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The speed of partial reactions of the uncoating ATPase Hsc70 depends on the source of coated vesicles

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SUMMARY

Hsc70 was previously isolated by its ability to catalyse the uncoating of clathrin-coated vesicles from bovine brain. We have recently shown that Hsc70 is more active towards coated vesicles from brain than those from other tissues. In order to gain information on the mechanistic reason for this difference we have examined the ability of brain and placental coated vesicles to stimulate partial reactions during a single round of ATP turnover. The Hsc70·ATP complex is turned over to Hsc70·ADP·P_i, from which phosphate is slowly released. The resulting Hsc70·ADP complex exchanges ATP for ADP. Dissociation of ATP or ADP from Hsc70 does not seem to occur under physiological conditions. The hydrolysis of ATP is accelerated by the presence of clathrin-coated vesicles, with vesicles from brain being about twice as effective as vesicles from placenta. Additionally, it appears that brain, but not placental, coated vesicles can also stimulate the exchange of ADP for ATP.

Key words: Heat shock protein, Hsc70, Reaction mechanism, Clathrin-coated vesicle

INTRODUCTION

Hsc70 is a protein with chaperone function that is involved in many cellular processes, including protein translation, folding, transport and assembly (Ahmad et al., 1990, Beckmann et al., 1990; Hartl et al., 1994; Haus et al., 1993; Imamoto et al., 1992; Sadis and Hightower, 1992; Schlossman et al., 1984; Shi and Thomas 1992; Vanbuskirk et al., 1989; Terlecky et al., 1991; Wiech et al., 1993). Hsc70 expression is essential for cell survival, and its sequence is highly conserved from bacteria to mammals (Ahmad et al., 1990; Haus et al., 1993; Gao et al., 1991; Perkins et al., 1990; Wooden and Lee, 1992; Zafarulla et al., 1992). Despite all this we have little knowledge on its mechanism of action.

The original model of Rothman and Schmid (1986), in which Hsc70 uses the energy of ATP hydrolysis to break protein-protein interactions during the uncoating of clathrin-coated vesicles, is no longer feasible, since non-hydrolysable ATP analogues are also, at least partially, active (Heuser and Steer, 1989; Buxbaum and Woodman, 1995; E. Buxbaum and P. G. Woodman, unpublished data). From those data it seems that Hsc70 function is at least in part driven by the energy of ATP binding rather than of ATP hydrolysis, and that the hydrolysis may be required only to release products or/and thermodynamically drive the reaction in the desired direction. This would also agree with the function of Hsc70 in other contexts, where Hsc70 prevents the formation of unfavourable protein-protein interactions rather than breaking them (Hartl et al., 1994).

Other data show that binding and release of substrate peptides are fast processes, while ATP hydrolysis by Hsc70 is notoriously slow. It is also a matter of debate whether peptide binding to Hsc70 accelerates ATP hydrolysis (Flynn et al., 1989) or ADP/ATP exchange (Sadis and Hightower, 1992). On the other hand, nucleotide binding clearly influences the binding of substrate proteins (Heuser and Steer, 1989; Palleros et al., 1991). It is therefore not clear at present whether there is indeed a 1:1 relationship between protein and ATP turnover in Hsc70; that is, whether ATP hydrolysis is actually required for protein turnover or may fulfil another, for example regulatory, function. Notwithstanding these findings, it is clear that ATP hydrolysis does play an important role in the reaction cycle of Hsc70, since release of clathrin from coated vesicles is more efficient under conditions where ATP hydrolysis occurs (Heuser and Steer, 1989; Buxbaum and Woodman, 1995).

To answer these questions it seems necessary to break down both the ATP and the protein turnover into partial reactions and to find out whether or not these are interrelated; that is, how they influence each other. We have used the uncoating reaction with clathrin-coated vesicles as a model for Hsc70 action, because coated vesicles are readily available in pure form, the reaction is comparatively well characterized and coated vesicles are a particular good substrate for Hsc70, stimulating its ATPase activity about 10-fold (simple peptide substrates stimulate only 2- to 3-fold). Additionally, we have recently shown, that there are qualitative and quantitative differences in the effect of Hsc70 towards clathrin-coated vesicles from brain

and other tissues like placenta and liver (Buxbaum and Woodman, 1995). Elaborating the reasons for these differences might teach us a lot about the reaction mechanism of Hsc70.

