High-level expression of soluble rat hsc70 in *Escherichia coli*: purification and characterization of the cloned enzyme

Chung WANG* and Miau-Rong LEE
Institute of Molecular Biology, Academia Sinica, Taipei, Taiwan

We have cloned the cDNA of rat hsc70 (clathrin-uncoating ATPase) into a T7 expression system and have expressed this enzyme in *Escherichia coli*. The recombinant clathrin-uncoating ATPase is in the cytosolic fraction of the bacterium and is soluble. It was purified to homogeneity by DEAE-cellulose and ATP–agarose column chromatography. From 1 litre of bacterial culture (0.3–0.4 g of proteins), 5–20 mg of pure recombinant clathrin-uncoating ATPase was routinely obtained. The cloned enzyme is capable of dissociating clathrin from bovine coated vesicles. Furthermore, it is not methylated on basic amino acid residues and is not blocked at the N-terminus, indicating that these modifications on hsc70 are not essential for uncoating of clathrin. Binding of [α-^{32}P]ATP by purified recombinant hsc70 was analysed by Scatchard plot. The results indicate that there is one high-affinity binding component with a K_d (dissociation constant) of 0.2–0.3 μM. The peptide-stimulated ATPase activities of recombinant hsc70 at 37 °C with respect to S-peptide, peptides P3a and GT4 at a concentration of 1.2 mM are 142 ± 6, 214 ± 8 and 362 ± 5 pmol/h per μg of hsc70 protein respectively. The EC_{50} values of hsc70 ATPase for S-peptide, peptides P3a and GT4 are 2, 0.67 and 0.17 mM respectively. On the other hand, the dissociation constants of S-peptide, peptides P3a and GT4 for recombinant hsc70 are 7.6, 13 and 100 μM respectively. Thus peptide GT4 is the only peptide examined for which the binding constant is comparable with the EC_{50} for stimulation of ATPase activity, albeit it has the lowest affinity for hsc70.

INTRODUCTION

A considerable amount of evidence supports the view that correct folding and subunit assembly of proteins *in vivo* may be mediated by a class of proteins collectively known as 'molecular chaperones' [for reviews, see Ellis and van der Vies (1991) and Gething and Sambrook (1992)]. Members of the 70 kDa heat-shock proteins, including their constitutively expressed 'cognates' (hsc70s), are among the most well characterized chaperone proteins [for a review, see Hightower (1991)]. The cytosolic hsc70 of mammalian cells has been identified as clathrin-uncoating ATPase (Ungewickell, 1985; Chappell et al., 1986). This enzyme, originally purified from bovine brain (Schlossman et al., 1984), catalyses the removal of clathrin from coated vesicles. This uncoating reaction requires ATP hydrolysis and the presence of clathrin light chains (Schmid et al., 1984; DeLuca-Flaherty et al., 1990). More recently, it was demonstrated that, besides clathrin uncoating, hsc70 of mammalian cells may have other functions. For instance, it appears to play an essential role in the transport of proteins into organelles, such as nucleus (Shi and Thomas, 1992) and lysosomes (Terlecky et al., 1992). Chymotrypsin digestion of clathrin-uncoating ATPase yields a 44 kDa fragment which is located within the highly conserved N-terminal region of the hsp70 family and which hydrolyses ATP in an uncoupled fashion (Chappell et al., 1987). The three-dimensional structure of this N-terminal domain was recently determined by X-ray crystallography (Flaherty et al., 1990), and a cleft for ATP binding can be visualized. It is clear that the N-terminal portion of this enzyme contains the ATP-binding site, but the C-terminal portion is essential for peptide-binding activity. The specific peptide sequence recognized by hsc70 has been determined in some cases. The pentapeptide, KFERQ, has been identified as the recognition site for the transport of certain cytosolic proteins into lysosomes for degradation (Terlecky et al., 1992). However, this sequence bears little resemblance to that of LC_a which is responsible for hsc70 binding (DeLuca-Flaherty et al., 1990). The consensus sequences, if any, for hsc70 binding among various peptides remain to be elucidated.

Earlier studies on hsc70 proteins from tissue cultured cells indicate that this protein is methylated at both lysine and arginine residues. This post-translational modification is irreversible and occurs during or very soon after translation of the polypeptides (Wang et al., 1981, 1982; Wang and Lazarides, 1984). Furthermore, the level of arginine methylation is reduced after treatment of these cells with sodium arsenite (Wang and Lazarides, 1984; Wang et al., 1992), which is known to induce increased synthesis of hsc70. The functional significance of methylation of hsc70 is as yet undetermined. In order to obtain a better understanding of the role of methylation of the peptide substrate specificity of hsc70, we decided to express rat hsc70 in *Escherichia coli* and to characterize the recombinant enzyme. In this report, we describe the results of our investigation. It is clear that recombinant hsc70 is enzymically active, even though the protein is not methylated at basic amino acid residues. Moreover, peptide stimulation of the ATPase activity of hsc70 does not necessarily correlate with the affinity of the peptide for hsc70.

EXPERIMENTAL

Construction of the plasmid and expression of the hsc70 protein

To express rat uncoating ATPase in *E. coli*, we subcloned the coding sequence of hsc70, originally in pRC62 (O'Malley et al., 1985), into p56, a T7 expression vector (Hwang et al., 1989). Figure 1 shows the scheme used to construct this expression plasmid, pHSC70. An N-terminal fragment of hsc70 cDNA containing an *Nde*I restriction site at the initiation ATG was produced by PCR. Primer I (5′-TGGGCCTACACGCAAGCACATATG) is identical with the 5′-non-coding region upstream

Abbreviations used: IPTG, isopropyl β-thiogalactoside; PEI–cellulose, poly(ethyleneimine)–cellulose.
* To whom correspondence should be addressed.

