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Crystal structure of the C-terminal three-helix bundle subdomain of *C. elegans* Hsp70

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Abstract

Hsp70 chaperones are composed of two domains; the 40 kDa N-terminal nucleotide-binding domain (NDB) and the 30 kDa C-terminal substrate-binding domain (SBD). Structures of the SBD from *Escherichia coli* homologues DnaK and HscA show it can be further divided into an 18 kDa β-sandwich subdomain, which forms the hydrophobic binding pocket, and a 10 kDa C-terminal three-helix bundle that forms a lid over the binding pocket. Across prokaryotes and eukaryotes, the NBD and β-sandwich subdomain are well conserved in both sequence and structure. The C-terminal subdomain is, however, more evolutionary variable and the only eukaryotic structure from rat Hsc70 revealed a diverged helix–loop–helix fold. We have solved the crystal structure of the C-terminal 10 kDa subdomain from *Caenorhabditis elegans* Hsp70 which forms a helical-bundle similar to the prokaryotic homologues. This provides the first confirmation of the structural conservation of this subdomain in eukaryotes. Comparison with the rat structure reveals a domain-swap dimerisation mechanism; however, the *C. elegans* subdomain exists exclusively as a monomer in solution in agreement with the hypothesis that regions out with the C-terminal subdomain are necessary for Hsp70 self-association.

Keywords: Hsp70; Chaperone; *C. elegans*; Domain-swap; Three-helix bundle

Seventy kiloDalton heat-shock proteins (Hsp70s) are essential molecular chaperones involved in numerous protein folding processes [1]. They function via the repetitive transient association with exposed hydrophobic patches on client proteins in an ATP-dependent manner. Hsp70s are composed of two intimately related but functionally distinct domains; the 40 kDa N-terminal nucleotide-binding domain (NBD), which binds and hydrolyses ATP, and the 30 kDa C-terminal substrate-binding domain (SBD) [2]. The SBD can be further divided into an 18 kDa β-sandwich subdomain which forms the hydrophobic binding pocket and a 10 kDa helical-bundle subdomain which forms a lid over the binding pocket [3].

Substrate binding and release is an allosteric process. ATP binding in the NBD favours an open low-affinity conformation characterised by rapid substrate association and dissociation. ATP hydrolysis, triggered by substrate binding in synergy with J domain co-chaperones, shifts the equilibrium towards a closed high-affinity state, holding the substrate in the binding pocket [4]. The conformational changes involved are not precisely understood but involve regions of the β-sandwich subdomain surrounding the substrate-binding pocket and movement of the 10 kDa helical-lid.

Compared to the NBD and β-sandwich, the helical subdomain is less well conserved. Although not essential for chaperone activity, it does play an important role in stabilising the closed state, especially under stress conditions [5]. The structure of the complete SBD from *Escherichia coli* DnaK [3] revealed the lid encapsulates the bound substrate with a conformational change necessary to allow dissociation. The exact mechanisms of this remain unclear and models involving a small hinge movement [3], a pivot of the whole subdomain or local unfolding of regions of the helix immediately covering the binding pocket [6,7] have been

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proposed. In addition, the C-terminal subdomain is important for binding co-chaperones, including Hsp40/DnaJ [8] and a family of TPR domain containing co-chaperones [9] which bind to the extreme C-terminal EEVD motif.

The C-terminal 10 kDa subdomain is also implicated in Hsp70 oligomerisation. Hsp70 predominantly exists as a monomer but can also dimerise and further oligomerise in a concentration-dependent manner [10]. The SBD is both necessary and sufficient for self-association [11,12]; however, there are conflicting views on the exact mechanisms and both the β-sandwich and helical-lid subdomains have been proposed to mediate oligomerisation. Fouchaq et al. showed that the β-sandwich subdomain of bovine Hsc70 oligomerised in a substrate-sensitive manner comparable to the whole protein and also that oligomerisation of a 60 kDa fragment, lacking the C-terminal helical-lid, was both peptide and ATP sensitive [13]. Conversely, Hsiao and colleagues observed a dimer of the C-terminal subdomain from rat Hsc70 in the crystal state and confirmed that this domain was both necessary and sufficient for oligomerisation in solution [14]. Finally, a recent study has implicated regions of both domains to be necessary for dimerisation of human Hsp70 [15].

