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35. A clone of the *ERG13* gene, encoding HMG-CoA synthase was provided by K. Jarman and J. Proffitt (Amoco Corp.). A Bam HI fragment containing the yeast *HIS3* gene was inserted into a Bgl II site in the HMG-CoA synthase coding region to create a disruption allele, which was then used to disrupt the chromosomal *ERG13* locus, rendering the cell a mevalonate auxotroph.
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Peptide Binding and Release by Proteins Implicated as Catalysts of Protein Assembly

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Two members of the hsp70 family, termed hsc70 and BiP, have been implicated in promoting protein folding and assembly processes in the cytoplasm and the lumen of the endoplasmic reticulum, respectively. Short hydrophilic (8 to 25 residues) synthetic peptides have now been tested as possible mimics of polypeptide chain substrates to help define an enzymatic basis for these activities. Both BiP and hsc70 have specific peptide binding sites. Peptide binding elicits hydrolysis of adenosine triphosphate, with the subsequent release of bound peptide.

BECAUSE MANY PROTEINS REFOLD AFTER DENATURATION (*1*), it has long been assumed that protein folding and assembly occurs spontaneously in cells. However, recent and diverse studies of protein folding, unfolding, and related processes (such as translocation across membranes) in vivo suggest that in many instances these events may be catalyzed. If so, then these catalysts can determine the nature of folding pathways and their location in cells, defining in essence what folded states consist of and coordinating protein assembly in relation to existing cellular organization. How this works is unknown and has been difficult to determine for lack of a well-defined in vitro system. Here we describe an assay system that may mimic essential steps in this process.

Two distinct families of adenosine triphosphate (ATP)–dependent proteins have been implicated as catalysts, namely members of the groEL family (termed chaperonins) found in bacteria, mitochondria, and chloroplasts (*2, 3*); and members of the hsp70 family, found both in the cytoplasm and in the lumen of the endoplasmic reticulum (ER) (*4–6*). The heat shock protein hsp70 family consists of several members (ranging from 70 to 78 kD) that are induced at elevated temperatures (*4, 5*), and of other members that are constitutively expressed (*6*). These constitutively expressed proteins include hsc70, also known as the uncoating ATPase for clathrin-coated vesicles (*7*) and a binding protein BiP (*8*), also known as

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Table 1. Summary of the kinetic parameters of hsc70 and BiP-dependent ATPase activity. ATPase assays were performed as described in Fig. 7. Peptides A to D are from the vesicular stomatitis virus glycoprotein (VSV-G). Peptides F, G, and I are from the influenza hemagglutinins. The peptide E sequence is from a Ras homolog (Rab 1) identified in rat brain. ND, peptide-stimulated ATPase activity was not detectable at the indicated concentration. Abbreviations for the amino acid residues are: A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; and Y, Tyr.

Peptides	Source	Residue	BiP		Hsc70	
			K_m (μM)	V_{max} (nmol^{-1} min^{-1} mg^{-1})	K_m (μM)	V_{max} (nmol^{-1} min^{-1} mg^{-1})
(A) KRQIYTDLEMNRLGK	VSV G (IND)	1–15*	900	1.7	770	2.9
(B) LSSLFRPKRRPIYKS	(NJ)	9–23*	35	1.0		
(C) KLIGVLSSLFRPK	(NJ)	16–28*	150	2.0	55	3.0
(D) RRPIYKSDVGMAHFR	(NJ)	1–15*	170	1.6		
(E) CKIQSTPVKQS	Rab 1	6–15*	150	1.6		
(F) YHCDGFQNE	HA 1	75–83	ND (1.5 mm)			
(G) EGMIDGWYGFRHQNC	HA 2	15–29	90	1.2		
(H) VGIDLGTTYSC	Hsp70	7–17	330	1.0		
(I) SNGSLQCRIC	HA 2	2–11*	12	1.6		

*These peptides are numbered from the COOH-terminus to the NH_2-terminus.