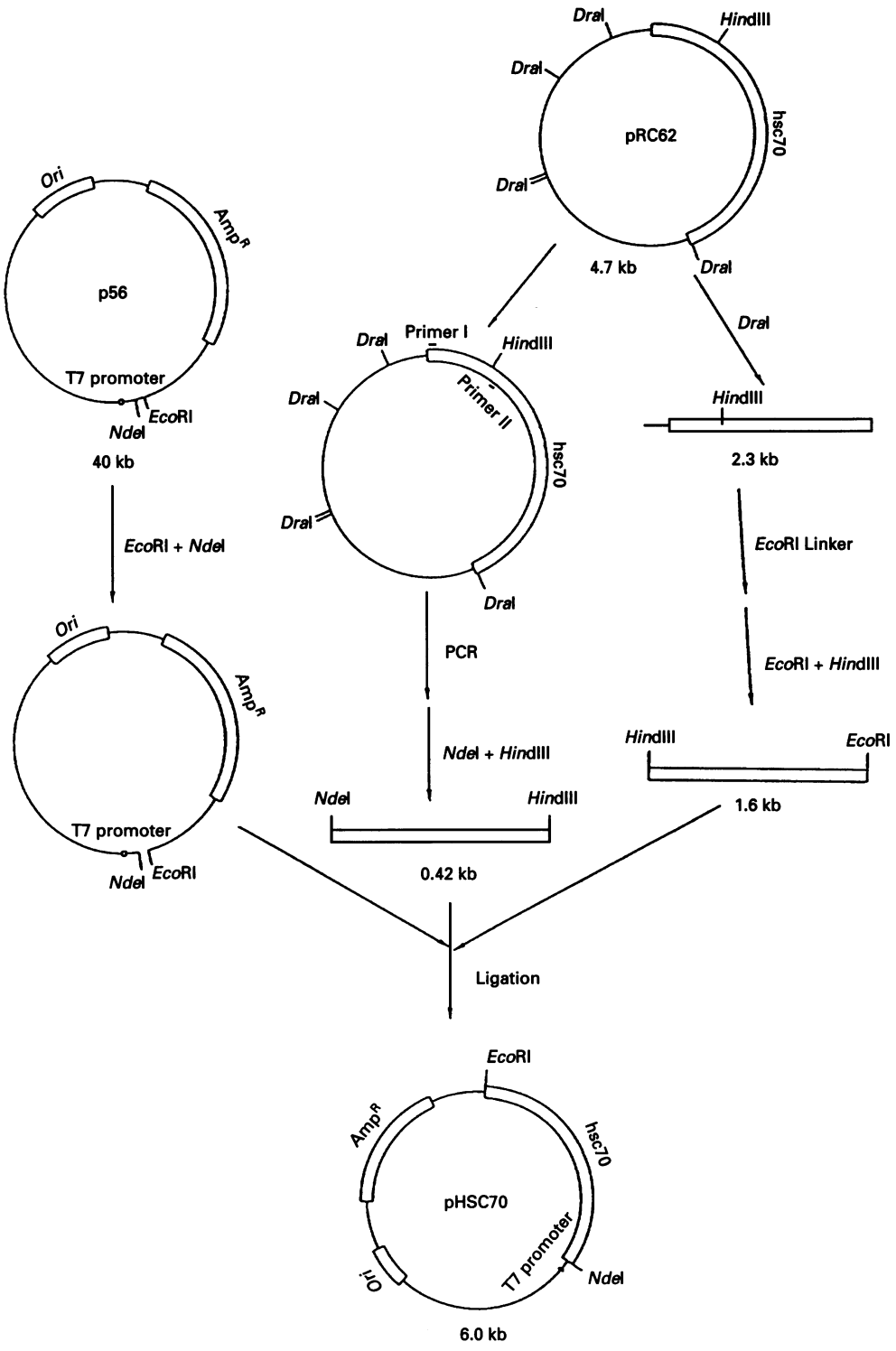

Figure 1 Construction of plasmid pHSC70 for expression of rat hsc70 in *E. coli*

Details of the construction of pHSC70 are described in the Experimental section. The synthesis of rat hsc70 mRNA from pHSC70 is driven by T7 RNA polymerase. The sizes of the plasmids and linear DNA fragments are not drawn to scale. Only restriction sites relevant to the construction are shown.

of the initiation ATG, except that there is an *Nde*I site at the 3′ end of this primer. Primer II (5′-AGCTGGCACGGTGACCAC-GGC) is complementary to the hsc70 sequence and is located 23 bp downstream of the unique *Hin*dIII site of hsc70 cDNA. The PCR product was sequenced by the dideoxynucleotide chain termination method (Sanger et al., 1977). One of the guanosines was found to be changed to an adenosine at a wobble position thus not affecting the amino acid sequence. The product was then doubly digested with *Nde*I and *Hin*dIII to produce the N-terminal fragment (0.42 kb). To generate the C-terminus of

hsc70 cDNA, we first digested pRC62 with restriction enzyme *Dra*I, followed by addition of *Eco*RI linkers to the 2.3 kb fragment containing the coding region of rat hsc70 cDNA. A *Hin*dIII–*Eco*RI fragment (1.6 kb) was subsequently produced by double digestion with these two restriction enzymes. Finally, the *Nde*I–*Hin*dIII and *Hin*dIII–*Eco*RI fragments were directionally ligated into p56, which had previously been digested with both *Nde*I and *Eco*RI, to obtain pHSC70 (6.0 kb). To rule out the possibility that a minor contaminant of the PCR product might have been cloned into the pHSC70 plasmid, the segment of the plasmid encoding for the hsc70 protein was sequenced by using a set of synthetic oligonucleotide primers. No additional base alteration was found in the coding region for hsc70.

The pHSC70 plasmid was transformed into *E. coli* BL21 (DE3) and the synthesis of recombinant hsc70 was induced by treatment with isopropyl β-thiogalactoside (IPTG) as described by Studier et al. (1990). Briefly, single colonies of plasmid-containing bacteria were used to inoculate a culture. IPTG was added to a final concentration of 0.4 mM immediately after the A_{600} had reached 0.3. Incubation was allowed to continue for 2 additional hours. At the end of the incubation, a small portion of the cell suspension was withdrawn, centrifuged (12000 *g*), the pellet dissolved in SDS sample buffer and then analysed by PAGE to evaluate the efficiency of hsc70 expression.

Purification of recombinant clathrin-uncoating ATPase

The procedures originally used to purify clathrin-uncoating ATPase from bovine brain by Schlossman et al. (1984) were followed with some modifications. After IPTG induction, the bacteria were harvested by centrifugation and resuspended in 0.1 vol. of a solution containing 25 mM Tris/HCl (pH 7.0), 1 mM EDTA, 250 mM sucrose and 0.2 mg/ml lysozyme. The suspension was gently stirred on ice for 15 min. The mixture was then slowly poured into 4 vol. of ice-cold water to lyse the cells. The lysate was centrifuged at 50000 *g* for 30 min, and the supernatant was applied to a DEAE-cellulose column pre-equilibrated with 25 mM Tris/HCl (pH 7.0). The column was washed with 2 vol. of 25 mM Tris/HCl (pH 7.0), followed by 1 vol. of the same buffer containing 50 mM NaCl. hsc70 was eluted with 1.5 vol. of 25 mM Tris/HCl (pH 7.0) containing 120 mM NaCl. Then $MgCl_2$ was added to a final concentration of 5 mM before application to an ATP–agarose column (2 ml of resin) equilibrated with buffer A (120 mM NaCl, 5 mM $MgCl_2$ and 25 mM Tris/HCl, pH 7.0). The resin was washed with 2 vol. of buffer A, followed by 10 vol. of 0.6 M NaCl in buffer A. The bound proteins, predominantly hsc70, were eluted with 5 mM ATP in buffer A or with 10 mM EDTA in a buffer containing 120 mM NaCl and 25 mM Tris/HCl, pH 7.0.

Purification of high-affinity ATP-binding proteins from *E. coli*

Bacteria were grown, harvested and subjected to osmotic shock. After the addition of $MgCl_2$ and NaCl to final concentrations of 5 mM and 120 mM respectively, the lysate was centrifuged at 50000 *g* for 30 min. The supernatant was then applied to an ATP–agarose column pre-equilibrated with buffer A. The column was washed extensively and the bound protein was eluted with 5 mM ATP in buffer A as described above.

