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Binding of ATP and ATP analogues to the uncoating ATPase Hsc70 (70 kDa heat-shock cognate protein)

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Nucleotide binding to the 70 kDa heat-shock cognate protein (Hsc70) from mung bean seeds and pig brain was investigated, as well as the clathrin uncoating activity of Hsc70 in the presence of these nucleotides. The two enzymes were found to behave identically. ATP bound to two different forms of Hsc70, with dissociation constants of $1.1 \pm 0.1\ \mu M$ and 1.4 ± 0.7 mM respectively at 25 °C. This corresponds to $\Delta G^{0\prime} = -34$ and -16 kJ/mol respectively. From the temperature-dependence of the dissociation constant of the high-affinity site, $\Delta H^{0\prime}$ was calculated to -36 ± 2 kJ/mol. This gives $\Delta S^{0\prime} = 6.7$ J/mol per K. Adenosine 5′-[γ-thio]triphosphate, ADP, adenosine 5′-[β,γ-imino]triphosphate and adenosine 5′-[β,γ-methylene]triphosphate showed dissociation constants of 2.3, 11, 31 and 284 μM respectively. The order of affinities corresponded to the order of effectiveness in uncoating of pig brain coated vesicles. The implications of these findings for the mechanism of Hsc70 action are discussed.

INTRODUCTION

Hsc70 was first described by Schlossman et al. [1] as an *N*-ethylmaleimide-insensitive clathrin-coat-dependent ATPase of 70 kDa. It is a constitutively expressed member of the 70 kDa class of heat-shock proteins (Hsp70) [2], and is involved in a number of cellular processes [3]. Hsc70 can be digested with chymotrypsin into two domains: an N-terminal 44 kDa ATP-binding domain and a C-terminal 30 kDa peptide-binding domain. The ATP-binding domain is highly conserved between various members of the Hsp70 family of proteins, while the peptide-binding domain is less well conserved. The whole Hsc70 molecule has only a small basal ATPase activity, which is stimulated up to 10-fold by the presence of substrate proteins, but the isolated 44 kDa ATP-binding fragment has full ATPase activity even in the absence of peptide substrates [4].

The three-dimensional structure of the ATP-binding domain of bovine brain Hsc70 has been determined by X-ray diffraction [5], but the structure of the whole Hsc70 molecule, and the mode of energy transmission between the N-terminal ATPase and the C-terminal peptide-binding domains of Hsc70 are unknown.

Despite the obvious importance of this enzyme, its precise mechanism of action during vesicle uncoating has not been elucidated. According to Rothman and Schmid [6,7], a molecule of Hsc70 binds to a central position on the clathrin triskelia within coats in a light-chain-dependent manner, and uses the energy of ATP hydrolysis to displace one leg of a clathrin triskelion from the coat. After this displacement reaction, Hsc70 is transferred to a distal position in the triskelion, which was previously engaged in clathrin–clathrin interactions. Binding to this site ('capture') is independent of light chains and uses ATP catalytically (i.e. without hydrolysis). Once all three legs have been freed by successive (possibly co-operative) binding of three Hsc70 molecules, the triskelion comes out of the cage. Heuser and Steer [8] proposed an essentially similar model, but with the Hsc70 in the final complex occupying a central and ventral position on the triskelion, in agreement with electron microscopic observations. They were also the first to notice the partial effectiveness of adenosine 5′-[γ-thio]triphosphate (ATP[S]) in the uncoating reaction, and found that Hsc70–clathrin complexes are stable only under conditions where ATP hydrolysis cannot occur.

Their finding that non-cleavable analogues such as ATP[S] can to a limited extent support the Hsc70-promoted uncoating of clathrin-coated vesicles has recently been confirmed and extended to other ATP analogues [9]. It is also known that ATP binding to Hsc70 leads to the release of substrate peptides, and that the ADP-bound form of Hsc70 has a higher affinity for peptide than the ATP-bound form [10]. Similar observations have been made for DnaK, a close homologue of Hsc70 found in *Escherichia coli*, where protein release is initiated by ADP/ATP exchange, itself catalysed by binding of the nucleotide exchange factor GrpE [11].

One could, therefore, imagine another possible mechanism, which may apply both to the uncoating reaction and to other activities catalysed by Hsc70. If the Gibbs free energy of binding of ATP to Hsc70 were high enough, then this binding could 'charge' the Hsc70 and open the protein-binding site, thereby releasing any bound protein. This would free Hsc70 to bind new substrate proteins. Hydrolysis of ATP would serve to stabilize the Hsc70–protein complex, until ADP/ATP exchange allowed protein release, thereby closing the catalytic cycle (in this context, it is significant that ATP and ADP can leave their binding site in Hsc70 only by nucleotide exchange, not by dissociation [12]). In the case of clathrin-coated vesicles, binding of Hsc70–ATP to the triskelia could occur to legs of clathrin freed spontaneously, since

Abbreviations used: Hsc70, 70 kDa heat-shock cognate protein (uncoating ATPase); $p[CH_2]ppA$, adenosine 5′-[β,γ-methylene]triphosphate; p[NH]ppA, adenosine 5′-[β,γ-imino]triphosphate; ATP[S], adenosine 5′-[γ-thio]triphosphate; BiP, immunoglobulin heavy chain binding protein (Grp78).