GRP78 (*9–11*). Both are ATP-binding proteins and are similar to each other (*7, 10*). However, BiP, containing a signal sequence, is found in the lumen of the ER (*12*); hsc70, lacking a translocation signal, is found in the cytoplasm (*10, 13*). Timed labeling studies of the assembly of immunoglobulins have shown that BiP is transiently associated with the heavy chains until light chains are bound. If light chains are absent, BiP remains bound permanently and the heavy chains are retained in the ER (*14*). BiP is also transiently bound to influenza hemagglutinin (HA) subunits prior to their trimerization, and is bound to mutant or underglycosylated HA's that are unable to fold properly (*15*). Synthesis of BiP is induced by the accumulation of misfolded proteins in the lumen of the ER, whether a primary consequence of mutation in the nascent protein or a secondary consequence of misfolding due to insufficient glycosylation, caused by the absence of glucose or the presence of inhibitors (*16–18*). The cytoplasmic homolog of BiP, hsc70, is required for posttranslational translocation of diverse proteins across ER and mitochondrial membranes (*19*).

The groEL gene product of *Escherichia coli* is required for assembly of bacteriophage capsids (*20*). Timed labeling experiments of the assembly of ribulose carboxylase in chloroplasts revealed that a protein closely related to groEL, and analogous to BiP, is transiently bound to unassembled protomers (*2*). Mutants in the groEL-related protein of mitochondria are able to import subunits into mitochondria, but functional (oligomeric) enzymes are not formed (*21*). As is the case for BiP (*10*), complexes of groEL with substrates also dissociate in the presence of ATP (*22*). Altogether, such studies offer indirect evidence that members of the hsp70 and groEL families catalyze ATP-dependent protein assembly and disassembly reactions (*23*).

Such activities, to be useful as a general pathway, would require the interaction of the catalysts with diverse polypeptide chains. We do not know, for example, (i) what features of a newly made or misfolded protein initiate interactions with a folding enzyme, (ii) how these proteins facilitate folding, unfolding, or assembly, and (iii) exactly what role ATP plays. We have now addressed these issues with the use of a well-defined and purified enzyme system consisting of the catalyst ATP and a protein substrate. The simplest mimic of a segment of native or an unfolded protein is a short peptide. We find that both BiP and hsc70 have sequence-specific binding sites for peptides. Peptide-binding elicits hydrolysis, and ATP hydrolysis is a necessary prerequisite for peptide release.

Binding of a peptide to BiP. To determine whether BiP would bind a short synthetic peptide, we incubated BiP, purified from bovine liver microsomes (*24, 25*), with ^{3}H-labeled peptide A (Table 1) and then isolated BiP together with any bound peptide by gel filtration (Fig. 1). Peptide A, a 15–amino acid fragment with four positive and two negative residues, was chosen for initial studies because of its availability. The purity of the BiP preparation is demonstrated in Fig. 3A, lane 2.

The peptide bound to BiP in a saturable fashion with a K_d of approximately 12 μM (Fig. 1). At saturation, about 0.85 mole of peptide was bound per mole of BiP (78-kD monomer), based on Scatchard analysis of these data.

BiP dissociates from complexes with incompletely assembled or

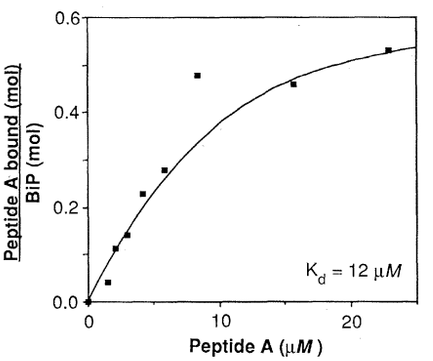

Fig. 1. BiP binding to soluble peptide A. Purified BiP (2.0 μg) was incubated with ^{3}H-labeled peptide A (0 to 1150 pmole) in buffer containing 50 mM tris-HCl (pH 7.5), 200 mM NaCl, and 1 mM Na_2EDTA (50 μl, final volume) for 30 minutes at 37°C. The free peptide was removed from the BiP-peptide complex by a centrifugation at 100g by desalting through a 1-ml Sephadex G-50 column, for 2 minutes (*31*). To prevent binding to the resin, columns were first treated with 100 μl of bovine serum albumin at 1 mg/ml in the same buffer and centrifuged as described above. The bound peptide, in the void volume, was quantified by liquid scintillation spectroscopy. The small amount of BiP-independent background radioactivity (50 cpm) was subtracted for each peptide concentration. The amount of BiP recovered in the void volume was quantitated by the intensity of stain in densitomitry after SDS-PAGE with pure BiP as standards. On the basis of 78 kD being the mass of BiP, 4.7 pmole of BiP passed through the columns. Peptide A was labeled with tritium by reductive methylation (*32*). Briefly, a 200-μl solution of peptide A (10 mg/ml) in 0.2M sodium borate buffer (pH 8.9) was incubated with 15 μl of 0.2M formaldehyde and 10 μl of 0.12M $NaB[^3H]_4$ (5 Ci/mmole, ICN) for 10 minutes at 4°C; and labeling was then terminated by the addition of 10 μl of 0.5M $(NH_4)_2SO_4$. Labeled peptide was separated from contaminating radioactivity and unreactive reagents by gel filtration with Sephadex G10 in 25 mM Hepes (pH 7.0), 200 mM NaCl. The first major peak of radioactivity was rechromatographed over a separate Sephadex G10 column resulting in a single peak. This peak of radioactivity coincided exactly with the peak of peptide as measured by ninhydrin (*33*). For binding assays, the tritiated peptide was diluted with unlabeled peptide A in the appropriate buffer to an activitiy of 480 cpm per picomole of peptide. Data are presented as moles of peptide A recovered bound per mole of BiP recovered.

