We have carried out studies on HscA (Hsc66; 66 kDa) from *Escherichia coli*, a constitutively expressed Hsp70 that participates in the biosynthesis of iron–sulfur proteins (Seaton & Vickery, 1995; Vickery *et al.*, 1997). HscA binds IscU, a 12 kDa protein proposed to serve as a molecular scaffold for [Fe–S] cluster assembly, and specifically recognizes a conserved sequence motif, LPPVK (Hoff *et al.*, 2000, 2002; Silberg *et al.*, 2001). The structure of the HscA SBD (residues 389–616) was recently solved in complex with an IscU-derived peptide ELPPVKIHC (Cupp-Vickery *et al.*, 2004) and revealed molecular interactions in the peptide-binding cleft that contribute to HscA peptide-recognition specificity.

Our initial efforts to crystallize the mature full-length HscA protein (residues 2–616) for structure determination have been unsuccessful. To facilitate crystallization, we have investigated the removal of potentially flexible regions at the N- and C-termini of HscA that might lead to mixed populations of solution structures and

prevent crystallization (Fig. 1). HscA contains an N-terminal extension of 17 amino acids on the NBD that is not found in other Hsp70s, suggesting that it might be possible to delete this segment without major effects on chaperone activity. A second site for truncation is located between the α- and β-subdomains of the SBD. HscA lacking the lid-like α-subdomain (residues 506–616) retains allosteric coupling between the NBD and peptide-binding β-subdomain of the SBD (Silberg *et al.*, 2001). We therefore created a truncated form of HscA containing residues 17–505 for crystallization trials. Here, we report the expression, crystallization and X-ray diffraction analysis of HscA(17–505) containing both the NBD and SBD in complex with an IscU-derived recognition peptide.

2. Experimental results

The recombinant plasmid pTrcHscA(17–505) was created by PCR, cloning a fragment from pTrcHsc66 (Vickery *et al.*, 1997) into pTrc99A (Amersham Pharmacia). To facilitate cloning and translation, an *Nco*I site was introduced at residue 17 and the codon corresponding to Asp506 was mutated to a stop codon. Insertion of the *Nco*I site created an initiation methionine (Met17) and changed Arg18 to alanine. Initial attempts at protein expression in DH5α cells using pTrcHscA(17–505) as described for pTrcHsc66 yielded ~100-fold lower expression than for the full-length protein as assessed by SDS–PAGE. As low expression can result from a reduction in ribosome-binding capacity owing to secondary-structure formation at the start site within mRNA (Kozak, 1999), guanine and cytosine bases in the initiation region were replaced with adenine and thymine as synonymous substitutions (acc atg gct AgA TtA) following a motif presented by Gold *et al.* (1981). These changes improved expression levels by approximately tenfold. HscA(17–505) was expressed and purified as described previously for the wild-type protein (Vickery *et al.*, 1997). The purified protein was stored in 50 m*M* Tris–HCl pH 8.0, 0.5 m*M* EDTA and 1 m*M* DTT at ~20 mg ml^{-1}. HscA(17–505) exhibits slightly elevated ATPase activity relative to the full-length protein as found for HscA(2–505) owing to the removal of the lid-like α-subdomain (Silberg *et al.*, 2001) and retains allosteric communication between the NBD and SBD (unpublished results).

Crystallization occurred in the presence of an IscU-recognition peptide, WELPPVKI (Tapley & Vickery, 2004). Initial crystallization conditions of HscA(17–505) with the peptide were found by the hanging-drop vapor-diffusion method using Hampton Crystal Screen I (2.0 *M* ammonium sulfate). Crystallization conditions were refined at room temperature to 1.6 *M* ammonium sulfate pH 5.2 as the mother liquor with 3 µl mother liquor and 3 µl protein solution in the hanging drop. The protein solution contained equimolar concentrations (200 µ*M*) of HscA(17–505) and peptide. Using these conditions, crystals suitable for X-ray diffraction analysis were obtained within one week by microseeding a pre-equilibrated drop. The crystals were dipyramidal in shape, with dimensions up to 0.1 mm. Prior to data collection, crystals were transferred to a cryoprotectant solution consisting of 25%(*v*/*v*) glycerol containing 1.6 *M* ammonium sulfate and were flash-frozen in liquid nitrogen.