The complex between Hsc70 and ATP or ADP is very stable, in fact so stable that special methods are required to generate nucleotide-free Hsc70 (Gao et al., 1993; E. Buxbaum and P. G. Woodman, unpublished data). This can be used to precharge Hsc70 with labelled ATP, separate it from unbound nucleotides, and then study the turnover of the label under different conditions, for example with or without unlabelled ATP and with or without protein substrate. By using ATP labelled with ^{32}P in either the α- or the γ-position one can follow a single round of turnover of both the terminal phosphate and the ADP while the enzyme is working under steady state conditions.

Interpretation of the results is complicated by the fact that in all these experiments we are actually measuring the sum of several processes. Release of label from [α-^{32}P]ATP can in theory occur by dissociation of ATP, ATP/ATP exchange, and after hydrolysis by dissociation of ADP or by ADP/ATP exchange. Release of label from [γ-^{32}P]ATP can occur by dissociation of ATP, ATP/ATP exchange or ATP hydrolysis. The appearance of P_i in the medium is simpler to interpret as it can occur only by hydrolysis of bound ATP.

However, it is possible to choose experimental conditions in such a way that only some of the above mentioned processes can occur. For example, ATP/ATP and ADP/ATP exchange can only occur in the presence of free ATP. If one does the measurements both with and without free ATP, then the difference in results will give information on the extent of the exchange reactions. By performing experiments under different conditions it is therefore possible to deconvolute the various processes and gain information about their relative rates.

MATERIALS AND METHODS

Hsc70 from mung bean seeds and clathrin-coated vesicles from pig brain and human placenta were prepared as described (Buxbaum and Woodman, 1995; E. Buxbaum and P. G. Woodman, unpublished data). Protein was determined by the method of Bradford (1976) with bovine IgG as standard.

Preloading of Hsc70 with radioactive ATP

A 5 mg (139 μM) portion of Hsc70 was incubated in 0.5 ml of uncoating buffer (25 mM Hepes-KOH, pH 7.0, 20 mM KCl, 10 mM $(NH_4)_2SO_4$, 1 mM $MgCl_2$) with 10 mM MgATP, containing about 100 μCi of either [α-^{32}P]ATP or [γ-^{32}P]ATP (ICN) for 10 minutes on ice. The mixture was passed first over a NAP-5, then over a PD-10 gel filtration column (Pharmacia). Ice-cold uncoating buffer was used for elution. Remaining $[ATP]_{free}$ was less than 0.5 μM.

Monitoring the fate of labelled ATP on Hsc70

A 20 μl sample of a mixture of 200 μg/ml (2.8 μM) Hsc70·ATP in ATPase buffer (20 mM Mes-KOH, pH 5.9, 20 mM KCl, 10 mM $(NH_4)_2SO_4$, 4.5 mM $MgCl_2$, 1 mM Na_3VO_4) with or without 1 mg/ml coated vesicles and with or without 50 μM unlabelled ATP were incubated for the specified time at room temperature. Afterwards, bound nucleotide was determined by filtration (E. Buxbaum and P. G. Woodman, unpublished data) or released $^{32}P_i$ by extraction (Buxbaum and Woodman, 1995).

Evaluation of data

Count rates were converted to relative concentrations (i.e. mol per mol of Hsc70) using the specific activity of the ATP used and averaged over several independent experiments. Curve fitting to these data was performed using the least sum of squares criterion with the simplex algorithm of Caceci and Cacheris (1984), error margins for the parameters were estimated by Monte Carlo simulation (Straume and Johnson, 1992).

RESULTS

Stability of the Hsc70·ATP complex

The complex between Hsc70 and [γ-^{32}P]ATP decays slowly over several days, as shown in Fig. 1. However, the complex was used within the first 4 hours after preparation to ensure complete loading of the Hsc70. This figure also shows that the apparent sum of bound ATP and liberated phosphate is larger than than the total amount of radiophosphorus present at early time points. This is probably caused by Hsc70·$^{P_i}_{ADP}$. This species will be counted both in our binding and in the extraction assay.