Labelling of recombinant clathrin-uncoating ATPase with L-[*methyl*-^{3}H]methionine and amino acid analysis

Bacteria harbouring the plasmid pHSC70 were grown in Luria broth medium to A_{600} 0.3. IPTG and L-[*methyl*-^{3}H]methionine (DuPont-NEN; specific radioactivity = 80 Ci/mmol) were added to 0.4 mM and 50 μCi/ml respectively, and the incubation was continued for 3 h. The cultures were then centrifuged (12000 *g*), and the resulting pellets boiled in SDS sample buffer before being subjected to PAGE. The protein bands corresponding to recombinant hsc70 were excised from the gels after staining with Coomassie Blue. The protein was then recovered from the gel slices, hydrolysed with 6 M HCl, and subjected to amino acid analysis as described previously (Wang et al., 1982; Wang and Lazarides, 1984).

Dissociation of clathrin by recombinant hsc70

Dissociation of clathrin from coated vesicles by recombinant clathrin-uncoating ATPase was performed essentially by the methods of Greene and Eisenberg (1990). Briefly, 1 μg of enzyme was mixed with 20 μg of bovine coated vesicles in buffer A containing 2 mM ATP, 15 mM phosphocreatine and 6 units of creatine kinase in a total volume of 200 μl. The reaction mixtures were incubated at room temperature for 30 min and then centrifuged at maximum speed in a TLA 100.2 rotor for 15 min at 4 °C in a TLA 100 centrifuge (Beckman). Samples of supernatant were subjected to SDS/PAGE. Clathrin heavy and light chains appear in the supernatant after dissociation.

Determination of dissociation constant (K_d) of ATP for recombinant clathrin-uncoating ATPase

The purified recombinant clathrin-uncoating ATPase was immobilized by coupling to CNBr-activated Sepharose CL-4B at a ratio of 1 mg of protein/ml of packed resin. After termination of the coupling reaction, the resin was extensively washed and equilibrated with buffer A. To measure the affinity of ATP for the enzyme, portions of hsc70–Sepharose (5 μl) were resuspended in buffer A containing different concentrations of [α-^{32}P]ATP in a total volume of 100 μl. The mixture was rocked at room temperature for 30 min and centrifuged (4000 *g*) at the end of the incubation. A portion of the supernatant was withdrawn to quantify the amount of free ATP. The pellet was quickly rinsed twice with 1 ml of ice-cold buffer A, and the ATP bound to hsc70–Sepharose was determined by measuring the Cerenkov radiation. The results were analysed by Scatchard plot. Alternatively, the dissociation constant of ATP to hsc70 was measured by displacement of [α-^{32}P]ATP by non-radioactive ATP. Briefly, samples of hsc70–Sepharose were mixed with 50 nM [α-^{32}P]ATP, and various amounts of non-radioactive ATP were added as a competitor. After the mixture had been incubated at room temperature for 30 min with constant rocking, it was centrifuged and the pellet washed once with ice-cold buffer A. The amount of [α-^{32}P]ATP bound to hsc70–Sepharose was quantified. The data were analysed as described by Wang and Brennan (1988). In both cases, the binding of [α-^{32}P]ATP in the presence of 1 mM non-radioactive ATP was taken as non-specific and subtracted.

Assay of peptide-stimulated ATPase activity of recombinant clathrin-uncoating ATPase

The effect of peptides on the ATP-hydrolytic activity of clathrin-uncoating ATPase was determined as described by DeLuca-Flaherty et al. (1990) unless otherwise specified. Three peptides were used in this investigation. The first, P3a (FAILDGGAPGP-QAHGEPPGGPDAVD), is a fragment of clathrin light chain LC_a, previously shown to be the sequence recognized by hsc70 (DeLuca-Flaherty et al., 1990). The second was S-peptide, which is known to bind hsc70 (Terlecky et al., 1992). The third, GT4

(ARKSLKRLTGWADVC), is a fragment of the glucose transporter in insulin-responsive tissues (James et al., 1989).

Measurement of the binding constants of peptides to recombinant hsc70

Peptide P3a and S-peptide were first labelled with ^3H by the methods of Flynn et al. (1989), except that the specific radioactivity of NaB^3H_4 (Amersham) was 500 mCi/mmol. After labelling, ^3H-labelled peptides were estimated by ninhydrin analysis of acid hydrolysates. To measure the dissociation constants of peptide P3a and S-peptide for hsc70, various amounts of ^3H-labelled peptides were mixed with hsc70–Sepharose in buffer B (75 mM KCl, 5 mM magnesium acetate, 40 mM Hepes, pH 7.0) to a total volume of 100 μl. The suspension was rocked at room temperature for 1 h before centrifugation. A portion of the supernatant was saved to quantify the free peptide, and the pellets were quickly rinsed once with 0.5 ml of ice-cold buffer B. The resin was then incubated with 0.3 ml of 0.1% SDS in 100 mM NH_4HCO_3 at 70 °C for 15 min before liquid-scintillation spectrophotometry. The results were analysed by Scatchard plot. The dissociation constants of S-peptide and peptide GT4 from recombinant hsc70 were also determined by measuring displacement of ^3H-labelled peptide P3a by these two peptides. Briefly, hsc70–Sepharose (10 μl) was incubated with 4 μM of ^3H-labelled peptide P3a and various amounts of S-peptide and peptide GT4. The mixture was incubated at room temperature for 1 h and then the ^3H-labelled peptide P3a bound to the resin was quantified. The ^3H radioactivity associated with the resin in the presence of 1.2 mM non-radioactive peptides was regarded as non-specific and was subtracted during analysis.

Other methods

Bovine clathrin-uncoating ATPase was purified by the methods of Schlossman et al. (1984). Bovine brain coated vesicles were purified by the methods of BarZiv and Branton (1986). The amount of recombinant hsc70 protein at each purification step was determined by quantification of Coomassie Blue bound to hsc70 protein bands. Known quantities of purified recombinant hsc70 [assayed by the method of Lowry et al. (1951)] were used as standards. Briefly, portions of bacterial lysate, soluble cytosol and DEAE-cellulose chromatography fractions together with standards were subjected to SDS/PAGE. After staining with Coomassie Blue and destaining with acetic acid/ethanol, the protein bands corresponding to hsc70 were excised from the gels. The proteins were eluted from gel slices by incubation with 0.7 ml of 1% SDS in 150 mM NH_4HCO_3 at 37 °C overnight. A_{650} was measured and used to quantify hsc70 protein. Phosphorylation *in vitro* on purified proteins was performed by the methods of Cegielska and Georgopoulos (1989). Two-dimensional gel electrophoresis was carried out as described by O'Farrell (1975). Peptide synthesis was performed by t-Boc chemistry on an ABI model 430 peptide synthesizer. After cleavage with HF, the peptide was desalted by gel filtration on Sephadex G-25 (peptide P3a and S-peptide) or Sephadex G-10 (peptide GT4). The concentration of peptide P3a was measured by ninhydrin analysis of intact peptide, whereas S-peptide and peptide GT4 were assayed by ninhydrin analysis of acid hydrolysates. Known quantities of phenylalanine were used as standard. The N-terminal amino acid sequence of recombinant hsc70 was determined by Edman degradation on an ABI gas–liquid-phase peptide sequencer (model 470A) with an on-line phenylthiohydantoin amino acid analyser.