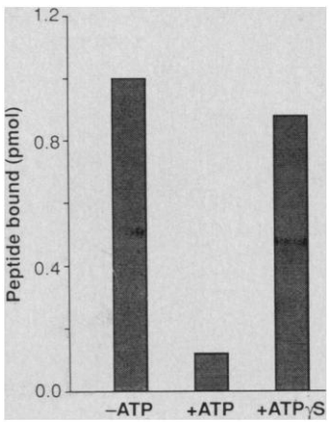

Fig. 2. ATP hydrolysis prevents peptide A binding to BiP. The binding reaction was carried out with 125 pmole of tritiated peptide A, in the presence of 50 μ*M* ATP, 50 μ*M* ATPγS, or with no added nucleotide as described in Fig. 1. A background of 55 cpm was subtracted for all values.

misfolded protein upon incubation with ATP (*10*). The peptide-BiP complex isolated in Fig. 1 was formed in the absence of ATP. The inclusion of ATP (50 μ*M*), but not ATPγS, eliminated this complex, either by preventing its formation or facilitating its dissociation (Fig. 2). That the latter is the case is shown in Figs. 4 and 5.

BiP is present in the lumen of microsomal vesicles (*12*) (derived mainly from the ER) and comprises at least 0.4 percent of the microsomal protein, calculated from the amount of purified BiP we obtain from bovine liver. To test whether BiP (in preference to other ER proteins) binds to peptide A, we passed a detergent extract of salt-stripped, bovine liver microsomes through a column to which peptide A had been linked. A control column was prepared in which no peptide was used. A single major protein component of about 78 kD (along with numerous minor components) remained bound after the peptide A column was washed with 1*M* NaCl (Fig. 3A, lane 3; compare with the microsomal extract, lane 1). The 78-kD component bound to the peptide columns was indeed BiP. The two proteins migrate identically on SDS–polyacrylamide gel electrophoresis (PAGE) (Fig. 3B, lanes 1 and 2) and their partial proteolysis patterns are identical (Fig. 3B, lanes 3 and 4). Only the minor components bound to the control column (Fig. 3A, lane 4). One minor component binding to peptide A coupled to Affigel migrates at a position corresponding to a molecular mass of approximately 94 kD (Figs. 3A and 4). A likely candidate for this protein is GRP94. Both GRP78 (BiP) and GRP94 are luminal ER proteins and are induced by malfolded proteins, suggesting related roles (*16*). The fact that BiP is affinity-purified as a result of peptide-Affigel chromatography implies that peptide binding is an unusual property that occurs in BiP and hardly any other microsomal proteins. Purified BiP also binds to peptide A columns.

ATP hydrolysis is required to dissociate BiP and hsc70 from peptide. The release of BiP from complexes with endogenous substrates (isolated from cell extracts) is ATP-dependent (*10*). BiP is not released with 1*M* NaCl, but dissociates rapidly on incubation with 100 μ*M* MgATP at 25°C (Fig. 4, lane 2; compare with lane 1 representing what had been bound). Little release was observed with magnesium in the absence of ATP (lane 4), or 100 μ*M* MgATPγS (lane 5). Moreover, a 40-fold (molar) excess of ATPγS over ATP inhibited ATP-dependent release of BiP (Fig. 4, lane 6). These results demonstrate that ATP hydrolysis is required for dissociation of BiP from peptide, and explain why ATP (but not ATPγS) prevented the isolation of soluble peptide-BiP complexes (Fig. 2).