Release of radiolabel from Hsc70

When Hsc70 was preloaded with [α-^{32}P]ATP and incubated in the absence of free ATP with or without coated vesicles, no release of label occurred at least over 40 minutes (Fig. 2a). If the same experiment was repeated with Hsc70·[γ-^{32}P]ATP, slow release of label occurred, which was accelerated upon addition of clathrin-coated vesicles (Fig. 3a).

In the presence of 50 μM unlabelled ATP, release of label from Hsc70·[α-^{32}P]ATP was observed (Fig. 2b), which again was accelerated by the addition of coated vesicles. Release of label from Hsc70·[γ-^{32}P]ATP was also more effective if unlabelled ATP was present (Fig. 3b).

In all cases clathrin-coated vesicles isolated from brain were more effective than those isolated from placenta. This agrees

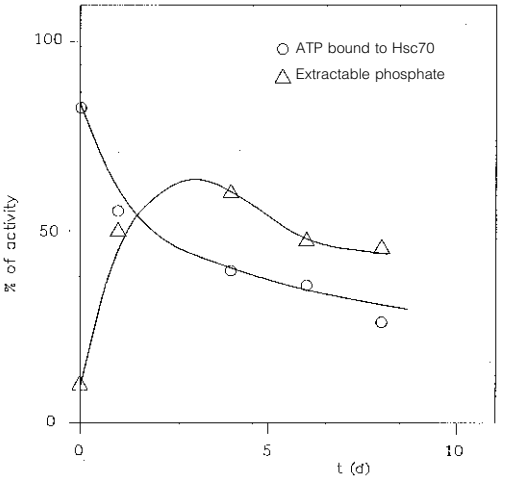

Fig. 1. Stability of Hsc70·[γ^{32}P]ATP on ice. A 10 μl sample of a solution of Hsc70 in uncoating buffer preloaded with [γ-^{32}P]ATP was used for determination of total radioactivity, radioactivity extractable as phosphomolybdate (triangles) and radioactivity bound to Hsc70 (by filtration; circles). 100% corresponds to 450,000 cpm on day 0. t(d), time in days.

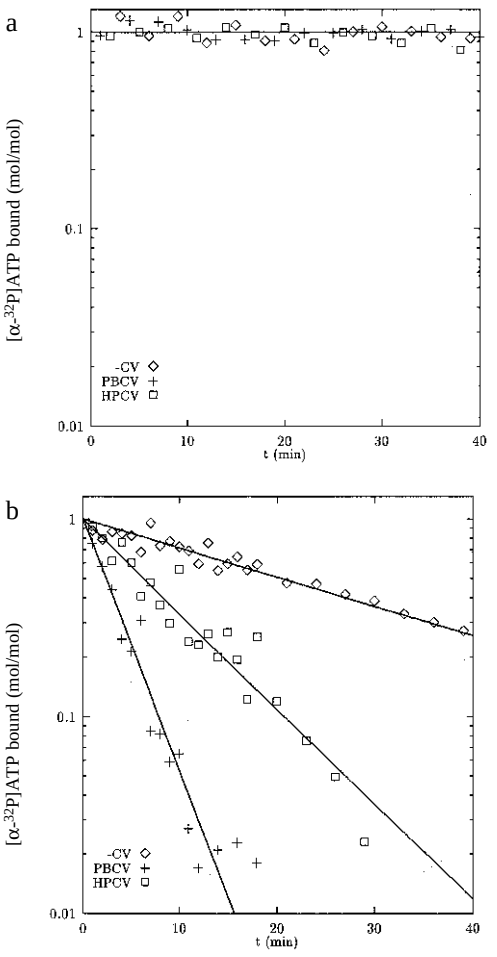

Fig. 2. Release of radioactivity from 2.8 μM Hsc70 preloded with [α-^{32}P]ATP. (a) Incubated in the absence of free ATP. (b) Incubated in the presence of 50 μM of free unlabelled ATP. Incubation was performed without coated vesicles (diamonds), with 1 mg/ml pig brain coated vesicles (crosses) and with 1 mg/ml human placental coated vesicles (squares).

well with the fact that placental vesicles are less effective in stimulating the ATPase activity of Hsc70 than brain vesicles (Buxbaum and Woodman, 1995). The apparent first order rate constants for all cases are given in Table 1.