RESULTS

Starting with a cDNA clone encoding rat clathrin-uncoating ATPase (pRC62), we constructed a plasmid, pHSC70 (Figure 1), suitable for the expression of this enzyme in *E. coli*. Total cell lysates were prepared from the bacteria with or without induction and were analysed by SDS/PAGE. The results (Figure 2a) show that the amount of a 72 kDa polypeptide increases dramatically after IPTG induction. The mobility of this induced protein on SDS/polyacrylamide and two-dimensional gels is identical with that of clathrin-uncoating ATPase purified from bovine brain (results not shown), indicating that this protein is recombinant hsc70. To determine if the induced hsc70 is soluble, spheroplasts were prepared and then lysed by osmotic shock. Recombinant hsc70 appears in the high-speed supernatant after centrifugation (results not shown). Therefore we prepared the soluble cytosolic fraction from induced cells in order to purify recombinant clathrin-uncoating ATPase. As shown in Figure 2(b), the protein eluted from the ATP–agarose column is predominantly hsc70. A summary of the steps undertaken to purify recombinant hsc70 is shown in Table 1. In this particular case, about 10% of the total protein is recombinant hsc70. Using 0.8 l of bacterial culture, we obtained 5.2 mg of highly pure recombinant hsc70 with a yield of 19%. A substantial loss of hsc70 (40%) in the first step of purification may have been the result of poor preparation of spheroplasts from *E. coli*. Higher recovery of hsc70 at the DEAE-cellulose step was achieved by eluting the column with 150 mM NaCl. However, at this concentration, a contaminant protein of molecular mass 75 kDa (also see below) copurified with hsc70, and therefore a lower concentration of NaCl (120 mM) was later used. From the results of several prepar-

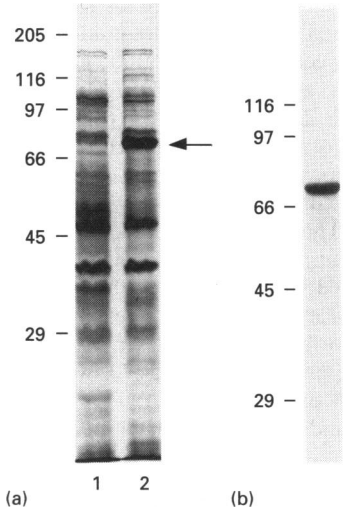

Figure 2 (a) induction of rat hsc70 in *E. coli* and (b) purified recombinant hsc70

(**a**) Recombinant *E. coli* cultures in early exponential phase were treated with IPTG for 2 h. Then 200 μl of cell suspension was withdrawn and analysed by SDS/PAGE. The Coomassie Blue-stained gels are shown here. Lanes 1 and 2 are control and IPTG-treated cells respectively. The intense band of molecular mass 72 kDa appearing only in lane 2 is recombinant hsc70 (arrow). Molecular mass standards are myosin heavy chain (205 kDa), β-galactosidase (116 kDa), phosphorylase (97 kDa), BSA (66 kDa), ovalbumin (45 kDa) and carbonic anhydrase (29 kDa). (**b**) Recombinant hsc70 purification was performed as described in the Experimental section. Proteins (7 μg) eluted from the ATP–agarose column were analysed by SDS/PAGE. As can be seen on the Coomassie Blue-stained gel shown here, recombinant hsc70 is the only predominant band. Molecular-mass standards are β-galactosidase, phosphorylase, BSA, ovalbumin and carbonic anhydrase.

Table 1 Recovery of recombinant hsc70 from *E. coli*

A volume of 800 ml of bacterial culture was used for this purification. The total protein at each purification step was assayed by the method of Lowry et al. (1951). The amount of hsc70 was quantified by measuring Coomassie Blue bound to protein bands corresponding to hsc70 on SDS/polyacrylamide gels, with known quantities of purified recombinant hsc70 as standards.

Purification step	Total protein (mg)	hsc70 (mg)	Purification (fold)	Yield (%)
Bacterial lysate	285	27	1	100
Soluble cytosol	152	16	1.1	59
DEAE-cellulose	45	7	1.6	26
ATP–agarose	5.2	5.2*	11	19

* All the protein recovered from the ATP–agarose column was assumed to be hsc70.

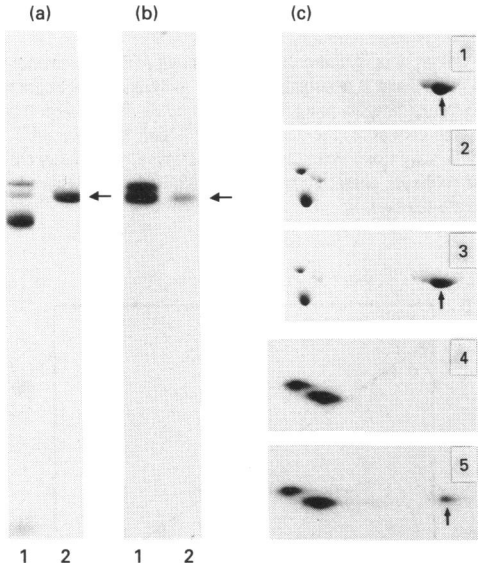

Figure 3 Comparison of high-affinity ATP-binding proteins of *E. coli* with recombinant hsc70

Proteins were purified, phosphorylated *in vitro* and then subjected to SDS/PAGE. The Coomassie Blue-stained gel (**a**) and the corresponding autoradiogram (**b**) are shown. Lanes 1 and 2 are high-affinity ATP-binding proteins isolated from *E. coli* BL21 (DE3) and purified recombinant hsc70 respectively. The samples were also subjected to two-dimensional gel electrophoresis (**c**). Panels 1, 2 and 3 are Coomassie Blue-stained gels of recombinant hsc70, high-affinity ATP-binding proteins and a combination of both respectively. Panels 4 and 5 are the autoradiograms showing the phosphoproteins of panels 2 and 3 respectively. Arrows indicate the location of recombinant hsc70 on the gels. The acidic end of the gel is on the left hand side. Only the relevant regions are shown. Clearly, purified recombinant hsc70 is not contaminated with endogenous bacterial ATP-binding proteins to any significant extent.

ations, we estimated that 5–10% of the total protein was recombinant hsc70, and that 5–20 mg of purified hsc70 was obtained from 1 litre of culture.