In the preceding experiments (Fig. 4), we used saturating concentrations of ATP and measured the maximum amount of release over long periods; hence the effects of ATP on the initial rate of release are underestimated. A more complete analysis of the rate of release (Fig. 5) shows both the time course and nucleotide concentration dependence of this reaction. The rate of release is about half-maximal at 10 μ*M* nucleotide. This low requirement for ATP is comparable to that for ATP-dependent clathrin release from coated vesicles catalyzed by hsc70 (*13*).

Hsc70 is present in the cytosolic fraction. To test whether hsc70, like BiP, is selectively bound by peptide A, bovine brain cytosol was passed through peptide A resin. Three major bands (82, 70, and 55 kD) were bound (Fig. 6A). The 70-kD band reacts with an antibody to a peptide containing a sequence conserved in all members of the hsp70 family (*7*) (Fig. 6B). The 70-kD protein, or hsc70, can be specifically released from the peptide resin by incubation with ATP and Mg^{2+} (Fig. 6A, lanes 3 to 6), as was the case for BiP. Little hsc70 was released with ATPγS and Mg^{2+}, or with Mg^{2+} alone (Fig. 6A, lanes 5 and 6). The identities of the 55-kD and the 82-kD bands are unknown.

Peptide-dependent ATPase activity. In efficient enzyme reactions, the hydrolysis of ATP is coupled to the work performed, with little hydrolysis occurring in the absence of all substrates. The peptide binding results imply that BiP can bind peptide in an ATP-independent fashion, and that ATP hydrolysis drives the release of the peptide. Thus, ATP should be hydrolyzed at a rate determined by the concentration of peptide.

The initial rate of ATP hydrolysis by BiP is a function of the

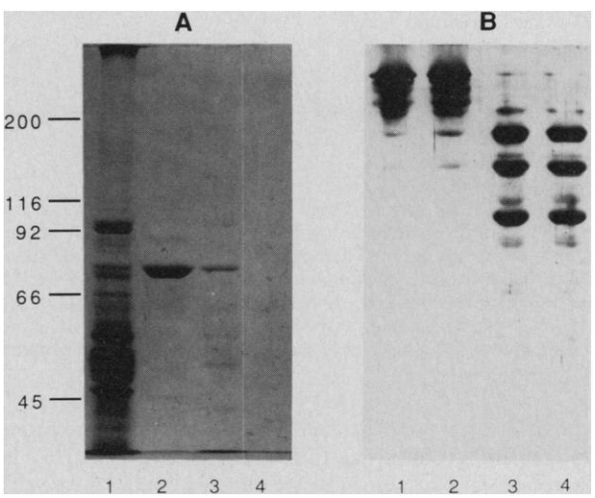

Fig. 3. (**A**) Peptide affinity of microsomal proteins. Microsomes (lane 1) were prepared from bovine liver (*34*) with some modifications (*35*). The microsomes were disrupted with an equal volume of buffer A, containing detergent (100 m*M* tris-HCl, *p*H 7.5, 0.4*M* NaCl, 10 m*M* Na_2EDTA, and 2 percent sodium cholate), then diluted to a final protein concentration of 5 mg/ml with buffer B (50 m*M* tris-HCl, *p*H 7.5, 0.2*M* NaCl, 5 m*M* Na_2EDTA, and 1 percent sodium cholate). The suspension was clarified (100,000*g*, 60 minutes, 4°C), and the soluble extract was passed over the peptide-Affigel columns (*36*) at 40 ml/hour per milliliter of resin (800 mg of microsomal protein per milliliter of resin). The columns were washed with five column-volumes of buffer B, ten column-volumes of buffer B containing 1*M* NaCl, and two column-volumes of buffer B without Triton X-100, in succession. Bound material was eluted with 8*M* urea, precipitated with trichloroacetic acid (*37*), and analyzed by SDS-PAGE (*38*). All chromatography was performed at 4°C. The composition of proteins bound to the peptide A–Affigel resin is shown in lane 3. Proteins that bind to a control column lacking conjugated peptide A are shown in lane 4. Lane 2 demonstrates the homogeneity and relative mobility of BiP (*24*) purified by a modification of the method of Welch and Feramisco (*25*). (**B**) Proteolytic digestion of BiP and the 78-kD peptide binding protein. BiP and the 78-kD peptide binding protein were excised from a 7.5 percent SDS-polyacrylamide gel and digested with V8 protease (*39*) and separated on a 15 percent SDS-polyacrylamide gel. (Lanes 1 and 3) Three micrograms of BiP; lanes 2 and 4, 3 μg of the peptide binding protein. In lanes 3 and 4, 50 ng of V8 protease was added at the start.