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clathrin–clathrin interactions are not very strong at neutral pH. Binding of Hsc70 would, therefore, prevent the re-formation of the clathrin–clathrin bond and shift the equilibrium towards dissociation of the coat. Upon hydrolysis of ATP the equilibrium would be shifted even further, because the affinity of Hsc70 for clathrin increases. For such a model to be correct, we would expect it to fulfil four predictions: (1) ATP binding must be able to impart sufficient energy to Hsc70 to charge the protein; (2) the ability of ATP analogues to support Hsc70 action would be determined in part by their binding energy; (3) factors which accelerate ATP hydrolysis would be expected to drive uncoating towards completion; and (4) factors which destabilize clathrin–clathrin interactions should favour uncoating.

The Gibbs free energy of the binding of ATP to Hsc70 can be determined from the equilibrium constant of this reaction. Unfortunately, reported values for this K_D range from 10 nM [13] to 100 μM [14], 1 μM [1,15,16] and 10 μM [10]. This corresponds to values for $\Delta G^{0\prime} = -\boldsymbol{R}T\ln(1/K_D)$ from -45.6 to -28.6 kJ/mol, which should be compared with a standard energy of ATP hydrolysis of -34 kJ/mol. These differences can be explained, at least in part, by the fact that Hsc70 prepared by the standard method [1] is a mixture of nucleotide-containing monomeric, and nucleotide-free dimeric, Hsc70. We therefore measured K_D for the dissociation of ATP from nucleotide-free Hsc70. We also measured the temperature-dependence of this constant, as this gives a value for the heat of reaction, $\Delta H^{0\prime}$, one of the components of $\Delta G^{0\prime}$. The other component, the reaction entropy ($\Delta S^{0\prime}$), can then be calculated from $\Delta G^{0\prime} = \Delta H^{0\prime} - T\Delta S^{0\prime}$.

EXPERIMENTAL

Preparation of Hsc70

Hsc70 was prepared from pig brains or mung bean seeds. Brains were obtained as soon as possible after slaughter and transported on ice. Upon arrival in the laboratory they were used immediately. Mung bean seeds were soaked in tap water overnight. Each tissue was homogenized in 3 vol. of ice-cold homogenization buffer (20 mM Hepes/KOH, pH 7.0, 1 mM dithiothreitol, 0.2 mM PMSF) in a food processor. The homogenate was spun for 10 min at 1600 $\boldsymbol{g}$ in a JA10 rotor (Beckman), and the resulting pellet was homogenized and spun again as described. Combined supernatants were spun for 30 min at 18000 $\boldsymbol{g}$. The supernatant from this spin was brought to 40% saturation with $(NH_4)_2SO_4$ and centrifuged for 15 min at 18000 $\boldsymbol{g}$. This step removes some turbidity, which is difficult to pellet by centrifugation alone, and a factor that inactivates ATP–agarose. The supernatant was brought to 80% saturation with $(NH_4)_2SO_4$ and spun again to remove nucleotides. The pellet of this step was dissolved in buffer C of [1] [20 mM Hepes/KOH, pH 7.0, 25 mM KCl, 10 mM $(NH_4)_2SO_4$, 2 mM magnesium acetate, 1 mM dithiothreitol] to the original volume of the cytosol and passed at 2 ml/min through a 26 mm × 150 mm column of ATP–agarose (Sigma; A2767). The column was washed with 200 ml each of buffer C, buffer C plus 150 mM KCl and buffer C again, and then eluted with 1 mM ATP in buffer C. Protein-containing fractions were combined (about 150 ml), passed over a 26 mm × 130 mm column of Bio-Gel HTP (Bio-Rad; a beaded hydroxyapatite) and eluted with a 200 ml gradient from 0 to 500 mM potassium phosphate in buffer C. Hsc70-containing fractions were combined, supplemented to 10 mM with EDTA and concentrated to 5 ml in an Amicon ultrafiltration device. Protein was then precipitated by addition of $(NH_4)_2SO_4$ to 75% saturation. The mother liquor was removed and the protein was dissolved in 2 ml of binding buffer [20 mM Hepes/KOH, 25 mM KCl, 10 mM $(NH_4)_2SO_4$ and 1 mM EDTA] and run on a Superdex 200 column (Pharmacia HiLoad 16/60) in binding buffer. The fractions containing dimeric nucleotide-free Hsc70 were combined, run on a 1 ml HiTrap Q column (Pharmacia), and eluted with a 20 ml gradient from 0 to 1 M KCl in binding buffer. Hsc70-containing fractions were concentrated and the protein precipitated with $(NH_4)_2SO_4$. Hsc70 was stored in this form at 4 °C and found to be stable for several months. The absence of nucleotide was verified by using [α-^{32}P]ATP and [γ-^{32}P]ATP (ICN) at 0.5 c.p.m./pmol for the elution from ATP–agarose and monitoring the radioactivity during purification.

The 44 kDa ATPase domain of Hsc70 was obtained by the method of [4], starting with nucleotide-free Hsc70.