Structures of the C-terminal helical subdomain are only available for *E. coli* homologues DnaK [3,16] and HscA [17], and rat Hsc70 [14]. Despite structural conservation of the NBD and β-sandwich, the helical subdomains were observed to adopt alternative conformations; DnaK and HscA formed three-helix bundles whilst rat Hsc70 formed a dimeric helix–loop–helix. We have determined the crystal structure of this subdomain from *Caenorhabditis elegans* Hsp70 which shows a three-helix bundle similar to the distantly related bacterial homologues. This represents the first direct evidence of the structural conservation of this subdomain in eukaryotes. Comparison with the divergent rat structure reveals a putative domain-swap dimerisation mechanism though we show that the isolated *C. elegans* domain exists exclusively as a monomer in solution.

Materials and methods

Cloning, expression, and purification. The C-terminal 10 kDa subdomain of *C. elegans* Hsp70 homologue Hsp70A (now denoted ceHsp70-CT) was cloned, expressed, and purified as described [18]. Briefly, cDNA corresponding to residues 542–640 of Hsp70A was cloned into expression vector pET-28a (Novagen) and expressed in Rosetta2(DE3) *E. coli* (Novagen) at 37 °C for 4 h. His-tagged ceHsp70-CT was enriched using a Ni–NTA superflow (Qiagen) column prior to passage over a Sephacryl-200 HR (Pharmacia) gel-filtration column. Protein was stored at 4 °C in buffer A (25 mM Hepes, pH 7.5, 50 mM KCl, and 1 mM DTT).

Crystallisation and data collection. Crystallisation of an orthorhombic crystal form belonging to space group $I2_12_12_1$, with unit-cell dimensions $a = b = 196.9$, $c = 200.6$ Å, was previously described [18]. A new tetragonal form diffracting to 3.2 Å was subsequently produced using hanging drop vapour diffusion at 17 °C from drops consisting of an equal mixture of protein (15 mg ml^{-1}) and reservoir solution (55% saturated ammonium sulphate, 0.5% PEG 400, and 0.1 M sodium citrate, pH 6.0). Crystals were flash-cooled in liquid nitrogen directly from well solution prior to data collection at 100 K using station BM14, ESRF, Grenoble, France. 120° of data was collected using a 1° oscillation. Data were indexed and integrated using MOSFLM [19] and scaled using SCALA [20]. Crystals belong to space group $P4_22_12$ with unit-cell dimensions $a = b = 138.9$ Å, $c = 100.6$ Å.

Structure determination. Phases for the orthorhombic crystal form were derived using multiwavelength anomalous dispersion (MAD) with data collected from a mercury derivative crystal [18]. Native data to 3.5 Å were used to build a preliminary model containing 24 monomers in the asymmetric unit arranged as four hexamers related by translational non-crystallographic symmetry. Self-rotation Patterson analysis of the new tetragonal crystal form indicated the same general packing and the unit cell volume suggested an asymmetric unit with one hexamer observed in the orthorhombic solution. Molecular-replacement with PHASER [21] using one hexamer as a search model was successfully employed. Model-building and refinement was continued with COOT [22] and REFMAC [20]. TLS refinement [23] with one TLS group per monomer was used. Hydrogen atoms were included in riding positions.

The final model contains six protomers in the asymmetric unit and was refined to an R_{cryst}/R_{free} of 26.8/28.2. Tight NCS restraints were applied throughout refinement and all protomers are identical with RMSDs <0.05 Å. Most residues are well modelled except the first 12 N-terminal amino acids, belonging to the recombinant 6×His tag, and the last 26 C-terminal residues. Six sulphate ions are included but water molecules were not added due to the rather low resolution of the data. The strereochemical quality was checked with PROCHECK [24] with all parameters within or better than the expected range for data of this resolution. Diffraction data, refinement statistics, and model parameters are given in Table 1. The coordinates and structure factors have been deposited in the RSCB Protein Data Bank under Accession Code 2P32. Figs. 1, 2B, and 3 were generated with PyMol (http://www.pymol.org), Fig. 2B generated with ESPript [25].