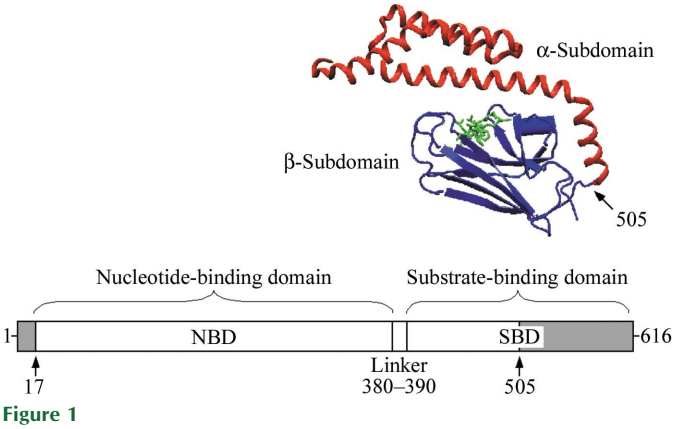

Figure 1
Schematic representation of the HscA protein domain and subdomain structure. The construct used for crystallization studies contained residues 17–505 and the deleted regions (1–16 and 506–616) are shaded. The structure of the HscA substrate-binding domain with bound ELLLVKIHC peptide (Cupp-Vickery *et al.*, 2004) is shown above the scheme with the β-subdomain colored blue, the lid-like α-subdomain colored red and the peptide shown in green.

Table 1
Summary of crystallographic data.

Values in parentheses refer to the outermost shell (2.90–3.05 Å).

Beamline (ALS)	5.0.1
Wavelength (λ)	1.0
Space group	$P2_12_12_1$
Unit-cell parameters (Å)	a = 158.37, b = 166.16, c = 168.24
Resolution (Å)	100–2.9
Total No. reflections	486133
No. unique reflections	98858
Multiplicity	4.9 (5.0)
Completeness (%)	100 (100)
$I/\sigma(I)$	14.1 (2.3)
R_{sym}† (%)	10.9 (65.2)

† $R_{sym} = 100\sum|I_i - \langle I\rangle|/\sum I_i$, where I_i is the observed intensity and $\langle I\rangle$ is the statistically weighted average intensity of multiple observations of symmetry-related reflections.

Diffraction data from a single HscA(17–505) crystal were collected to 2.9 Å under cryogenic conditions (100 K) at the Lawrence Berkeley Laboratory (ALS). Integration and scaling of the diffraction data was accomplished with *MOSFLM* (Leslie, 1998) and *SCALA* (Weiss, 2001) from the *CCP*4 crystallographic program suite (Collaborative Computational Project, Number 4, 1994). The crystals belong to the orthorhombic space group $P2_12_12_1$, with unit-cell parameters a = 158.35, b = 166.15, c = 168.26 Å. The statistics of the crystallographic data collection are summarized in Table 1.

Using the Matthews equation (Matthews, 1968) and assuming a solvent content of ~45–70%, the number of molecules was estimated to be 5–9 molecules per asymmetric unit. Subsequent analysis of the self-rotation function revealed peaks at κ = 120 and κ = 180°, suggesting threefold and twofold symmetry, respectively. Taken together, the Matthews equation and self-rotation function suggest that the asymmetric unit contains six molecules arranged as dimers related by a threefold rotation.

Initial phases were determined using likelihood-based molecular replacement with *PHASER* v.1.2 (Storoni *et al.*, 2004). The β-subdomain (residues 389–505) of the HscA(SBD)–ELPPVKIHC complex (Cupp-Vickery *et al.*, 2004; PDB code 1u00) was used as an SBD search model. The DnaK NBD (Harrison *et al.*, 1997; PDB code 1dkz; residues 6–383; 42% sequence homology with HscA) was used as an NBD search model; alanine was substituted for non-conserved residues in the DnaK model. The relationship between the NBD and SBD models was not known and molecular replacement was performed using the DnaK NBD and HscA SBD as separate probes. Based on the assumptions above, there are six molecules in the asymmetric unit; however, the NBD and SBD search models account for only 12.5 and 3.8%, respectively, of the atoms in the asymmetric unit. Because of the small fraction of atoms represented by the search models, identification of the molecular-replacement solution was a difficult multi-step process. A partial solution was found using the DnaK NBD model and five NBD molecules could be identified in the asymmetric unit. This solution was then used to search for the SBD

and three SBD domains could be located. The model containing five NBD and three SBD models was subjected to rigid-body refinement and used to generate initial phases (*CNS*; Brünger *et al.*, 1998). Examination of the electron-density map generated using the initial phases allowed us to identify the relationship between the NBD and SBD. This information was used to construct a model that contained both the NBD and SBD and this model was used to perform additional molecular-replacement trials in *PHASER* (Storoni *et al.*, 2004). A clear solution that contained six HscA molecules was identified. The six molecules are arranged as three dimers and the non-crystallographic symmetry of the model corresponded to the peaks found in the self-rotation function. Rigid-body refinement in *CNS* (Brünger *et al.*, 1998) yielded an initial crystallographic *R* factor of 47% and an $R_{\rm free}$ of 48%. This model will be used as the starting structure for model building and refinement.

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