Nucleotide binding to Hsc70

A 20 μg sample of Hsc70 was incubated for up to 30 min at the desired temperature in 100 μl of uncoating buffer [20 mM Hepes/KOH, pH 7.0, 25 mM KCl, 10 mM $(NH_4)_2SO_4$ and 1 mM magnesium acetate] with various concentrations of nucleotides in the presence of 100000 c.p.m. of radioactive nucleotide. A 95 μl aliquot of the mixture was passed by vacuum suction over a hydrophobic Durapore membrane (Millipore; 13 mm diameter; 0.2 μm pore size; presoaked in methanol and washed with water), which was then washed with 5 × 500 μl of uncoating buffer (nucleotide free) at the same temperature as was used during the incubation. The whole filtration step took less than 1 min. Filters were transferred to vials, submerged in scintillation cocktail and counted for radioactivity after they had become translucent. Filtration was carried out with samples in the order of both increasing and decreasing concentrations, to monitor for possible effects of hydrolysis. Samples without Hsc70 and samples containing 100 mM ATP were used to determine background radioactivity (about 100 c.p.m.).

Monomerization of Hsc70 by nucleotides

Samples of 0.25 mg of Hsc70 in a total volume of 0.1 ml of binding or uncoating buffer were incubated for 10 min with the desired concentration of nucleotide (all nucleotides from solutions neutralized with KOH). Then a 40 μl aliquot of 20 mg/ml Dextran Blue 2000 (Pharmacia; a marker for void volume) was added and the sample was injected on to the Superdex 200 column. The column was eluted with uncoating or binding buffer, and 2 ml fractions were collected. The protein concentration in these fractions was measured by the method of Bradford [17], and the protein amounts in the monomer and dimer peaks were calculated by integration.

Other methods

Isolation of coated vesicles and Triton X-100-extracted coated vesicles from pig brain and human placenta, and uncoating of clathrin-coated vesicles (20 μg of coated vesicles and 50 μg of Hsc70 in 200 μl), were performed as described [9]. Protein was determined by the method of Bradford [17], using ready-made reagent and IgG standard solutions (Bio-Rad). SDS/PAGE was performed by the method of Laemmli and Favre [18] on 5–20% gradient gels.

Nucleotide-free Hsc70 from pig brain was also prepared by the method of Gao et al. [13].

Curve fitting was done using the simplex algorithm [19], with error estimation by the Monte-Carlo simulation [20].

To monitor ATP binding by autoradiography, we incubated 2 mg/ml Hsc70 with 11 μM [α-^{32}P]ATP to selectively label the high-affinity site, and cross-linked the bound ATP to the enzyme

with UV light (from an unfiltered mercury lamp) on ice. Cross-linking was complete after 10 min. Labelled Hsc70 was separated from free nucleotides by gel chromatography on a NAP-5 column (Pharmacia), SDS/PAGE was performed on a 5–10 % gradient gel and autoradiography on Kodak X-Omat AR film.

RESULTS

Preparation of nucleotide-free Hsc70

The described method yielded about 100 mg of a nucleotide-free dimeric Hsc70 per kg of tissue, which could be used for binding assays without interference from bound nucleotides. Using radioactive ATP for elution of Hsc70 from the ATP–agarose column, we could follow the release of nucleotide during the purification (Table 1). Bound nucleotide was not removed from Hsc70 by $(NH_4)_2SO_4$ alone, but only in combination with the Mg^{2+} chelator EDTA (which did not release nucleotide by itself; see later). Even under these conditions removal of nucleotide was incomplete, so an additional step of gel filtration to separate the monomeric nucleotide-containing enzyme from the dimeric nucleotide-free enzyme had to be included. Purity was assessed by SDS/PAGE (results not shown). Hsc70 was also prepared from mung beans [21]. The properties of Hsc70 from pig brain and from mung bean were indistinguishable by any assay we performed. We shall report only the results obtained with enzyme from mung bean, except were specifically stated otherwise.

Nucleotide binding

Once the Hsc70 was nucleotide-free, rebinding of nucleotides could be studied quantitatively by our filtration assay. The assay makes use of the very tight binding of ATP (and ADP) to Hsc70, which allows unbound nucleotides to be washed away without effect on those bound. In this context it is important to distinguish between high-affinity ($k_{off}/k_{on} = K_D$ low) and tight (k_{off} low) binding. Binding of ATP to Hsc70 is tight in the sense that bound ATP is released with a half-life of about 7 days, as determined by dialysis against a suspension of activated charcoal (results not shown). Under these conditions free ATP is removed completely after 3 h. Binding of protein by the Durapore membranes was near-quantitative, since 95 % of the radioactivity from $[^{125}I]$iodinated Hsc70 was bound to the filters under the conditions described, independent of the protein amount in the range tested (5–50 μg; results not shown), while free nucleotides passed through the membrane unhindered. This compares favourably with nitrocellulose filters, to which Hsc70 binds incompletely and in a concentration-dependent manner. ATP binding occurred at two different sites, with dissociation constants (at room temperature) of 1.0 ± 0.1 μM and 1.4 ± 0.6 mM respectively. The total binding capacity was 1 mol/mol, with the high-affinity binding accounting for 30–50 % of the capacity in different preparations (Figure 1a). [α-^{32}P]ATP and [γ-^{32}P]ATP gave identical results. There was no difference in binding between samples incubated for 2 min and up to 6 h, indicating fast binding of ATP to Hsc70 (results not shown).