To evaluate whether bacterial protein of similar molecular mass, such as dnaK, was present in the recombinant hsc70 preparation, we purified the high-affinity ATP-binding proteins from *E. coli* BL21(DE3) and used them for comparison with recombinant hsc70. We obtained 0.5–1 mg of ATP-binding proteins from 3 litres of bacterial culture. Indeed, one of them has an apparent molecular mass virtually indistinguishable from that of hsc70 (Figure 3a). Phosphorylation of these purified proteins *in vitro* was also performed. The results shown in Figure 3(b) indicate that two of the high-affinity ATP-binding proteins from *E. coli* can be phosphorylated. Moreover, low-level phosphorylation can be detected in recombinant hsc70. Therefore two-dimensional gel electrophoresis was used to determine if the phosphorylated species in the recombinant hsc70 preparation was contaminated with bacterial proteins (Figure 3c). It is clear that the ATP-binding proteins from *E. coli* are more acidic than recombinant hsc70, and the purified hsc70 preparation contains little bacterial protein (panels 1, 2 and 3). Moreover, recombinant hsc70 is phosphorylated *in vitro* (panels 4 and 5), albeit at a relatively low level. We have also determined the N-terminal sequence of recombinant hsc70. The first 39 amino acids (SKGPAVGIDLGTTYSCVGVFQHGKVEIIANDQGNRT-TPS) are as predicted from the nucleotide sequence. Interestingly, the N-terminus of native bovine brain hsc70 is blocked.

As hsc70 is methylated at both lysine and arginine residues in mammalian and avian cultured cells, we first asked whether or not recombinant hsc70 is methylated. If methylation occurs in recombinant hsc70, metabolical labelling of the bacteria with [*methyl*-^{3}H]methionine will result in incorporation of [^{3}H]methyl groups into these methylated amino acid residues. Therefore we grew *E. coli* with L-[*methyl*-^{3}H]methionine and then isolated hsc70 for amino acid analysis. The elution profile shown in Figure 4(a) indicates that methylation at lysine and arginine residues occurs in hsc70 from NIH 3T3 cells. The methyl-lysines (peak II), dimethylarginine (peak III) and monomethylarginine (peak IV) comprise 14, 3.5 and 6.4% of the total ^{3}H radioactivity respectively. However, the elution profile of recombinant hsc70 shows only one radioactive peak corresponding to [*methyl*-^{3}H]methionine (Figure 4b). Therefore we conclude that recombinant hsc70 is not methylated at these residues.

The second question addressed in this study was does the cloned rat hsc70 retain the ability to remove clathrin from bovine coated vesicles. To perform the uncoating assay, coated vesicles were incubated with or without purified recombinant hsc70. Dissociation of clathrin by the enzyme was observed by ultracentrifugation and then followed by SDS/PAGE. The coated vesicles appeared to be stable in solution containing ATP, as little clathrin appeared in high-speed supernatant in the absence of hsc70 (Figure 5, lane 1). In contrast, when incubated with recombinant hsc70, there was a significant amount of dissociated clathrin in the supernatant (Figure 5, lane 3). Under identical experimental conditions, the uncoating activity of recombinant hsc70 is as high as, if not higher than, the native enzyme purified from bovine brain (results not shown). Evidently, recombinant hsc70 is enzymically active in dissociation of clathrin from bovine coated vesicles, even though the protein is not methylated and is not modified at the N-terminus.

The third question considered was what is the affinity of ATP for recombinant clathrin-uncoating ATPase. The purified enzyme was first immobilized by coupling to CNBr-activated resin. Subsequently, hsc70–Sepharose was used for the binding assay. The results were analysed by Scatchard plot (Figure 6a). It appears that the binding of ATP to hsc70–Sepharose is one component with $K_d = 0.2\ \mu M$, which is one to two orders of magnitude lower than that reported by Schmid et al. (1985) ($K_d = 1\ \mu M$) and by Palleros et al. (1991) ($K_d = 9.5\ \mu M$). The total number of binding sites (27 pmol) was also determined from Figure 6(a). Assuming that the coupling efficiency of hsc70 to CNBr-activated Sepharose is 100%, we calculated that 0.4 ATP molecules bind to each polypeptide at saturation. A similar

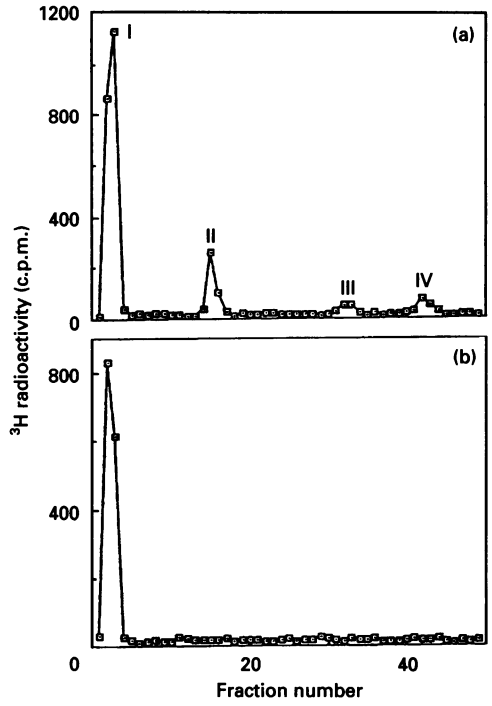

Figure 4 Elution profiles of hydrolysed [^{3}H]hsc70 from NIH 3T3 cells and from recombinant *E. coli*

(**a**) NIH 3T3 cells were grown in RPMI medium supplemented with 10% fetal calf serum (Gibco). After the cells reached confluence, the medium was replaced with 5 ml of Dulbecco's minimal essential medium containing 150 μCi of L-[*methyl*-^{3}H]methionine. The incubation was continued for 4 h and then the cells were harvested and subjected to two-dimensional gel electrophoresis. The labelled hsc70 was excised from the gels and hydrolysed with 6 M HCl. The hydrolysates were analysed on a PAA211 column (Pierce). The ^{3}H radioactive peaks are methionine (I), methyl-lysines (II), $N^G N^G$-dimethylarginine (III) and N^G-monomethylarginine. (**b**) ^{3}H-labelled recombinant hsc70 was isolated and hydrolysed by 6 M HCl. The hydrolysates were analysed by ion-exchange chromatography. There is only one ^{3}H radioactive peak corresponding to that of methionine.

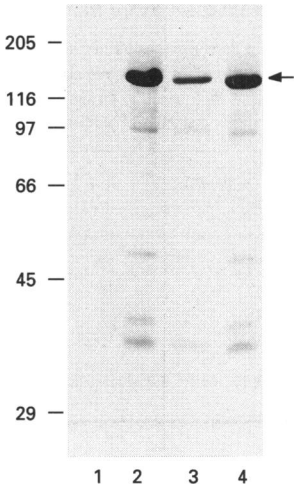

Figure 5 Dissociation of clathrin from bovine coated vesicles by recombinant rat hsc70

Bovine coated vesicles were incubated in buffer containing ATP in the absence (lanes 1 and 2) or presence (lanes 3 and 4) of recombinant hsc70. At the end of the incubation, they were centrifuged and the supernatants (lanes 1 and 3) and pellets (lanes 2 and 4) were analysed by SDS/PAGE. The Coomassie Blue-stained gels are shown here. The arrow indicates the location of clathrin heavy chain. Molecular-mass standards are myosin heavy chain (205 kDa), β-galactosidase (116 kDa), phosphorylase (97 kDa), BSA (66 kDa), ovalbumin (45 kDa) and carbonic anhydrase (29 kDa).