ATP hydrolysis

If the increase of free phosphate was measured by extraction instead of the decrease of bound radioactivity being measured by filtration, the situation became slightly more complicated. While the filtration data could be described by a simple exponential function ($\hat{y}$=exp(k∗t)), the extraction data had to be fitted to $\hat{y}$=max−max∗exp(k∗t)+background. This was because some of the radioactivity bound to Hsc70 could be extracted even at time 0, indicating limited cleavage of ATP with both ADP and P_i still bound to the protein. This actually led to the strange situation, that the sum of radioactivity bound to filters (label bound to Hsc70) and the radioactivity extractable as free phosphate was higher than the total radioactivity present, as this phosphate bound to Hsc70 was counted in both assays (Fig. 1).

In the absence of free ATP this background was lower (about 38% of total), in the presence of 50 μM free ATP it increased to about 54% (Fig. 4a and b).

In the absence of free ATP the maximum release was 58% of total, so that, given enough time, virtually all bound activity would be released as free phosphate (background + maximum = 96%). This occurred in the absence and in the presence of coated vesicles, though the rate was increased by coated vesicles (Fig. 4a).

In the presence of 50 μM of free ATP this was no longer the case. The total percentage of releasable phosphate decreased, as the speed of release decreased, i.e. while in the presence of brain coated vesicle release was fast and virtually complete, in the presence of placental vesicles, and even more in the absence of vesicles, release of phosphate was slower and less complete. Hence, slowing down the release of phosphate from Hsc70·ATP increases the opportunity for ATP/ATP exchange (Fig. 4b).

The parameters for these data are shown in Table 2. Since the release of $^{32}P_i$ at time 0 for the experiments with ATP (Fig. 4b), and both the release of $^{32}P_i$ at time 0 and the maximum release for the experiments without ATP (Fig. 4a) did not depend on the

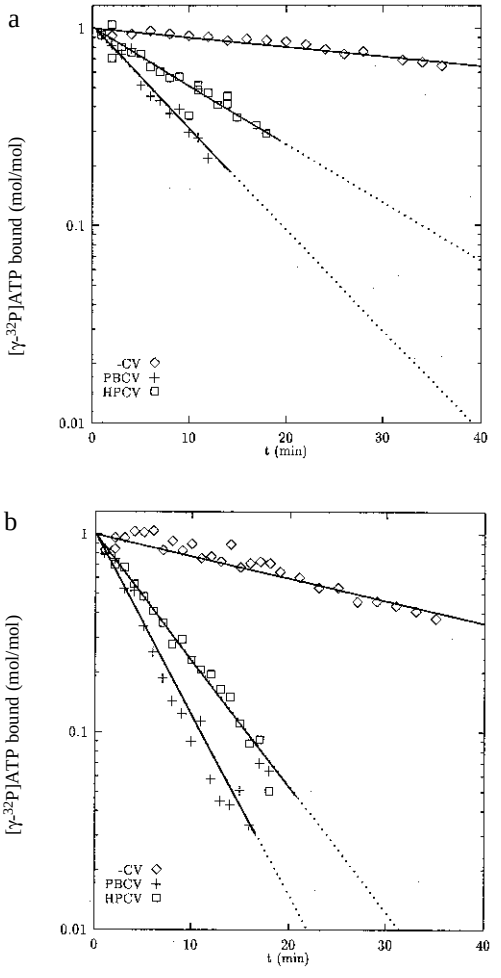

Fig. 3. Release of radioactivity from 2.8 μM Hsc70 preloded with [γ-^{32}P]ATP. (a) Incubated in the absence of of free ATP. (b) Incubated in the presence of 50 μM of free unlabelled ATP. Incubation was performed without coated vesicles (diamonds), with 1 mg/ml pig brain coated vesicles (crosses) and with 1 mg/ml human placental coated vesicles (squares).