Table 1
Data collection and refinement statistics

A. Data collection	
Wavelength (Å)	0.978
Space group	$P4_22_12$
Unit-cell parameters (Å)	$a = b = 138.9$, $c = 100.6$
Resolution range (Å)	36–3.2
No. observations	146865 (21737)
No. unique reflections	16809 (2399)
Completeness (%)	99.9 (100)
Redundancy	8.7 (9.1)
R_{sym}[a] (%)	13.6 (93.6)
$R_{p.i.m}$[b] (%)	5.1 (33.2)
$I/\sigma(I)$	12.9 (2.0)
B. Structure refinement	
Protein atoms	3966 (6 molecules)
Sulphate ions	6
Resolution range	36–3.2
R_{cryst}[c]/R_{free}[d] (%)	26.8/28.2
Average *B*-factor (Å^2)	89
RMSD bonds (Å)/angles (deg.)	0.018/1.76
Ramachandran plot	
Most favoured (%)	80
Additionally allowed (%)	15.5
Generously allowed (%)	4.5

Values in parentheses are for the highest resolution bin.

[a] $R_{sym} = \sum_{hkl}\sum_i|I_i(hkl) - \langle I(hkl)\rangle|/\sum_{hkl}\sum_i|I_i(hkl)|$.

[b] $R_{p.i.m} = \sum_{hkl}[1/N-1]^{1/2}\sum_i|I_i(hkl) - \langle I(hkl)\rangle|/\sum_{hkl}\sum_i|I_i(hkl)|$, where $I_i(hkl)$ and $\langle I(hkl)\rangle$ are the observed individual and mean intensities of a reflection with indices *hkl* respectively, Σ_i is the sum over the individual measurements of a reflection with indices *hkl*, Σ_{hkl} is the sum over all reflections, and *N* is redundancy.

[c] $R_{cryst} = \sum_{hkl}||F_{obs}| - |F_{calc}||\sum_{hkl}|F_{obs}|$, where F_{obs} and F_{calc} are the observed and calculated structure factors, respectively.

[d] R_{free} as R_{cryst} but summed over a 5% test set of reflections.