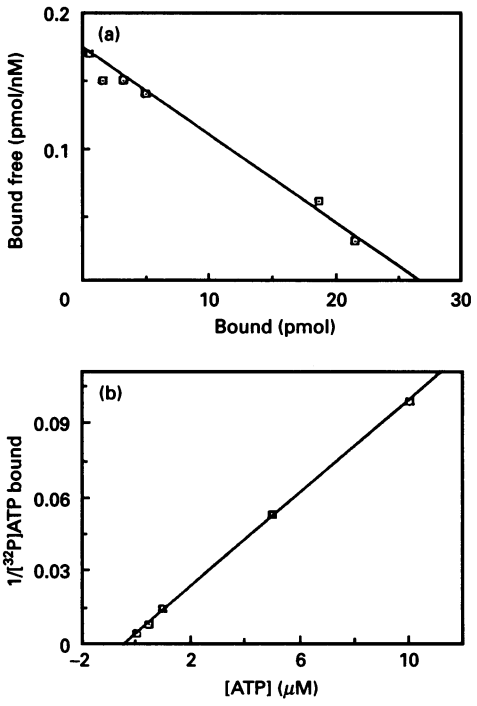

Figure 6 Affinity of ATP for immobilized hsc70

Purified recombinant hsc70 was coupled to CNBr-activated resin, and hsc70–Sepharose was used for the binding assay. The association constant was determined by either Scatchard analysis (**a**) or displacement of radioactive ATP with non-radioactive ligand (**b**). Each point is the average of duplicate determinations with a deviation of less than 2%. ATP concentrations were calculated by counting samples of known amounts of [α-^{32}P]ATP. Non-specific binding (usually less than 1% of the total) was subtracted. In (**a**), K_d and R_0 (total number of binding sites) are 0.2 μM and 27 pmol respectively. The amount of protein used for these assays was 5 μg (70 pmol). In (**b**), K_d = 0.32 μM.

stoichiometry was obtained for peptide-binding sites per hsc70 molecule (see below). The substoichiometric binding of ATP or peptide to hsc70 might be caused by either inactivation of hsc70 during the coupling or other as yet undetermined reasons. As ATP hydrolysis may occur during the course of the binding assay, one may speculate that the ^{32}P radioactivity bound to hsc70–Sepharose resin might be [α-^{32}P]ADP. Indeed, Palleros et al. (1991) showed that hsc70 binds ADP with a high affinity. Therefore a competition assay with a fixed amount of [α-^{32}P]ATP and variable quantities of non-radioactive ATP was performed. Under these conditions, the dissociation constant of the competitor (non-radioactive ATP) was measured. The results shown in Figure 6(b) also indicate that there is one binding component with a dissociation constant of 0.3 μM, which is in good agreement with the result of the Scatchard analysis.

The fourth question investigated was is the peptide-stimulated ATPase activity of recombinant hsc70 identical with that of the native bovine enzyme. We assayed the ATPase activity of hsc70 in the presence of various concentrations of peptide P3a, a fragment of clathrin light chain LC_a specific for hsc70 binding (DeLuca-Flaherty et al., 1990). We confirmed that this peptide fragment does indeed stimulate the ATP-hydrolytic activity of bovine clathrin-uncoating ATPase in a concentration-dependent

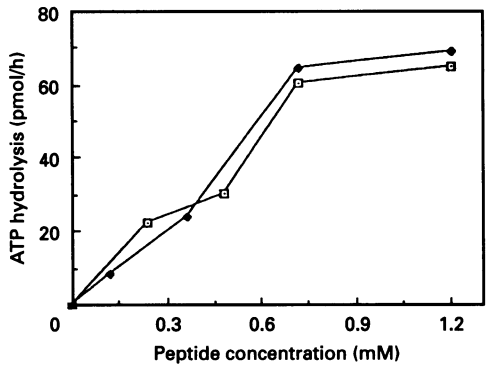

Figure 7 Peptide concentration-dependent ATP hydrolysis of hsc70

Either bovine brain hsc70 (◆) or rat recombinant hsc70 (□) was incubated with various concentrations of peptide fragment in buffer (5 μl) containing 0.4 mM [γ-^{32}P]ATP at 37 °C for 1 h. Purified enzymes (0.35 μg) were used for these assays. Then 0.4 μl of the reaction mixture was spotted on poly(ethyleneimine) (PEI)–cellulose sheets and developed in a solution containing 0.5 M LiCl and 1.0 M formic acid. The plates were dried and subjected to autoradiography. The spots corresponding to [^{32}P]ATP and [^{32}P]phosphate were excised from the plates and quantified by measurement of Cerenkov radiation. ATP hydrolysis was expressed as the formation of [^{32}P]phosphate from total ^{32}P radioactivity/unit time. The amount of ATP hydrolysed in the absence of peptide was subtracted.

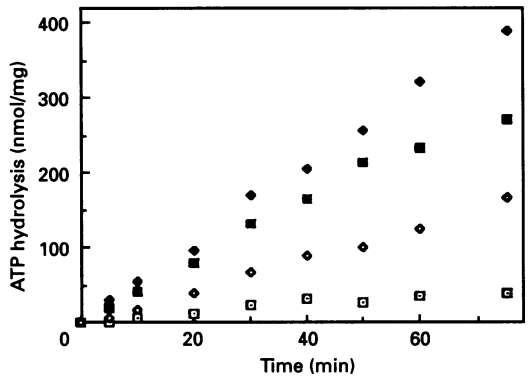

Figure 8 Time-course of ATP hydrolysis by hsc70

Recombinant rat hsc70 or bovine brain hsc70 was incubated in buffer containing [α-^{32}P]ATP with or without 1.2 mM peptide. At given time intervals, small portions were withdrawn, spotted on PEI–cellulose sheets and developed in a solution containing 1 M LiCl and 0.5 M formic acid to separate [α-^{32}P]ATP from [α-^{32}P]ADP. ATP hydrolysis was measured as described in the Experimental section. □, ◇, ATP hydrolysed by bovine brain hsc70 and recombinant hsc70 respectively in the absence of peptides. ■, ◆, ATP hydrolysis by native bovine hsc70 and cloned hsc70 respectively in the presence of peptides. It is evident that recombinant hsc70 has a higher basal ATPase activity.