Table 1. Apparent first order rate constants for the loss of radioactivity from ATP prebound to Hsc70 (min^{-1})

	[α-^{32}P]ATP		[γ-^{32}P]ATP	
Vesicle	–ATP	+ATP	–ATP	+ATP
Without	0.000	–0.034±0.007	–0.011±0.001	–0.026±0.010
HPCV	0.000	–0.111±0.006	–0.068±0.005	–0.147±0.003
PBCV	0.000	–0.294±0.003	–0.118±0.005	–0.211±0.004

For methods see text.

presence of coated vesicles, only the averages for these parameters are given and the rate constants have been refitted to these parameters. This reduction in degrees of freedom increases slightly the power of the curve-fitting routine.

DISCUSSION

The described properties of Hsc70 can be explained by the following model for the sequence of events during the hydrolysis of ATP (Fig. 5): Hsc70 will, under physiological conditions, always have ATP or ADP bound to it. Free Hsc70 without bound nucleotide can be generated only in the absence of Mg^{2+} under mildly denaturing conditions (Schlossman et al., 1984). Hsc70·ATP will use water to hydrolyse the terminal phosphodiester bond in ATP, giving Hsc70·$^{P_i}_{ADP}$. From our data it appears that a portion of this phosphate is initially bound tightly enough to survive our filtration assay, but this complex can be destroyed upon addition of acid as required for the extraction of phosphate, indicating that the release of P_i is relatively slow. This explains the background of extractable phosphate at time zero that remains bound to the enzyme (Fig. 4a and b). This agrees with the finding that Hsc70 crystallized in the presence of ATP contains bound ADP and P_i (Flaherty et al., 1994). Uncoating is also inhibited more by ADP and P_i than by ADP alone (Greene and Eisenberg, 1990), which would suggest that release of P_i is a reversible process. The background of extractable phosphate is the same with or without vesicles, but it is increased in the presence of free ATP. At present we have no explanation for the latter observation.

Hsc70·$^{P_i}_{ADP}$ will slowly lose phosphate to form Hsc70·ADP. Both the release of radioactivity from Hsc70 loaded with [γ-^{32}P]ATP and the appearance of free phosphate are accelerated by the presence of coated vesicles. Coated vesicles from brain are about twice as effective as coated vesicles from placenta. The stimulation of phosphate liberation could be a direct effect of the interaction with the substrate clathrin, and/or it could be caused by the presence of a hypothetical stimulatory factor (acting like DnaJ on DnaK, the *Escherichia coli* homologue of Hsc70). Such a factor could explain why coated vesicles from brain stimulate the ATPase more than those from placenta, from which it may be absent, present in lower concentrations or replaced by a less effective isoform. It is known that effective uncoating requires an additional protein factor (Prasad et al., 1993). Additionally, brain coated vesicles have a different isoform of clathrin light chains (Jackson et al., 1987), which may influence the uncoating reaction.

Placental vesicles are not only less effective in stimulating phosphate release from Hsc70·ATP, they are also uncoated three to five times less efficiently than brain vesicles (Buxbaum and Woodman, 1995) and are less efficient in stimulating the ATPase activity of Hsc70. They are also more stable against changes in environmental conditions like pH. This intimate connection between coat stability, stimulation of ATPase activity and rate of uncoating has important implications for the reaction mechanism of Hsc70.

There is no measurable release of radioactivity from Hsc70 loaded with [α-^{32}P]ATP in the absence of free ATP. This has been observed before and ascribed to a rapid dissociation-rebinding equilibrium (Gao et al., 1993). However, this explanation is unlikely to be correct, because the concentration of free nucleotide, and therefore the rate of rebinding, is near zero.