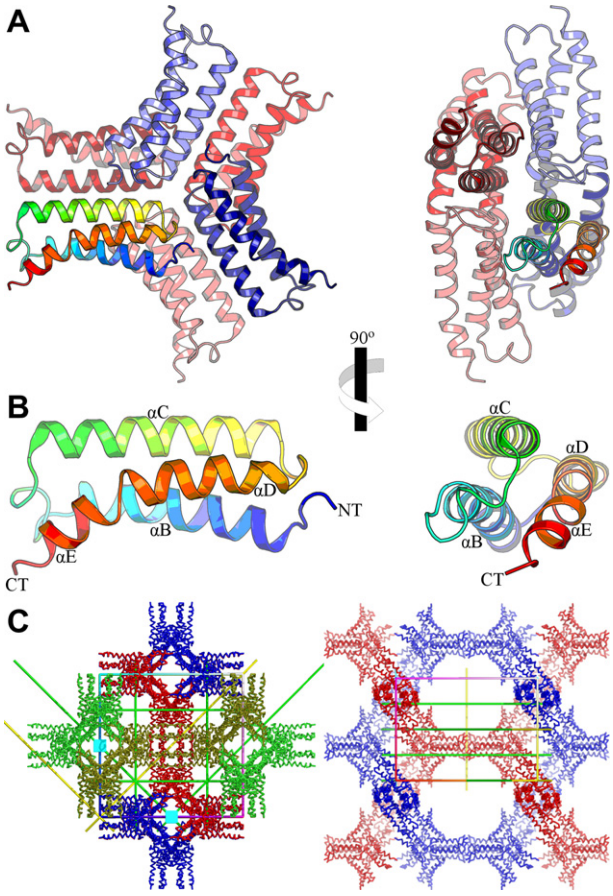

Fig. 1. (A) Structure of the ceHsp70-CT asymmetric unit viewed down the threefold NCS axis (left) and the orthogonal twofold NCS axis (right). The asymmetric unit consists of six protomers arranged as back-to-back trimers, coloured red and blue. One monomer coloured in a gradient from N-terminal (blue) to C-terminal (red). (B) Monomeric structure of ceHsp70-CT. Coloured in a gradient from N-terminal (blue) to C-terminal (red). Helices αB–αD form a compact three-helix bundle with helix αE kinked across one end. The complete sequence of the construct used was *mgsshhhhhhssGLVPRGSHMAS*GLESYAFNLKQTIEDEKLKD KISPE DKKKIEDKCDEILKWLDSNQTAEKEEFEHQQKDLEGLANPIISK LYQSaggappgaapggaaggaggptieevd; recombinant tag residues in italic, disordered residues in lowercase. (C) Crystallographic packing viewed down the c-axis (left) with each sub-lattice coloured red, blue, green or olive; or b-axis (right) showing two sub-lattices intertwined in a left-handed double-helix running down unit-cell c-axis. Blue panel indicates 4_2-screw axis, 2-fold and 2_1-screw axes yellow and green, respectively. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Gel-filtration. Gel-filtration was carried out on an AKTA explorer FPLC using a Superdex 75 HR 30/10 column (Amersham Bioscience) at 4 °C. Two-hundred microlitres of ceHsp70-CT (2, 5, and 80 μM) in buffer A was applied to the column equilibrated in the same buffer and run at 0.5 ml min^{-1}. The column was calibrated with protein standards with sizes ranging from 16.4 Å (13.7 kDa) to 85 Å (669 kDa).

Results and discussion

ceHsp70-CT forms a compact three-helix bundle

The C-terminal three-helix bundle (residues 538–607) from the complete *E. coli* DnaK SBD structure was described as a relatively stable functional unit with a well-defined hydrophobic core [3]. The same region from *C. elegans* Hsp70 (residues 542–640) was crystallised as a recombinant protein incorporating a 23 residue N-terminal 6×His tag. The asymmetric unit contains six molecules with 32 point group symmetry arranged as a pair of back-to-back trimers (Fig. 1A). The crystal packing is particularly elegant with crystal symmetry generating four distinct sublattices, each forming left-handed single-stranded helices extending parallel to the c-axis. These overlay generating double-stranded left-handed helices running down the c-axis (Fig. 1C).

Each monomer is comprised of four α-helices, αB–αE ((−5)542–554, 565–585, 590–603, and 605–612; named in accordance with *E. coli* DnaK [3] and numbered according to Hsp70A, see Fig. 2A; helix αB includes five N-terminal tag residues). The helices form an anti-parallel three-helix bundle, with helices αB–αD arranged in an anti-clockwise up-down-up topology. Helix αE is contiguous with helix αD but kinked 32° at Ala^{604} and extends under the loop connecting helices αB and αC (Fig. 1B). The helices have a classical amphipathic nature with a well-defined hydrophobic core and are stabilised by intra- and inter-chain electrostatic interactions. Nine residues belonging to the recombinant tag are visible in the electron density, five of which form the N-terminal region of helix αB.

In accordance with solution studies of *E. coli* DnaK [16] and the crystal structure of rat Hsc70 [14], the final 26 C-terminal residues were found to be disordered. This highly mobile region is enriched in glycine and proline residues in many Hsp70 family members [26] and contains the conserved co-chaperone binding EEVD motif at the extreme C-terminus.

The ceHsp70-CT structure suggests that the three-helix bundle is conserved in eukaryotes and prokaryotes

Structures of the NBD (cow, human, and *E. coli*) and the β-sandwich subdomain (cow, rat, and *E. coli*) reveal structural conservation from bacteria to mammals. Structures of the C-terminal 10 kDa subdomain are, however, limited to two prokaryotic homologues: *E. coli* DnaK [3] and HscA [17], solved as part of the complete SBD and exhibiting near identical structures; and one eukaryotic homologue: rat Hsc70, solved in isolation [14]. In contrast to the NBD and β-sandwich, the helical subdomains of DnaK/HscA and rat Hsc70 are significantly different with the bacterial isoforms adopting monomeric three-helix bundles and rat Hsc70 forming a dimeric helix–loop–helix.

