Characterization of nucleotide-induced changes on the quaternary structure of human 70 kDa heat shock protein Hsp70.1 by analytical ultracentrifugation

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INTRODUCTION

The 70 kDa heat shock protein (Hsp70) is a central protein in the ubiquitous chaperone system, which is the major machinery that protects cells from stress and facilitates cellular protein folding (1-3). This particular chaperone is the most highly conserved protein in evolution and in eukaryotic family members it is localized in the cytosol, endoplasmic reticulum and mitochondria (4). In fact, eukaryotes have more than one gene that encodes for Hsp70 proteins and the human Hsp70 family consists of at least eight gene products that differ in cellular location, functionality and type of induction, constitutive or stress-induced (5).

All types of Hsp70 proteins have a two-domain structure, an N-terminal ATPase domain that is 45 kDa (nucleotide-binding domain, NBD) and a C-terminal polypeptide-binding domain that is 25 kDa (substrate-binding domain, SBD), which is critical for the regulated interactions of this chaperone (Fig. 1). The affinity for client proteins is increased by several orders of magnitude upon hydrolysis of ATP to ADP and inorganic phosphate while substrate binding to the SBD stimulates ATP hydrolysis. However, Hsp70s in general have only low ATPase activity and the action of Hsp40 or Hsp40-like co-chaperones, which stimulate ATP hydrolysis by specific rate enhancement, is required (6, 7). A proposed model for this action is that Hsp40 proteins form complexes with unfolded proteins preventing their aggregation and delivering them to Hsp70. In this model the stable Hsp70-substrate complex is formed by a mechanism that involves an Hsp40 J-domain dependent conversion of ATP to ADP and inorganic phosphate. The dissociation of the Hsp70-

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Fig. 1. Schematic representation of Hsp70 domains and available high-resolution structures. 1S3X: human Hsp70.1 (locus HSPA1A) residues 2-382, PDB accession number 1S3X (18). 2P32: C. elegans Hsp70 residues 1-554, PDB accession number 2P32 (20). 1YUW: bovine (Bos taurus) Hsc70 (locus HSPA8) residues 1-554, PDB accession number 1YUW (17). 1DKX: E. coli DnaK residues 389-607, PDB accession number 1DKX (31). The position of the EEDV sequence is indicated in gray.
substrate complexes will then occur during regeneration of the Hsp70-ATP bound state.

Biochemical and crystallographic studies suggest that hydrolysis of ATP causes a conformational change in the ATPase domain of Hsp70 that is transmitted to the polypeptide binding site, which modulates the affinity of this chaperone for unfolded substrates (8). However, a full understanding of how the information is transmitted from the NBD to the SBD has not yet been established. Therefore, we used analytical ultracentrifugation (AUC) and dynamic light scattering (DLS) techniques to evaluate changes caused by adenosine nucleotide binding on the conformation of human stress-induced Hsp70.1 (locus HSPA1A). Our results suggest that ATP induced changes in the conformation of Hsp70 apparently by interfering with the relative movement of the two domains. The conformational change hypothesis discussed here is in good agreement with previous biochemical experiments.

RESULTS AND DISCUSSION

ATP had larger effect on the conformation of Hsp70 than ADP

Our results indicate that the effect of ATP on the conformation of Hsp70.1 is larger than that caused by ADP, a result that correlates well with previous biochemical studies (9). The aforementioned observation was supported by experiments using sedimentation velocity AUC. Sedimentation velocity AUC can be used to measure the sedimentation coefficient \( s \) (data not shown), which provides information on the shape of a macromolecule (10-12). The standard sedimentation coefficients \( s_{20w} \) at each protein concentration were used to estimate the \( s_{20w} \) at 0 mg/ml protein concentration \( s_{20w}^0 \) by extrapolation (Fig. 2). This procedure minimizes errors caused by temperature, solution viscosity, and molecular crowding (12). Therefore, the values obtained for \( s_{20w} \) can be used to investigate whether nucleotide binding had modified the shape of Hsp70.1. The \( s_{20w}^0 \) of Hsp70.1 in the absence of nucleotide binding was 4.40 ± 0.03 S, in the presence of ADP it was 4.53 ± 0.05 S, and in the presence of ATP it was 4.72 ± 0.03 S. Based on these results it is apparent that ADP had a low impact on the value of \( s \) whereas ATP caused a 5% increase. Since the sedimentation coefficient \( s \) is directly related to the shape of the molecule we conclude that ATP had a significant effect on the conformation of Hsp70.