Release is observed upon addition of 50 μM ATP. Loss of bound ADP from Hsc70·ADP therefore is likely to occur, not by dissociation, but by ADP/ATP exchange. Part of the reason may be the high Gibbs free enthalpy of binding between Hsc70 and ADP (about 28 kJ/mol as calculated from the dissociation constant of 10 μM; E. Buxbaum and P. G. Woodman, unpublished data). Such an exchange requires the existence of an Hsc70$^{ATP}_{ADP}$ complex. This complex presumably is very weak and will lose ADP to form Hsc70·ATP again.

Sadis and Hightower (1992) claim that substrate proteins stimulate the ADP/ATP exchange of Hsc70, but not the hydrolysis of ATP. They measured only the velocity of total ATPase and of ADP/ATP exchange and found the acceleration of exchange upon addition of substrate to be similar to the acceleration of total ATPase. Their rate constants are in good agreement with ours, but we cannot agree with their explanation for the effect of coated vesicles on this rate. If there is an

Table 2. Parameter for the appearance of $^{32}P_i$ in the medium from Hsc70 preloaded with [γ-^{32}P]ATP

	Without ATP			With 50 μM ATP		
Vesicle	*m* (mol/mol)	*k* (min^{-1})	*b* (mol/mol)	*m* (mol/mol)	*k* (min^{-1})	*b* (mol/mol)
Without	0.577±0.018	–0.050±0.001	0.381±0.015	0.226±0.016	–0.104±0.016	0.537±0.014
HPCV	0.577±0.018	–0.121±0.007	0.381±0.015	0.377±0.015	–0.178±0.016	0.537±0.014
PBCV	0.577±0.018	–0.235±0.014	0.381±0.015	0.498±0.014	–0.218±0.014	0.537±0.014

Model is $\hat{y} = m - m * \exp(k * t) + b$. For methods see text. *m*, maximum; *k*, apparent rate constant; *b*, background.

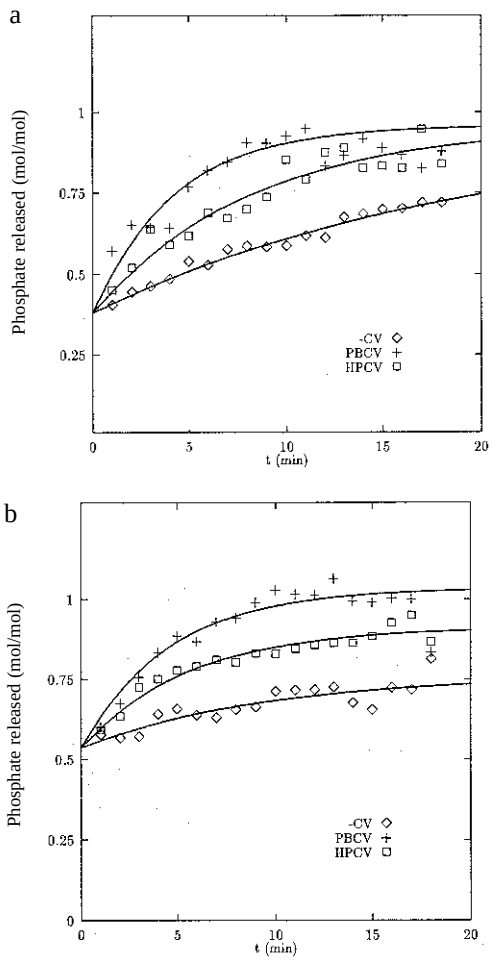

Fig. 4. Generation of free $^{32}P_i$ from 2.8 μM Hsc70 preloded with [γ-^{32}P]ATP. (a) Incubated in the absence of free ATP. (b) Incubated in the presence of 50 μM of free unlabelled ATP. Incubation was performed without coated vesicles (diamonds), with 1 mg/ml pig brain coated vesicles (crosses) and with 1 mg/ml human placental coated vesicles (squares).

equilibrium between Hsc70·ATP and Hsc70·ADP, then an increase in the rate of formation of Hsc70·ADP by hydrolysis will automatically increase the rate of exchange. Our results show that phosphate release is stimulated by the presence of vesicles even in the absence of free ATP, where ADP/ATP exchange can not occur. This suggests that stimulation of ATPase by coated vesicles occurs primarily by stimulation of ATP hydrolysis and phosphate release, though we cannot rule out the possibility that simple substrates like the apocytochrome *c* used by Sadis and Hightower (1992) behave differently.