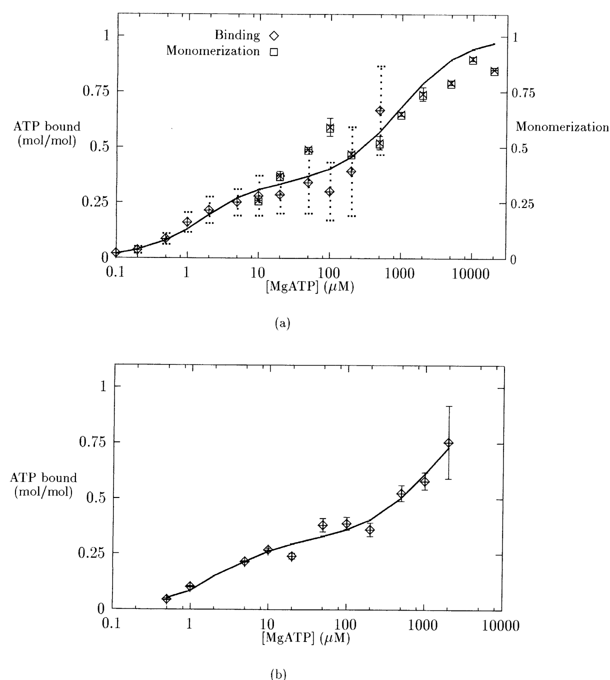

Figure 1 Binding of ATP to Hsc70, and binding-induced monomerization

Binding of ATP (◇) was determined by filtration (means ± S.D. of several experiments), and binding-induced monomerization (□) was determined by gel filtration (means ± S.D. of two independent experiments). The parameters for the two-step binding curve were estimated by non-linear least squares fitting to the combined data set, using the equation:

$$B_{exp} = \frac{n_1(T - B_{obs})}{K_1 + T - B_{obs}} + \frac{n_2(T - B_{obs})}{K_2 + T - B_{obs}}$$

where B_{exp} and B_{obs} are expected and observed concentrations of bound ligand respectively, n_1 and n_2 are binding capacities, T is total concentration of ligand, and K_1 and K_2 are dissociation constants. All data were then normalized to mol of ATP/mol of Hsc70. (**a**) Binding to mung bean Hsc70 prepared as described in the Experimental section; (**b**) binding to pig brain Hsc70 prepared by the method of Gao et al. [13].

In order to check whether our method of Hsc70 preparation had any adverse effects on the enzyme, which might account for these two steps in the binding curve, we also prepared nucleotide-free pig brain Hsc70 by the method described in [13] and measured the binding of ATP to this preparation. As shown in Figure 1(b), the results were very similar to those obtained with mung bean Hsc70 prepared by our method. A slightly higher value for the K_D of the enzyme prepared by the method of [13] is probably caused by incomplete removal of ATP (about 0.2 mol/mol of enzyme remained; results not shown).

The 44 kDa ATPase fragment showed a similar concentration-dependence of ATP binding as the complete molecule (Figure 2). However, the affinity for ATP of both binding sites appeared slightly greater.

It appears that there are two different forms of Hsc70 present in our preparation. Intriguingly, if Hsc70 is run on SDS/PAGE, a doublet band can be seen, providing that not too much enzyme is loaded. To test whether or not these two very closely spaced bands correspond to the two binding sites, we labelled the high-affinity site with 11 μM [α-^{32}P]ATP and cross-linked the bound

Table 1 Stability of the Hsc70–nucleotide complex during purification

Elution of Hsc70 from the ATP–agarose column was done in the presence of [α-^{32}P]ATP (0.5 c.p.m./pmol). For methods, see the Experimental section.

Treatment	Bound [α-^{32}P]ATP (mol/mol)
$(NH_4)_2SO_4$ without EDTA	0.90
$(NH_4)_2SO_4$ with EDTA	0.08
Dimer fraction after gel chromatography	0.004

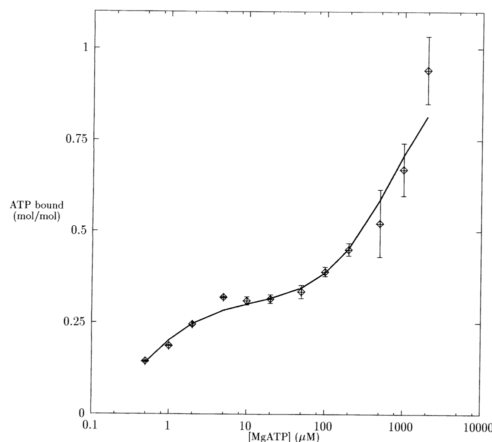

Figure 2 Binding of ATP to the 44 kDa chymotryptic fragment of Hsc70

For details of methods, see the Experimental section. Data points are means ± S.D. of several independent experiments.

nucleotide to the enzyme. SDS/PAGE (on a gel with a flat gradient for better resolution) and autoradiography revealed that both bands were labelled (Figure 3). Therefore these two bands both contain a high-affinity binding species. They are not caused by contamination with the endoplasmic reticulum homologue of Hsc70, BiP [immunoglobulin heavy chain binding protein (Grp78)], as this protein would run to a different position on the gel (results not shown). In this context it is also interesting that the 44 kDa fragment has only one band in SDS/PAGE (results not shown), but shows very similar ATP-binding behaviour to the full protein.