The sedimentation coefficient is sensitive to molecular mass and can change due to protein aggregation. However, the results of several experiments using different probes showed no sign of aggregation under the experimental conditions used here. First, gel filtration chromatography showed that Hsp70.1 behaves as a single species (13). Second, the diffusion coefficient \( D_{20w}^0 \) of Hsp70.1 obtained by dynamic light scattering was 5.7 ± 0.1 \( \times 10^{-7} \text{cm}^2\text{s}^{-1} \) in the absence of nucleotide, 5.9 ± 0.1 \( \times 10^{-7} \text{cm}^2\text{s}^{-1} \) in the presence of ADP, and 5.8 ± 0.1 \( \times 10^{-7} \text{cm}^2\text{s}^{-1} \) in the presence of ATP. Based on these results molecular masses were calculated using Equation 2 and were found to be the same, 72 ± 2 kDa, within error of the measurements, indicating that no aggregated species were present. The molecular masses were also measured by sedimentation equilibrium. The best fit to the experimental data for 250 \( \mu \text{g/ml} \) of unbound Hsp70.1 (in TKP buffer) is shown in Fig. 3 (bottom panel). The fit was consistent with a single species that had a molecular mass of 70 ± 2 kDa and the random distribution of the residuals for this model (Fig. 3, middle panel).
el) was indicative of a high quality fit.

The so-called Perrin or shape factor \( F \) (14), which provides information on the shape of the molecule, was used to investigate the change in shape of HSP70.1 caused by nucleotide binding. The Perrin factor represents a ratio of the measured frictional coefficient \( f \) to the frictional coefficient \( f_0 \) of a hypothetical sphere for which a hypothetical radius is calculated using its molecular mass. For the theoretical calculations, subunits were treated as globular proteins (both equatorial, \( a \), and polar, \( b \), radii are equal). In this way, the Perrin factor (\( f_0/f \)) can be used to determine the shape of a particle or protein because the closer it is to 1, the more spherical (globular) the shape is. Using this method, Hsp70.1 was found to be an asymmetric protein with a \( f_0/f \) ratio of approximately 1.40 ± 0.01. However, in the presence of ADP the \( f_0/f \) ratio for Hsp70 was approximately 1.36 ± 0.02, and in the presence of ATP it was approximately 1.30 ± 0.01. Therefore, the \( f_0/f \) ratio was closer to 1 in the presence of nucleotides, which indicates that nucleotide binding promotes a less elongated conformation of the chaperone. Movements that change the relative position of the domains could induce a more globular shape for Hsp70 (see discussion below). These results support a model in which ATP binding to Hsp70 causes a conformational change in the chaperone that is larger than that caused by ADP. This hypothesis was also supported by calculating the concentration-dependence coefficient \( k_F \) from Fig. 2 using Equation 3. The value of \( k_F \) is usually small for globular proteins and increases for elongated particles (15). In the presence of ADP the \( k_F \) value of HSP 70 was calculated to be 0.24 ± 0.03 mL/mg and in the presence of ATP it was calculated to be 0.16 ± 0.01 mL/mg. These results indicate that ATP-bound Hsp70 had a less elongated shape than ADP-bound Hsp70.

However, contrary to the aforementioned for nucleotide-bound Hsp70.1 the sedimentation coefficients for the unbound protein seemed to be concentration independent (Fig. 2), indicating that a possible anomaly caused by viscosity effects occurred. Viscosity effects are known to severely affect the values of \( k_S \) (16) and therefore we believe this affected the sedimentation of unbound Hsp70 and caused this anomalous behavior. Although the explanation for this behavior is not simple and possibly involves changes in the hydration of the protein, altogether the results support the observation that nucleotide binding affected the conformation of Hsp70.

**What is the origin of the conformational change caused by ATP in Hsp70?**

The mechanism by which structural changes are induced by nucleotide-binding in Hsp70 is under intense investigation and has received special attention since recent findings suggest that Hsp70 proteins have an important role in human conformational diseases. Thus, obtaining information about these conformational changes has potential medical applications. However, a full-length crystallographic structure of Hsp70 is still not available as well as a detailed mechanism of how adenosine nucleotide binding induces conformational changes in the Hsp70 structure.