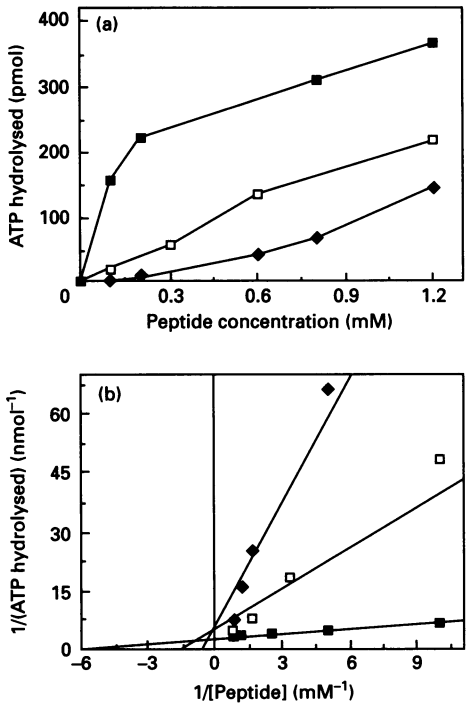

Figure 9 Stimulation of ATP hydrolysis by S-peptide and peptides P3a and GT4

(**a**) Different concentrations of S-peptide (◆) and peptides P3a (□) or GT4 (■) were incubated with 1 μg of purified recombinant hsc70 and 0.2 mM [γ-^{32}P]ATP at 37 °C for 1 h. Then 0.5 μl of the reaction mixture was spotted on PEI–cellulose chromatogram sheets and developed in a solution of 0.5 M formic acid and 1 M LiCl. ATP hydrolysis was measured as described in the Experimental section; ATP hydrolysed in the absence of peptides was subtracted. (**b**) Peptide-dependent hydrolysis of ATP in the presence of S-peptide, peptides P3a or GT4 was analysed by a Lineweaver–Burk plot. Each point represents the average of two determinations.

manner (Figure 7). Recombinant hsc70 produced a similar, if not identical, effect. The EC_{50} of peptide P3a for both native and recombinant enzyme is approximately 0.5 mM. The time course of hsc70-dependent ATP hydrolysis was investigated with or without the addition of peptide P3a. The ATP hydrolysis is linear for at least 75 min (Figure 8). A significant amount of ATP was hydrolysed by recombinant hsc70 in the absence of peptide P3a, and the basal level of ATPase activity was much lower for bovine brain enzyme than for recombinant hsc70. Addition of peptide P3a to the reaction mixture enhanced the ATP-hydrolytic activity of both bovine brain and recombinant hsc70 (Figure 8).

It was recently shown that S-peptide of ribonuclease is a good substrate for hsc70 binding (Terlecky et al., 1992). Therefore we decided to examine whether S-peptide affects the ATP hydrolysis by hsc70. An arbitrarily chosen peptide, GT4, was also tested for its ability to stimulate the ATPase activity of hsc70. The effect of increasing concentrations of these two peptides as well as peptide P3a on ATP hydrolysis by hsc70 is shown in Figure 9(a). The results indicate that peptide GT4 is most effective in activating ATP hydrolysis. The level of stimulation by peptide GT4 at 1.2 mM (362 pmol/h per μg of protein) is 170% of that by peptide P3a (214 pmol/h per μg). On the other hand, the level of activation by S-peptide at 1.2 mM (142 pmol/h per μg) is only 65% of that by peptide P3a. Identical results were obtained for purified bovine brain hsc70, i.e. peptide GT4 was most effective in stimulating ATPase activity, whereas S-peptide was least effective (results not shown). EC_{50} values were determined by double-reciprocal plots and are 2, 0.67 and 0.17 mM for S-peptide, peptides P3a and GT4 respectively (Figure 9b). The maximal level of peptide-stimulated ATPase activity was also obtained from this plot. The catalytic-centre activity at maximal velocity for peptide GT4 (0.5 pmol/min per pmol of hsc70) is almost twice that for S-peptide and peptide P3a (0.26 pmol/min per pmol of hsc70). The values obtained here are comparable with those reported previously (Flynn et al., 1989; DeLuca-Flaherty et al., 1990).

The next question considered was whether stimulation of ATP hydrolysis by the peptides could be correlated with their affinities for hsc70. We first measured the dissociation constants of peptide

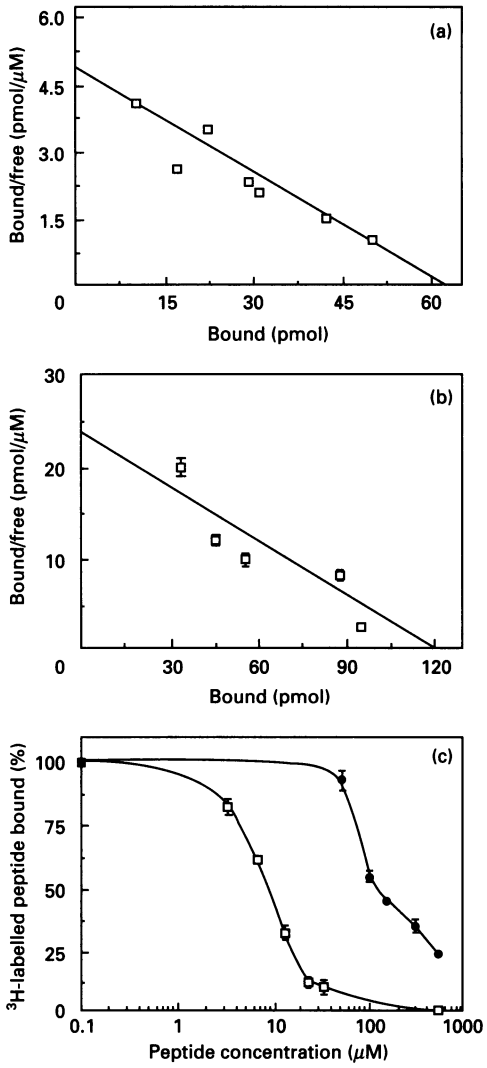

Figure 10 Affinity of peptides for hsc70–Sepharose

The affinity of peptide P3a (**a**) and S-peptide (**b**) for recombinant hsc70 was determined by Scatchard plot. Purified hsc70 was immobilized by coupling to CNBr-activated Sepharose (1 mg of protein/ml resin); 10 μl (**a**) and 20 μl (**b**) of the resin were used for each reaction. From the graph, K_d and R_0 can be deduced. For P3a and S-peptide, the K_d values are 13 and 5 μM, and R_0 values are 61 and 118 pmol respectively. The affinity of S-peptide and peptide GT4 for hsc70–Sepharose was estimated by competition with a fixed amount (4 μM) of ^{3}H-labelled peptide P3a (**c**). The quantity of ^{3}H-labelled peptide P3a bound to the resin in the absence of competitors is taken as 100%. The ^{3}H-labelled peptide P3a bound at various concentrations of S-peptide (□) and peptide GT4 (●) are plotted accordingly. The amount of ^{3}H-labelled peptide P3a bound to hsc70–Sepharose in the presence of 1.2 mM non-radioactive peptide P3a is regarded as non-specific and was subtracted. The peptide concentration at which 50% of the maximal binding is displaced is $K_{app.}$, and the values are 10 and 130 μM for S-peptide and peptide GT4 respectively. K_d can be calculated from $K_{app.}$ by using the equation $K_{app.} = K_d\{1 + ([P3a]/K_{d,P3a})\}$. Thus, $K_d = K_{app.}/1.3$.