ATP binding to Hsc70 was also measured by following the monomerization thereby induced by gel filtration (Figures 1a

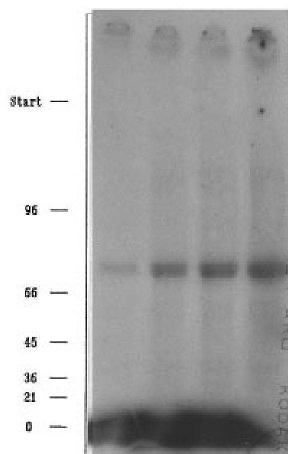

Figure 3 Autoradiogram of Hsc70 cross-linked to [α-^{32}P]ATP by UV irradiation

Hsc70 (4 mg/ml) and 11 μM [α-^{32}P]ATP were irradiated on ice with a mercury lamp for 60 min. After desalting on a NAP-5 column (Pharmacia), aliquots of 2, 4, 8 and 12 μg of the labelled protein were separated by electrophoresis on a 5–10% Laemmli gel (lanes from left to right). Note the two closely spaced bands of Hsc70, which are both labelled. The radioactivity at the front probably comes from bound, but not cross-linked, ATP.

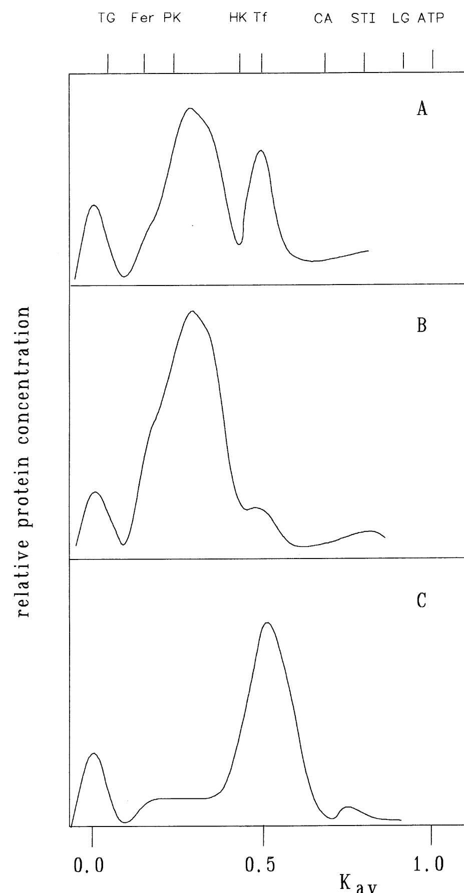

Figure 4 Gel chromatography of Hsc70 on Superdex 200

(**A**) Mixture of monomeric and dimeric Hsc70 after $(NH_4)_2SO_4$ precipitation; (**B**) purified dimer fraction; (**C**) dimer fraction after incubation with 10 mM MgATP. The peak positions of standard proteins are indicated at the top: TG, thyroglobulin (600 kDa); Fer, ferritin (440 kDa); PK, pyruvate kinase (237 kDa); HK, hexokinase (100 kDa); Tf, transferrin (75 kDa); CA, carbonic anhydrase (31 kDa); STI, soybean trypsin inhibitor (21 kDa); LG, β-lactoglobulin (14 kDa). The peak at $K_{av} = 0$ is Dextran Blue 2000, and $K_{av} = 1$ is defined as the peak position of ATP. For methods, see the Experimental section.

and 4). We have examined the effects of different nucleotides, bound both in the absence and in the presence of magnesium ions. The column was eluted with both binding buffer (containing EDTA) and uncoating buffer (containing magnesium acetate). A summary of the results can be found in Table 2. ATP induced monomerization when added together with Mg. This complex, once formed, was stable against EDTA, since the Hsc70 eluted as monomers even if the elution buffer contained this Mg chelator. ATP alone, without Mg, could not induce monomerization, and non-cleavable analogues of ATP, namely ATP[S] and adenosine 5′-[β,γ-imino]triphosphate (p[NH]ppA), were not effective even in the presence of Mg. This was also true for ADP, even in the presence of P_i. These results are similar to those obtained with BiP [22]. Mg^{2+} can be replaced in the monomerization assay by Co^{2+}, Mn^{2+}, Cu^{2+} or Ca^{2+} (results not shown). The latter is interesting, since Ca^{2+} cannot replace Mg^{2+} in the ATPase assay [23]. If radioactive ATP was used for the monomerization assay, all bound radioactivity was associated with the monomer and none with the dimer (results not shown).

Binding of ATP[S], ADP, adenosine 5′-[β,γ-methylene]triphosphate (p[CH_2]ppA) and p[NH]ppA to Hsc70 was monitored

Table 2 Monomerization of Hsc70 by various nucleotide analogues

Samples of 0.25 mg of dimeric Hsc70 were incubated in 100 μl of 50 mM Hepes/KOH, pH 7.0, with various nucleotides (10 mM each). The sample was then injected on to a Superdex 200 column and eluted with uncoating buffer (containing Mg^{2+}) or binding buffer (containing EDTA). For further details, see the text.