Another question is whether or not coated vesicles can stimulate ADP/ATP exchange in addition to to their effect on the rate of hydrolysis. To answer this, one can compare the rates of release of label in Table 1.

With placental coated vesicles the release of label from [γ-^{32}P]ATP is stimulated by a factor of 2.2 (from −0.068/min to −0.147/min) upon addition of unlabelled ATP. However, the rate of release from [α-^{32}P]ATP is only 0.111/min. Since release of α-label is slower than that of γ-label we would conclude that with these vesicles there is no stimulation of ADP/ATP-exchange with placental vesicles.

With brain vesicles the release from [γ-^{32}P]ATP upon addition of ATP is increased from −0.118/min to −0.211/min (factor of 1.8), but release of label from [α-^{32}P]ATP occurs with a rate of −0.294/min. This we would interpret as an indication for stimulation of the exchange reaction, although this will require further confirmation.

These differences between vesicles from different tissues are significant in the light of earlier observations that placental vesicles can stimulate only a single round of ATP hydrolysis by Hsc70, while brain vesicles can stimulate multiple rounds (Buxbaum and Woodman, 1995). It has also been shown that the first round of ATP hydrolysis in Hsc70 with brain vesicles as substrate is faster than subsequent rounds (Greene and Eisenberg, 1990). The simplest explanation for these data is that product release is rate limiting for ATP turnover even with brain vesicles. In placental vesicles multiple rounds of ATP hydrolysis cannot occur because they seem to be unable to stimulate ATP/ADP exchange.

If one postulates the existence of an $Hsc70^{ATP}_{ADP}$ complex, then one cannot a priori rule out the existence of an $Hsc70^{ATP}_{ADP}$ complex, and therefore the possibility of ATP/ATP exchange. In fact, this is observed experimentally: in the presence of unlabelled ATP the release of $^{32}P_i$ from [γ-^{32}P]ATP-charged Hsc70 is incomplete, and the amount released is inversely correlated to the speed of release. This indicates that ATP/ATP exchange is indeed occurring. Gao et al. (1993) have shown that the rate of release of labelled ADP from Hsc70 is also increased by free ADP. We would interpret this as evidence for ADP/ADP exchange and therefore the existence of an $Hsc7^{ATP}_{ADP}$ complex. The existence of such a second nucleotide binding site has so far not been demonstrated in direct binding studies. This may not be surprising, however, since the unusually high stability of the Hsc70·ATP complex allows the use of rigorous washing procedures in binding assays, which keep unspecific background binding low, but may also destroy binding to a specific, but less stable, site. One way of testing our model would therefore be the search for this proposed second ATP binding site, using methods that do not require rigorous separation of bound and unbound ligand.

Schmid et al. (1985) have shown that ATP can accelerate both the binding of clathrin to Hsc70 and its release from Hsc70, and that it did so without being hydrolysed, since ATPγS was effective. They interpreted their findings as

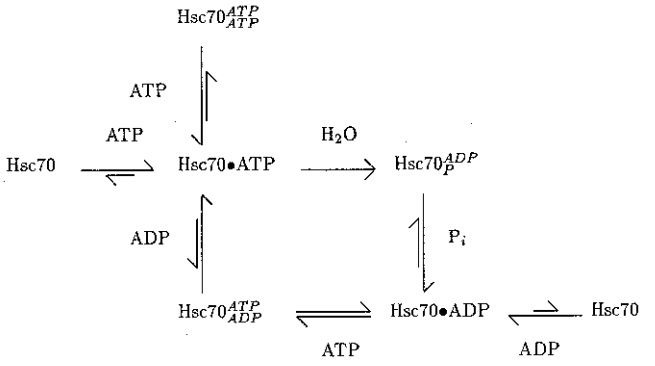

Fig. 5. Proposed reaction sequence of the ATPase reaction of Hsc70. For details see text.

evidence for a second 'catalytic' rather than 'hydrolytic' ATP binding site. Their distinction between hydrolytic and catalytic binding sites lost its appeal, when Heuser and Steer (1989) found that ATPγS was able to support the uncoating reaction. However, it appears quite possible that the catalytic effect of ATP described by Schmid et al. (1985) is caused by stimulation of nucleotide exchange.