Across the C-terminal 10 kDa subdomain *C. elegans* Hsp70 shares 69% sequence identity with rat Hsc70, 16% with DnaK, and only 5% with HscA (Fig. 2A). Interestingly, ceHsp70-CT is topologically well conserved with DnaK (residues 538–607) and the more distantly related HscA (residues 535–602) with backbone RMSDs of 2.3 and 2.5 Å, respectively (Fig. 2B). The biggest deviation is

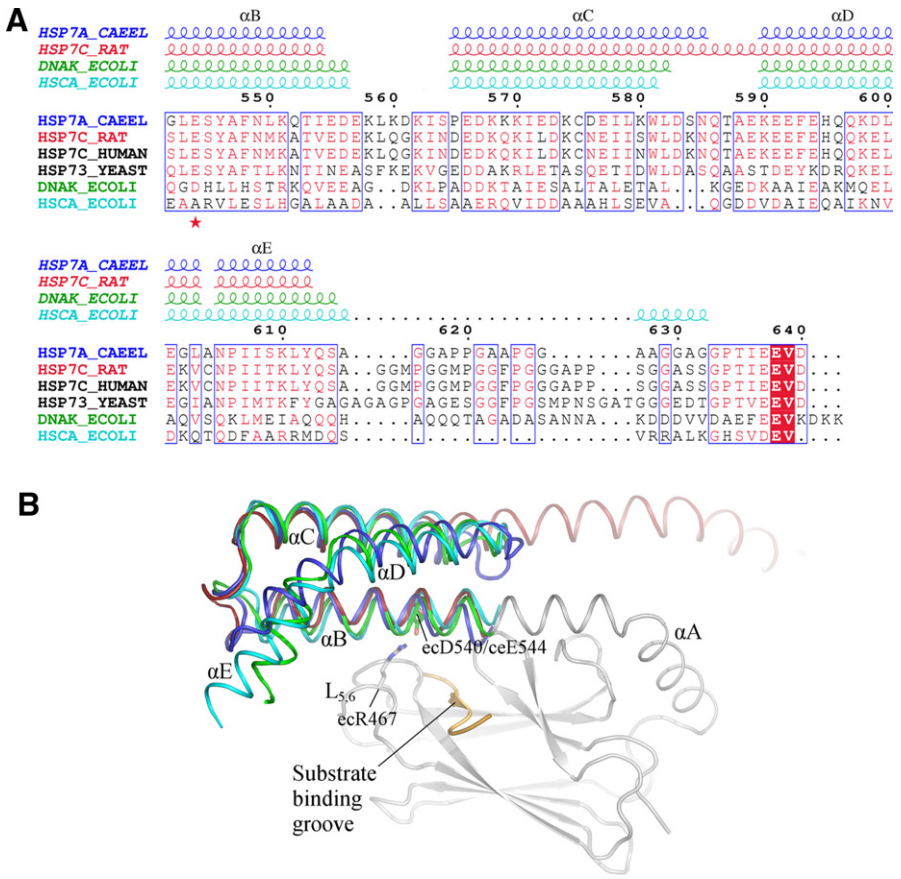

Fig. 2. (A) Multiple sequence alignment of the C-terminal subdomain. Secondary structure of homologues with known structure is indicated. Sequences are labelled with SWISS-PROT IDs, HSP7A_CAEEL is *C. elegans* homologue used in this study. (B) Structural alignment of the C-terminal domains from ceHsp70-CT, rat Hsc70, *E. coli* DnaK, and *E. coli* HscA. Coloured according to sequence alignment. The β-sandwich subdomain from *E. coli* DnaK is included to highlight the lid orientation. The latch interaction between DnaK Asp^{540} (ecD540) and Arg^{467} (ecR467) is shown, conserved *C. elegans* residue Glu^{544} (ceE544; indicated with red star in alignment) aligns structurally with *E. coli* Asp^{540}. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

the position of helix αE, with a more acute kink in the bacterial proteins (60–70°) compared to 32° in ceHsp70-CT.

The conserved fold of this subdomain in isolation demonstrates that this region is an independent folding unit, as was previously observed in an NMR study of the isolated C-terminal helical-bundle from *E. coli* DnaK [16]. The interaction of this subdomain with the outer regions of the SBD β-sandwich in eukaryotes is also inferred by comparison with the SBD from DnaK. The salt-bridge between DnaK Asp^{540} on helix αB and Arg^{467} on loop $L_{5,6}$ of the β-sandwich was proposed to be important in regulating access to the substrate-binding grove [3] and mutations in these residues in *E. coli* and eukaryotic Hsp70s disrupt substrate binding [27,28]. Both residues are conserved in *C. elegans* (Glu^{544} and Arg^{470}) with the position of Glu^{544} structurally conserved with DnaK Asp^{540} (Fig. 2).