Preincubation with:	Elution with:	Monomerization	
		2 mM $MgCl_2$	2 mM EDTA
ATP		−	−
MgATP		+	+
ATP[S]		−	−
MgATP[S]		−	−
p[NH]ppA		−	−
Mg-p[NH]ppA		−	−
ADP		−	−
MgADP		−	−
MgADP + P_i		−	−

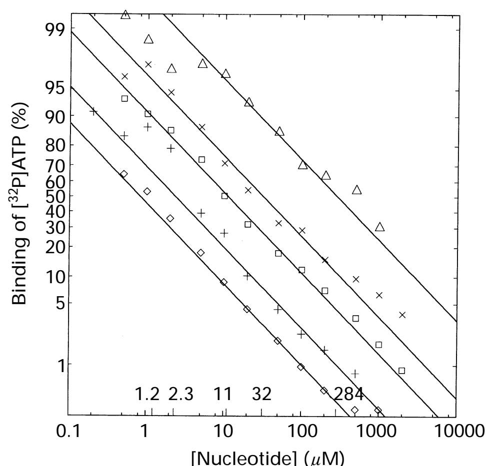

Figure 5 Binding of ATP analogues to Hsc70

Log–logit plot of the competition between [α-^{32}P]ATP and unlabelled ATP (◇), ATP[S] (+), ADP (□), p[NH]ppA (×) and p[CH_2]ppA (△) for binding to Hsc70. Results are means of triplicate experiments; all S.D.s were less than 10% of the mean. Values above the *x*-axis are the dissociation constants of the various nucleotides.

Table 3 Dissociation constants of Hsc70 for binding of ATP and non-cleavable ATP analogues, and their relative effectiveness for supporting the uncoating of brain coated vesicles

Uncoating experiments were carried out with 1 mM nucleotide, except for p[CH_2]ppA, where 2 mM was used. For further details, see the text.

Nucleotide	K_D (μM)	Uncoating (%)
ATP	1.2	87
ATP[S]	2.3	63
p[NH]ppA	32	50
p[CH_2]ppA	284	0

by competition with [α-^{32}P]ATP (Figure 5). Unfortunately, this type of assay is insensitive to the presence of a low-affinity binding site. However, the affinities for the high-affinity binding

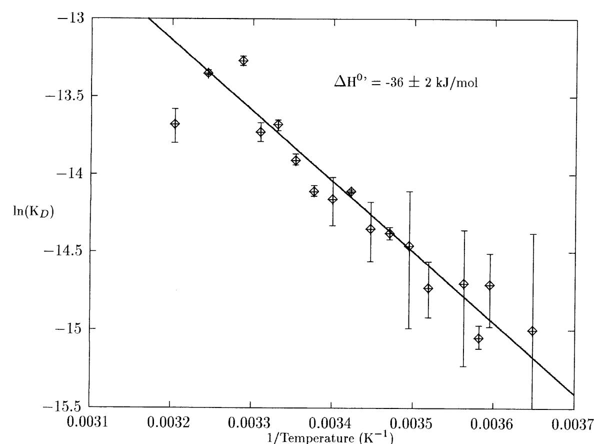

Figure 6 Effect of temperature on ATP binding to Hsc70

van't Hoff diagram of the effect of temperature on the dissociation constant of ATP from Hsc70. The $\Delta H^{0\prime}$ of binding can be calculated from the slope of the regression line, and $\Delta G^{0\prime}$ is obtained from the affinity at 25 °C.

site could be measured. There was a striking correlation between the affinity of Hsc70 for an ATP analogue and the ability of that analogue to support the Hsc70-promoted uncoating of clathrin-coated vesicles (Table 3). ATP has the highest affinity for Hsc70 (K_D $\sim$ 1 μM), and it is also the most effective agent in the uncoating assay. The K_D for ATP[S] is 2.3 μM, and it is also quite effective in uncoating (see also [9]). p[NH]ppA binds less well to Hsc70 ($K_D = 32$ μM) and its effectiveness in supporting the uncoating reaction is limited. p[CH_2]ppA binds only poorly, and cannot support the uncoating of coated vesicles.

We also tried to measure the binding of ATP[^{35}S] by the same method as used for ATP. However, this proved to be difficult, since binding of ATP[S] was less tight than the binding of ATP, and the extensive washing removed the bound ATP[S] quantitatively. We conclude that, although ATP[S] can bind to Hsc70 with high affinity, it does not bind as tightly as do ATP or ADP. The method of Gao et al. [13] for the preparation of nucleotide-free Hsc70 is based on the fact that p[NH]ppA behaves similarly to ATP[S], in that it can be removed from Hsc70 by dialysis.

Effect of temperature on ATP binding

The dissociation constant of the high-affinity site of Hsc70 was temperature-dependent (Figure 6); we have not evaluated the temperature-dependence of the low-affinity site, because of technical problems. At 25 °C the dissociation constant is 1.1 ± 0.1 μM, equivalent to $\Delta G^{0\prime} = -34$ kJ/mol. The slope of the van't Hoff plot gives us $\Delta H^{0\prime} = -36 \pm 2$ kJ/mol. From this we can calculate the change in entropy: $\Delta S^{0\prime} = 6.7$ J/mol per K.

DISCUSSION

As mentioned in the Introduction section, we wanted to measure the affinity of nucleotide-free Hsc70 for ATP and ATP analogues, and the efficacy of these analogues in supporting the uncoating of clathrin-coated vesicles by Hsc70, in order to find out whether the energy of nucleotide binding is important for Hsc70 to fulfil its function. We found a dissociation constant for ATP of 1.1 μM at 25 °C. Competition of unlabelled nucleotides and nucleotide analogues with [α-^{32}P]ATP for the binding site on Hsc70 gave us their respective dissociation constants (Figure 5).