P3a and S-peptide from immobilized recombinant hsc70 by Scatchard analysis (Figure 10a and 10b); K_d values for peptide P3a and S-peptide are 13 and 5 μM respectively. These values are two to three orders of magnitudes less than the corresponding EC_{50} values (Figure 9). From the total number of binding sites (Figure 10a and 10b), we calculated that, for both peptide P3a and S-peptide, there are 0.4 peptide-binding sites per hsc70 molecule. This value is in good agreement with the number of ATP-binding sites per hsc70 molecule calculated from Figure 6(a). A competition assay was then used to determine whether or not the binding sites for peptide P3a and S-peptide on hsc70 are independent of each other. If the binding sites are dependent, the bound ^{3}H-labelled peptide P3a should be displaced by S-peptide. The results shown in Figure 10(c) indicate that S-peptide is indeed capable of competing with ^{3}H-labelled peptide P3a for binding to hsc70. Peptide GT4, at higher concentrations, also displaces bound ^{3}H-labelled peptide P3a (Figure 10c). Similarly, binding of ^{3}H-labelled S-peptide to hsc70 can be completely displaced by 0.5 mM peptide P3a (results not shown). Therefore these peptides do not bind to independent sites on hsc70. The affinities of S-peptide and peptide GT4 for recombinant hsc70 can also be estimated by their effectiveness in displacing ^{3}H-labelled peptide P3a from hsc70–Sepharose. The values of $K_{app.}$ (the concentration that causes 50% displacement) for S-peptide and peptide GT4 are 10 and 130 μM respectively (Figure 10c). Thus the calculated K_d values for S-peptide and peptide GT4 are 7.6 and 100 μM respectively. The K_d value of S-peptide for recombinant hsc70 obtained here is virtually identical with that for bovine hsc70 (K_d = 8 μM) reported by Terlecky et al. (1992). Taken together, the results shown in Figures 9 and 10 indicate that the EC_{50} values for stimulation of hsc70 ATPase by peptides may or may not correlate with the affinities of the peptides for hsc70.

DISCUSSION

Rat clathrin-uncoating ATPase (hsc70) cDNA was cloned into a T7 expression vector (p56) and expressed in *E. coli*. After induction by IPTG, as much as 5–10% of the total bacterial protein was recombinant hsc70. However, even with such a high level of synthesis, this recombinant enzyme appears to be soluble. With a slight modification of the purification procedures for bovine brain hsc70, we have purified recombinant hsc70 to homogeneity (Figures 2 and 3). Enzymic properties of the purified recombinant hsc70 were then characterized. Several conclusions can be drawn from these investigations. In the first place, cloned hsc70 is active in dissociating clathrin from bovine coated vesicles (Figure 4). Therefore the cloned enzyme appears to be functional, and the small differences in amino acid sequences between rat and bovine hsc70 (DeLuca-Flaherty and McKay, 1990) do not affect the clathrin-dissociation activity.

The second conclusion is that, in contrast with hsc70s from tissue or cultured cells, recombinant hsc70 is not methylated on basic amino acid residues and is not blocked at the N-terminus. As the cloned enzyme appears to be active, these modifications do not appear to be essential for enzyme activity. In fact, the peptide-independent ATPase activity of recombinant hsc70 is higher than that of the native bovine enzyme (Figure 8). It is unlikely that the higher basal ATPase activity associated with recombinant hsc70 is due to contamination with as yet unidentified ATPases in the preparation, as a recombinant hsc70 mutant, with asparagine substituted for Asp-10, has a much lower basal ATPase activity when purified by identical procedures (Huang et al., 1993). There are several possible explanations for this observation. First, the observed difference in ATPase activity in the absence of peptides might be the results of species differences, because here bovine brain hsc70 is compared with recombinant rat hsc70. Secondly, the low ATPase activity of native bovine brain hsc70 may be the result of the N-terminus being blocked, which is not so for recombinant hsc70. Finally, the high ATPase activity of recombinant hsc70 may be due to lack of methylation at lysine and/or arginine residues. If this is the

case, methylation of recombinant hsc70 should bring about a decrease in peptide-independent ATP hydrolysis.

The third conclusion is that, for both ATP and peptides, there may be only one high-affinity binding site on immobilized recombinant hsc70. Analysis of the binding of ATP to hsc70–Sepharose demonstrates that the binding has one component (K_d = 0.2–0.3 μM) and is substoichiometric (0.4 ATP/hsc70). It is consistent with the view that there is only one binding site per hsc70 polypeptide. The results of Scatchard analysis of peptide binding also show that peptide binding appears to have one component and is substoichiometric (0.5 peptide/hsc70). Moreover, the peptide-binding site(s) are not independent because several peptides compete with each other for binding. Thus, despite the fact that different peptides have different affinities for hsc70, they appear to bind to the same site. The factor that determines the affinity of peptides for hsc70, however, remains to be elucidated.

The final conclusion is that binding of peptide of hsc70 *per se* is insufficient to stimulate ATP hydrolysis. Three peptides were used to address this issue. The first one, peptide P3a, was previously shown to contain the binding sequence of clathrin light chain LC_a to hsc70 (DeLuca-Flaherty et al., 1990). The second one, S-peptide of ribonuclease, was previously demonstrated to bind native hsc70 with high affinity (Terlecky et al., 1992). The third one, peptide GT4, was chosen for no obvious reason. Interestingly, of these three peptides, only peptide GT4 shows a correlation between its stimulation of ATPase activity (EC_{50} = 0.17 mM; Figure 9b) and its affinity for hsc70 (K_d = 0.1 mM; Figure 10c). In contrast, the affinity of peptide P3a (K_d = 13 μM; Figure 10a) is much lower than the EC_{50} value (670 μM; Figure 9b) for activation of hsc70 ATPase, and, although S-peptide binds hsc70 with high affinity (K_d = 5–8 μM; Figure 10b and 10c), at the concentrations around its K_d, it does not stimulate hsc70 ATPase activity at all (Figure 9a). A similar situation was discovered for immunoglobulin heavy-chain-binding protein (BiP) (Flynn et al., 1989), i.e. a peptide with high affinity for BiP stimulated the BiP ATPase activity poorly. The question of why binding of peptides to hsc70 does not lead to full stimulation of ATPase activity remains to be answered.

To summarize, we have overexpressed rat clathrin-uncoating ATPase (hsc70) in *E. coli*. The recombinant enzyme retains uncoating capability and acquires a high basal ATPase activity. It is not methylated and is not modified at its N-terminus, suggesting that these post-translational modifications play a role in regulating its intrinsic ATPase activity. Analysis of the binding data indicates that there is only one ATP-binding site per recombinant hsc70 polypeptide with a dissociation constant of 0.2–0.3 μM. Three peptides were examined with respect to their concentration effects on stimulating ATP hydrolysis by hsc70 and their affinities for hsc70. Peptides corresponding to previously identified hsc70 binding sequences appear to bind hsc70 with high affinity (K_d = 5–10 μM), but the EC_{50} values for stimulation of ATPase activity by these two peptides are much higher than their K_d values. Thus binding of peptides to hsc70 does not necessarily lead to activation of its ATPase. The structural basis and physiological significance of this observation remain to be determined.

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