A 3D domain-swap relates ceHsp70-CT and the rat Hsc70 C-terminal structure

ceHsp70-CT adopts an alternate conformation compared with the only other eukaryotic Hsp70 C-terminal structure from rat Hsc70 (Fig. 2A). Comparison of the two structures shows that the self-association of rat Hsc70 observed in the crystal structure is mediated via a domain-swap mechanism.

Corresponding residues from the *C. elegans* and rat structures superimpose with a backbone RMSD of 1.16 Å. Helices αB and αC (Leu^{543}-Asn^{585}) from ceHsp70-CT superimpose with the same region from rat Hsc70 chain A whilst helices αD and αE (Lys^{590}-Ser^{614}) superimpose with the equivalent residues from rat chain B (Fig. 3A). The most significant area of difference is loop 2 (Gln^{586}-Glu^{589}), the hairpin loop connecting helices αC and αD in ceHsp70-CT. This region, the hinge region for the domain swap, forms one helical turn in the rat structure resulting in elongated helix αC/D/E. This loop-helix transition leads to dimerisation via the exchange of helices αD and αE such that helices αB and αC of monomer A interact with helices αD' and αE' of monomer B and vice versa (Fig. 3A and B).

The closed interface—the interface found in both the monomer and domain-swapped oligomer—is well conserved with analogous hydrophobic packing in the core

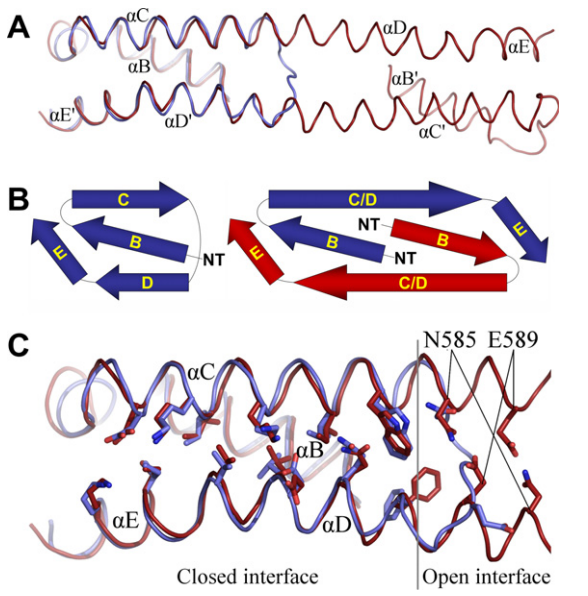

Fig. 3. (A) Superposition of ceHsp70-CT monomer (blue) and rat domain-swapped dimer (red). Structures superimpose with a backbone RMSD of 1.16 Å. (B) Topological representation showing packing of helices in the monomer and domain-swapped dimer. (C) Superposition of the ceHsp70-CT and rat structures illustrating the conservation of the closed interface and the newly formed interactions of the open interface. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

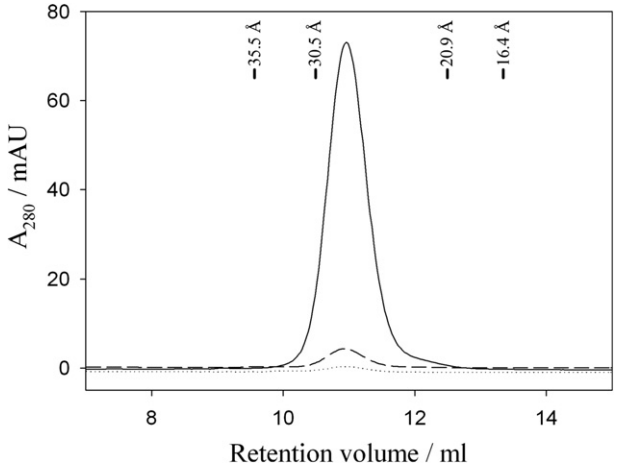

Fig. 4. Gel-filtration analysis of ceHsp70-CT. 80 μM (solid), 5 μM (dashed), and 2 μM (dotted) ceHsp70-CT were resolved on a superdex-75 HR column. Retention volumes of standards with known Stokes radius indicated. ceHsp70-CT elutes as a single peak (retention volume ~10.9 ml) at all concentrations with an estimated Stokes radius consistent with the dimensions of a single protomer in the crystal structure.