The dissociation constant of 1.1 μM for ATP at 25 °C corresponds to an energy of binding of $\Delta G^{0\prime} = -34$ kJ/mol, which is virtually the same value as the standard free energy of hydrolysis of the terminal phosphate group of ATP. This could mean that the mere binding of ATP to Hsc70 imparts sufficient energy to the enzyme to allow it to fulfil its protein-binding function, and that the hydrolysis of ATP is not required at this stage. It has to be kept in mind, however, that the actual values under physiological conditions of the free energy for the hydrolysis of ATP and for the binding of ATP to Hsc70 are quite different from the values under standard conditions, as the activities of the reactants will be different from 1 M. No information is presently available on the local activities of Hsc70, ATP, ADP and P_i at the site of vesicle uncoating. Nor do we know what effect regulatory proteins (such as eukaryotic DnaJ homologues) might have on the affinity of Hsc70 for ATP.

Indeed, non-cleavable analogues of ATP, namely ATP[S] and p[NH]ppA, are able to support the uncoating of clathrin-coated vesicles by Hsc70 [8,9]. The affinity of Hsc70 for these nucleotides and their ability to support the uncoating reaction are well correlated. However, we need to ask to what extent contamination of these analogues with ATP and ADP might affect our results. ATP[S] is unstable and is usually contaminated with 10–30 % ADP. However, since the affinity of Hsc70 for ATP[S] is greater than that for ADP, little interference is likely. In the case of p[CH_2]ppA, with its very high dissociation constant, it is possible that even a small contamination with ATP or ADP would produce a binding signal. This error, however, would be conservative, as the real dissociation constant would be even higher than that measured. For p[NH]ppA less than 0.1 % ATP contamination is specified by the manufacturer. This would rule out problems of ATP contamination at the concentration used. The affinity of Hsc70 for ADP is lower than that for ATP, which rules out problems of ADP contamination in this instance.

However, there is a difference between ATP and ADP on the one hand and ATP[S] and p[NH]ppA on the other. Both ATP and ADP bind not only with high affinity ($K_D = 1$ and 10 μM respectively) but also very tightly to Hsc70, so that preparations of Hsc70 usually contain stoichiometric amounts of bound nucleotides which have to be removed by special techniques. One is tempted to speculate that the enzyme somehow closes around the bound nucleotide, trapping it in the binding site. ATP[S] and p[NH]ppA do not show this behaviour. Palleros et al. [24] measured conformational changes in Hsc70 upon ATP binding. Although they did not require ATP hydrolysis, ATP[S] was found to be unable to support these conformational changes. Our data seem to confirm this, although the complete lack of activity of ATP[S] with respect to the conformational changes is somewhat unexpected in the light of its ability to support uncoating.

The formation of a substrate–Hsc70–ATP complex would prevent unfavourable interactions between substrate proteins (between clathrin triskelia in the context of uncoating). Since this complex would form spontaneously, without much change in energy from Hsc70–ATP and substrate protein, it would be fairly unstable and could dissociate again. Hydrolysis of ATP could lead to the formation of a more stable substrate–Hsc70–ADP complex [10], thus making the competition by Hsc70 for substrate binding more effective and explaining why Hsc70 is more effective in vesicle uncoating under conditions where ATP can be hydrolysed ([9], and Table 3). The affinity of ADP for Hsc70 is still rather high ($K_D = 10$ μM, equivalent to $\Delta G^{0\prime} = -28$ kJ/mol). One would therefore expect ADP dissociation from this complex to be impaired. This is in fact observed experimentally [12] (and by others [25], but with a different interpretation): ADP can be removed from this complex only by ADP/ATP exchange. It is also known that substrate protein cannot easily dissociate from the substrate–Hsc70–ADP complex, but is released upon ADP/ATP exchange [8,10].

An important constraint in this model would be the strength of substrate–substrate interactions. This might also explain the specificity of Hsc70 for coated vesicles rather than growing pits. A general model for vesicle coat assembly predicts that coat stability is controlled by GTPase cycles such that coats are destabilized by GTP hydrolysis after vesicle formation [26]. Another possibility for such regulation would be analogues of the Vps10p/Vps15p/Vps34p system described by Schu et al. [27] for vacuolar sorting, where Vps34 is a PtdIns 3-kinase believed to play a role in coat formation. The action of a (hypothetical) PtdIns3*P* phosphatase could destroy the PtdIns3*P* and thus destabilize the vesicle as a timed fuse.

Log–logit plots are not very sensitive to departures from the normal Henry–Michaelis–Menten behaviour. In order to investigate whether or not nucleotide binding to Hsc70 follows these kinetics, we expressed bound ATP in terms of mol of ATP per mol of Hsc70, and found that the ATP concentrations normally used did not lead to complete saturation of Hsc70 with ATP. Similar results have been found before: Schmid et al. [16] found a dissociation constant of 0.7 μM with a maximal occupancy of 0.4 mol/mol, while Wang and Lee [28] found a K_D of 0.2 μM and a capacity of 0.39 mol/mol. These two groups looked at binding only up to 15 and 10 μM respectively. We therefore increased the ATP concentration further and found low-affinity ATP binding with a dissociation constant of 1.4 mM. The sum of low- and high-affinity binding was 1 mol of ATP per mol of Hsc70. The low-affinity binding was specific, since: (1) it was saturatable; (2) the sum of low- and high-affinity binding was 1 mol of ATP per mol of enzyme; and (3) it led to monomerization.