How would this series of events tie in with the uncoating reaction? It is known that uncoating can proceed in the absence of ATP hydrolysis (Heuser and Steer, 1989; Buxbaum and Woodman, 1995). On the other hand, the 60 kDa chymotryptic fragment of Hsc70 shows coat-stimulated ATPase, but is unable to remove clathrin from coated vesicles (Tsai and Wang, 1994). The 40 kDa fragment has full ATPase activity, but can no longer bind clathrin (Chappell et al., 1987). There is therefore no direct causal relationship between hydrolysis of ATP and uncoating of clathrin coats.

However, we know that the presence of ATP accelerates binding and release of both clathrin to Hsc70 (Schmid et al., 1985; Prasad et al., 1994b) and fluorescently labelled model peptides to DnaK (Schmid et al., 1994). ADP has the opposite effect, preventing both binding and release (Heuser and Steer, 1989; Palleros et al., 1991). It seems that ATP binding to Hsc70 serves to open the peptide binding site, allowing substrate proteins to bind. Clathrin-coated vesicles are not particularly stable at neutral pH. Therefore one would expect an equilibrium between bound and unbound clathrin legs to exist. Binding of Hsc70 to the unbound legs would shift this equilibrium, and once all three legs have Hsc70 bound to them, the triskelion would fall off.

If this model is correct, then ATP hydrolysis could serve two different purposes. First, the released chemical energy of ATP hydrolysis would drive the whole process thermodynamically to completion. Once protein is bound, ATP hydrolysis is induced, leading to the formation of an protein·Hsc70·ADP complex. This complex has a closed protein binding site, with very low rates for protein binding and release (Heuser and Steer, 1989; Palleros et al., 1991). Since ADP cannot dissociate from this ternary complex, it will be stable until ADP/ATP exchange occurs, opening the protein binding site. This open protein site can not only release the bound protein, but also rebind another one. In DnaK considerable conformational changes have been found upon both ATP binding and hydrolysis (Banecki et al., 1992; Palleros et al., 1993).

A second purpose might be that both ATP hydrolysis and ADP/ATP exchange could be regulated by additional factors, as the *E. coli* homologue of Hsc70, DnaK, is regulated by DnaJ and GrpE. Both mitochondrial and cytosolic homologues of DnaJ have recently been identified (Cyr et al., 1994). In this context it is interesting that substrate proteins are not picked up from the cytosol by Hsc70, but released from supramolecular structures like coated vesicles, ribosomes or cytoskeleton. Those might be involved in regulation of ATP hydrolysis. A requirement for additional protein cofactors for the uncoating of clathrin-coated vesicles has been demonstrated (Prasad et al., 1993). It has been reported that this cofactor is the brain protein auxilin (Prasad et al., 1994a), which has some sequence similarity with DnaJ. By the same token, protein substrates of Hsc70 are not simply released into the cytosol, but transfered to other proteins. Clathrin is transfered to budding pits, actin and tubulin to the cytoskeleton and freshly translated proteins to either membrane transport systems or the foldosome GroES/GroEL. It is not unreasonable, that arrival at such a target could stimulate the exchange reaction. Locating those putative DnaJ- and GrpE-like factors on different structures in the cell would therefore result in vectorial transport of substrate proteins by Hsc70.

To test this model and increase our understanding of Hsc70 action, it will now be necessary to develop assays in which the various stages of the uncoating reaction (i.e. binding of Hsc70 to clathrin, breakage of clathrin-clathrin interactions, dissociation of Hsc70 and clathrin, and rebuilding of a new coated pit) can be linked to the stages of the ATPase reaction as defined in this paper. For example, our model was developed under the assumption that the ratio between ATP turnover and protein turnover is 1:1, but this has so far only been shown for the initial burst, not for the steady state reaction (Barouch et al., 1994).

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