of the structure and conserved intra-chain electrostatic interactions (Fig. 3C). In addition, domain-swapping results in the formation of a new open interface—interactions absent in the monomer—with two symmetrical inter-chain hydrogen bonded interactions between hinge residues Asn^{585} and Glu^{589} from opposite chains (Fig. 3C).

The role of the C-terminal 10 kDa subdomain in Hsp70 oligomerisation

Whether the domain-swap observed in rat Hsc70 represents a biologically relevant means of dimerisation is unclear. In addition to crystallising as a dimer, the C-terminal 10 kDa subdomain was shown to be necessary and sufficient for self-association of rat Hsc70 in solution [14]. The oligomeric state of ceHsp70-CT was investigated using gel-filtration. In contrast to rat Hsc70, ceHsp70-CT eluted as a single species over a range of concentrations (2–80 μM) with an estimated Stokes radius of 25–30 Å consistent with dimensions of a single protomer in the crystal structure (longest dimension ~45 Å) (Fig. 4). Corroborating this, analysis of the packing within the asymmetric unit with the web server PISA (http://www.ebi.ac.uk/msd-srv/prot_int/pistart.html) suggests that none of the interfaces are physiologically relevant.

Hsp70 proteins exist in equilibrium between open and closed states accompanied by significant conformational rearrangements [4,29]. Because substrate binding induces dissociation of Hsp70 oligomers, it was postulated that the rat structure could correspond to the open substrate-free state with refolding of the C-terminal helices accompanying the transition from monomer to dimer [14]. No evidence of different conformational states was observed with the *C. elegans* C-terminal subdomain in agreement with an NMR study of an isolated *E. coli* DnaK C-terminal subdomain [16]. Moreover, ATP binding, which allosterically triggers opening of the SBD and substrate release, has also been shown to induce dissociation of Hsp70 oligomers to the monomeric form [11,12]. Accumulating evidence implicates the β-sandwich subdomain in Hsp70 oligomerisation [11–13,15] and it has recently been proposed that the C-terminal dimerisation mechanism based on the rat Hsc70 dimeric structure needs to be re-evaluated [15]. The conserved three-helix bundle structure and monomeric behaviour of the *C. elegans* C-terminal subdomain also argues against such a dimerisation mechanism.

Non-biological domain-swaps are commonly observed in crystal structures, triggered by the non-physiological conditions required for crystallisation. Several examples of domain-swapping in isolated three-helix bundles have been reported [30,31]. The first structure of a cytoskeletal spectrin repeat showed a domain-swapped dimer analogous to the rat Hsc70 C-terminal structure although the three-helix bundle composite monomer was concluded to constitute the correct fold [30]. This was confirmed with the structure of two consecutive repeats showing two three-helix bundles connected by a helical linker [32]. Even when artificially induced, domain-swapped structures can provide insight into protein folding and flexibility. Folding pathways of three-helix bundles have been proposed to be populated by open two-helix intermediates suitable for domain-swapped dimer formation [33,34]. Thus, although unlikely to be relevant for Hsp70 function, the open conformation of the rat C-terminal subdomain and other

three-helix bundles domain-swaps may provide snapshots of folding intermediates.

In summary, the C-terminal 10 kDa subdomain from *C. elegans* Hsp70 is shown to form a three-helix bundle. Despite a high degree of sequence variability, the structural conservation of this domain amongst Hsp70s has been suggested [3,16] but this is the first direct evidence of this in a eukaryotic homologue. Comparison with rat Hsc70, the only other eukaryotic structure, reveals it dimerises via a domain-swap mechanism; however, the conserved structure and monomeric behaviour of the *C. elegans* subdomain supports the idea that regions out with the C-terminal 10 kDa subdomain are necessary for Hsp70 oligomerisation.

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