Since we obtained reliable experimental evidence that ATP binding caused a conformational change in Hsp70, we next wanted to determine if we could gain any useful insight into the mechanism of this conformational change. To address this question, we designed a model structure of Hsp70 with the most likely arrangement of the domains, based on the available high-resolution structures, see Fig. 1. Structural information regarding eukaryotic Hsp70 proteins is available mainly for the isolated domains both in the absence of ligands and in the presence of nucleotides or substrate peptides. The following available crystal structures were used in designing a Hsp70 model (Fig. 1). 1) The crystal structure of the housekeeping Hsc70 (locus \( HSPA8 \)) from bovine (\( Bos taurus \)), which covers most of the amino acid sequence (1-544) of an eukaryotic Hsp70, has modified charged surface residues and 100 amino acids deleted from the C-terminus (17). 2) The crystal structure of NBD (up to residue 382, Fig. 1) in the presence of ADP, which is the only available human Hsp70 structure (18, 19). 3) Crystal structure of SBD, which include the constitutive and housekeeping Hsp70 (locus \( HSPA8 \)) from \( Rattus novergicus \) (residues 386-561 (20)) and that of \( C. elegans \) (residues 533-614, Fig. 1 (21)).

Fig. 4 shows a model in which the structures of the four Hsp70 domains were arranged together: the bovine Hsc70 residues 1-536, which covers the NBD and a large part of the SBD in the absence of nucleotides, the bovine Hsp70 residues 4-381, which covers the NBD, \( E. coli \) DnaK residues 389-607 solved in the presence of a substrate peptide, which covers most of the SBD except for the C-terminus, is shown in blue, \( E. coli \) DnaK residues 389-607 in the presence of ADP, which is shown in green, bovine Hsc70 residues 1-536, which covers the NBD and a large part of the SBD in the absence of nucleotides, is shown in blue, \( E. coli \) DnaK residues 389-607 solved in the presence of a substrate peptide, which covers most of the SBD except for the C-terminus, is shown in green, bovine Hsp70 residues 1-536, which covers the NBD, is shown in blue, \( E. coli \) DnaK residues 389-607 solved in the presence of a substrate peptide, which covers most of the SBD except for the C-terminus, is shown in green, and \( C. elegans \) Hsp70 residues 533-614, which covers the C-terminus, is shown in red. See Fig. 1 caption for more details.
except for the C-terminal end, and the C. elegans Hsp70 residues 533-614, which covers the C-terminal end. The final arrangement of the model was in good agreement with an elongated protein structure and also with the results shown here for Hsp70.1. Based on the hydrodynamic analysis, a $s_{20w}$ of 4.26 S and $D_{20w}$ of 5.38 $\times 10^{-7}$ cm$s^{-1}$ was determined for the model (Fig. 4), which was in very good agreement with the values experimentally determined for unbound Hsp70.1 ($s_{20w}$ = 4.40 S and $D_{20w}$ = 5.70 $\times 10^{-7}$ cm$s^{-1}$). It is likely that the general form of the human Hsp70.1 had an elongated shape that was similar to the crystal structure of bovine Hsc70 (17).

We are aware that this is just a tentative model and the conclusions drawn from it should be carefully evaluated but we suggest that the results presented here support the hypothesis that ATP binding causes changes in the relative position of the domains. The data can also be explained by a different model; however, our hypothesis is supported by two recent studies where the structures of Hsp70 proteins containing short deletions were solved. In both of these studies the C-terminus of the proteins were deleted by 10-12kDa. More specifically, Revington et al. (22) conducted NMR studies on a truncated Thermopilus Hsp70 (residues 1-501, WT is 620 residues long) and Jiang et al. (17) solved the crystal structure of a truncated B. taurus cognate Hsc70 (residues 1-544, WT is 650 residues long). A few mutations were also introduced in these constructs, AT422 and A423E in T. thermophilus Hsp70 and E213A and D214A in bovine Hsc70. These mutations together with the deletion of the C-terminus were necessary to produce proteins that were sufficiently stable for the experiments conducted in these studies. Revington et al. (22) measured the NMR shifts of Hsp70 caused both by nucleotide and peptide binding. Based on these shifts they observed an allosteric effect of binding and suggested that the domains are moving in solution as a single unit and not as two individual structures connected by a flexible linker. They also showed that 28 peaks had significant chemical shift differences between the ADP bound and the apo form whereas this value doubles (sixty two) when the ATP bound form is compared with that of the unbound form. In addition, they found that the results with the whole protein were more dramatic than that for an isolated NBD. Revington et al. (22) suggested that this was due to SBD interactions, which was indicative of an allosteric protein. Therefore, these results provide strong evidence that the interface between the NBD and SBD is affected by nucleotide exchange.