The interpretation of our binding data hinges critically on the protein assay giving correct values. To control for this, we calculated the absorbance of Hsc70 at 280 nm from its amino acid composition by the method of Perkins [29]. For rat brain Hsc70 this gives $\epsilon_{280} = 31\,800$ $l \cdot mol^{-1} \cdot cm^{-1}$. A similar method published by Gill and von Hippel [30] gave 33 311 $l \cdot mol^{-1} \cdot cm^{-1}$. Hsc70 concentrations determined by A_{280} were only slightly lower (93 %) than those determined with Bradford reagent and IgG standard. Earlier reports of much higher ϵ_{280} values refer to Hsc70 with ATP bound, and are therefore not applicable to our preparations.

To confirm our findings on ATP binding, we also followed by gel filtration the monomerization induced by binding of MgATP to Hsc70. It is noteworthy that binding and monomerization assays are complementary, in that the binding assay is most precise at low ATP concentrations and the monomerization assay is most accurate at high ATP concentrations. The concentration-dependence of monomerization is superimposable on the data obtained in our binding assay. This could not be the case if the two-step curve in the binding assay was the result of one molecule of Hsc70 binding two molecules of ATP, as has been suggested by Schmid et al. [16]. It also rules out another possible explanation, namely that the two molecules forming a Hsc70 dimer are unequal, either by chemical composition or by unsymmetrical interactions between them. Rather, we would suggest that there are two completely independent populations of enzyme. These could, in theory, arise from different isoforms, different post-translational modifications or partial denaturation during isolation.

In a previous paper we showed that our preparation of Hsc70, once saturated with radiolabelled ATP, loses the label completely

and with monoexponential kinetics [12]. It is possible that removal of ATP leads to some denaturation of enzyme structure, which is reversible upon ATP binding and affects the k_{on} rate for binding of a proportion of the enzyme preparation. To confirm this, we also prepared nucleotide-free Hsc70 by the method of Gao et al. [13], which may appear to involve milder conditions than ours. As indicated by Figure 1(b), this preparation gave virtually identical results to the enzyme prepared by our method, which has economical advantages and also removes ATP more completely. In contrast to Gao et al. [13], we suggest that Hsc70 without bound nucleotide is unphysiological, and that such enzyme will be partly and reversibly denatured independent of the method of preparation. We agree, though, that nucleotide-free Hsc70 is stable during prolonged storage and can rebind ATP to obtain full activity. The 44 kDa chymotryptic fragment of Hsc70, which contains the ATPase domain, shows the same two-step binding curve as the parent molecule. However, the affinities are slightly greater.

Gao et al. [13] found a single affinity for ATP binding when they measured the K_D for ATP by exchange with p[NH]ppA. This confirms our own conclusion that the enzyme preparation is homogeneous if saturated with nucleotide. They also claimed that binding of p[NH]ppA and dATP to nucleotide-free Hsc70 gave linear Scatchard plots, indicating binding with a single affinity. A closer look at their Fig. 5(a) and especially their Fig. 5(b), however, reveals considerable curvature. This might explain the 10-fold difference in K_D for p[NH]ppA (2.5 versus 32 μM) compared with our data.

Recently, another paper dealing with ATP binding to Hsc70 has been published by Ha and McKay [14], who measured k_{on} and k_{off} rates of ATP binding to an Hsc70 preparation, from which they had tried to remove nucleotide by incubation with activated charcoal. Since ATP removal by this method is very inefficient (see the Results section), it appears that these authors may have measured the rates of ADP/ATP and ATP/ATP exchange (which are in reasonable agreement with those found by us in experiments described elsewhere [12]). It is, however, impossible to calculate K_D values from these exchange rates. In BiP the conversion of dimeric into monomeric enzyme has been described as part of the reaction cycle [22,31,32]. In the light of our results this is unlikely for Hsc70, and also by inference for BiP, for two reasons: (1) dimerization of Hsc70 requires removal of bound nucleotide and would not occur under physiological conditions; and (2) the monomerization reaction is supported not only by Mg^{2+}, but also by Ca^{2+}; this is not the case for the uncoating reaction [23].

In gel chromatographic experiments, all bound radioactivity from labelled ATP is associated with the monomeric fraction of Hsc70, during both removal and binding of ATP. This proves that: (1) as has been published before, it is the monomeric form of Hsc70 that contains ATP; the dimeric form is nucleotide-free [10,16]; and (2) the complex $Hsc70_2$–ATP, although it must exist, is shortlived and cannot be separated by gel chromatography.

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In summary, our results show that binding of ATP to Hsc70 imparts enough energy for the protein to fulfil its function. Taken together with our previous observations that destabilization of clathrin coats favours uncoating [9] and that there is a direct link between the rate of hydrolysis and the efficiency of uncoating [9,12], the model presented for the action of Hsc70 during uncoating appears at least to be feasible.

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