Jiang et al. (17) suggested that the conformational change caused by ATP binding would propagate throughout a flexible linker that is 10 amino acids longer and connects the NBD to the SBD. In fact, the linker became less protease sensitive upon ATP binding but not ADP (23), supporting the aforementioned hypothesis. Therefore, the linker will move to a less exposed region causing, according to Jiang et al. (17), a movement of the two domains to avoid steric clashes. This movement would trigger both the opening of the lid and the release of the substrate.

In conclusion, it is unlikely that the modifications caused by ATP binding would affect only the NBD and therefore we suggest that the main conformational change was caused by changes in the relative position of the domains. This hypothesis is in agreement with previous studies (22, 24), which showed that binding of ATP causes limited structural changes in the NBD but these changes were still transmitted to the SBD, in which the substrate was bound. Altogether, the results presented here with full-length Hsp70 in combination with those from previous studies fit to a model in which ATP binding causes a conformational change that is transmitted throughout the interface between the NBD and SBD domains.

**MATERIALS AND METHODS**

**Materials**

Cloning, purification and concentration measurements of human Hsp70 were described previously (13). The chaperone was maintained in TPK buffer (25 mM Tris-HCl, pH 7.5, 50 mM NaCl, 5 mM KCl, 5 mM Na$_3$PO$_4$ and 1 mM 2-mercaptoethanol) and folded and behaved as a single species, as previously described (13). All buffers used were of chemical grade and were filtered before use to avoid scattering from small particles.

**Analytical ultracentrifugation**

Sedimentation velocity (SV) and sedimentation equilibrium (SE) experiments were performed using a Beckman Optima XL-A analytical ultracentrifuge and analyzed as previously described (25-27). Briefly, SV experiments were carried out at 20°C and 25,000 rpm (AN-60Ti rotor) and SE experiments were carried out at 20°C and 6,000, 8,000 and 10,000 rpm (AN-60Ti rotor). Scan data acquisition were taken at 230 and 236 nm, at low and high protein concentrations, respectively. Hsp70.1 was tested at concentrations ranging from 150 to 1,000 µg/ml in TPK buffer, both in the presence and in the absence of 200 µM of MgADP or MgATP. The fitting of absorbance versus cell radius was done using SedFit (Version 9.3), which solves the Lamm equation in order to discriminate the spreading of the sedimentation boundary from diffusion (10, 11). The apparent sedimentation coefficient ($s$) and molecular mass ($M$) were obtained from $c$ ($s$) and $c$ ($M$) analyses, respectively.

SE analyses involved fitting a model of absorbance versus cell radius using nonlinear regression. The Self-Association method was used to analyze the SE experiments with several models of association for Hsp70.1. Distribution of the protein along the cell, obtained in the SE experiments, was fit with the following equation (20):
Dynamic light scattering
The diffusion coefficient parameter, D, was obtained from dynamic light scattering (DLS) using a DynaPro-MSX device (Protein Solutions) at 20°C and was used to calculate both high resolution sedimentation coefficient distributions c(s) and molecular mass distribution c(M). The ratio of the sedimentation coefficient (s) to D gives the molecular mass as indicated by the following equation:

\[ M = \frac{sRT}{D(1-V_{bar})} \]  

Equation 2

The software Sednterp (www.jphilo.mailway.com/download.htm) was used to estimate protein partial specific volume at 20°C (\(V_{bar} = 0.734 \text{ mL/g}\)), buffer density (\(\rho = 1.00198 \text{ g/mL}\)), buffer viscosity (\(\eta = 1.0161 \times 10^{-3} \text{ poise}\)), and both \(s_{\text{sphere}}\) and \(D_{\text{sphere}}\) for a globular protein of 72.2 kDa. This software was also used to estimate the standard sedimentation coefficient (s\(_{20,w}\)) at each protein concentration in order to estimate the s\(_{20,w}\) at a 0 mg/mL protein concentration (s\(_{0,20}\)) by extrapolation. This procedure minimizes errors caused by temperature, viscosity and molecular crowding (12). The concentration-dependent coefficient \(k_0\) was obtained from the following equation (29):

\[ s_{20,w} = s_{0,20}(1 - k_0 C) \]  

Equation 3

The frictional ratio (\(f/f_0\)), which is a measure of the shape asymmetry of a hydrated protein when compared to a globular protein, or sphere, of equal molecular mass (12, 30), was also estimated by Sednterp using the \(s_{0,20}\) value. Otherwise, the frictional ratio can be estimated either by the \(D_{\text{sphere}}/D_{\text{20,w}}\) ratio or by the \(s_{\text{sphere}}/s_{20,w}\) ratio (30).

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