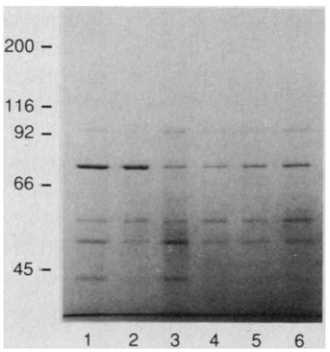

Fig. 4. ATP elution of BiP from peptide A columns. Detergent-solubilized microsomes were clarified and loaded onto 0.2 ml of peptide A–Affigel columns at 4°C. The columns were washed as described in the legend to Fig. 3A, then equilibrated in 50 m*M* tris-HCl (*p*H 7.5), 50 m*M* NaCl, and 3 m*M* $MgCl_2$ with or without added nucleotide. The columns were incubated for 10 minutes at 25°C, and the proteins were eluted with the same buffer. (Lane 1) The total protein bound was removed with 8*M* urea; (lane 2) proteins were eluted with buffer containing 100 μ*M* ATP; (lane 4) no added nucleotide; (lane 5) 100 μ*M* ATPγS; (lane 6) 2 m*M* ATPγS and 100 μ*M* ATP. (Lane 3) The material that was removed from the peptide column with urea after the elution with 100 μ*M* ATP.

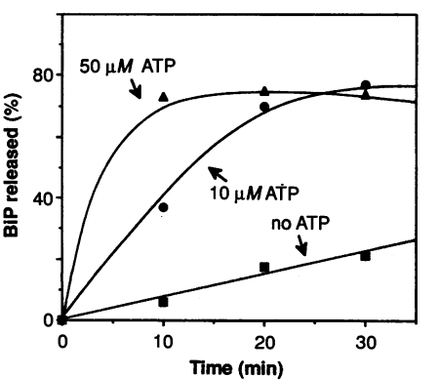

Fig. 5. Time course of the elution of BiP with ATP. Detergent extracted and clarified membranes, containing 1.3 g of total protein at 5 mg/ml (Fig. 3, lane 1) were slowly (1 ml/min) pumped through a 2-ml peptide A–Affigel column and washed as described in Fig. 3. The resin was then suspended in buffer (50 m*M* tris-HCl, *p*H 7.5, containing 50 m*M* NaCl) and divided into equal portions of 100 μl of resin. After centrifugation (500*g*, 1 minute), the resin portions were suspended 1 ml of the same buffer containing Mg^{2+} and the ATP concentration indicated on the figure. The resins were incubated for the indicated times at 25°C with gentle shaking, and then rapidly cooled on ice. Eluted material was removed from the resin after centrifugation at 4°C. Since the peptide-Affigel portions could not be made absolutely uniform, the protein eluted was expressed as a fraction of the total BiP present in the sample (*40*).

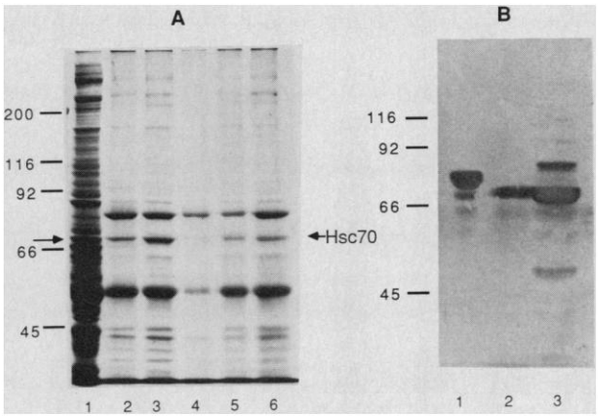

Fig. 6. Peptide affinity and ATP elution of hsc70. (**A**) Bovine brain cytosol (60 ml, 10 mg/ml) was clarified (100,000*g*, 60 minutes, 4°C), then passed over a 2.4-ml peptide A–Affigel column at 0.5 ml/min. The resin was washed with 5 ml of 50 m*M* tris (*p*H 7.5), 200 m*M* NaCl, and 1 m*M* Na_2EDTA, and then with a similar buffer containing 1*M* NaCl. After equilibration in the original wash buffer, the resin was suspended and divided into four equal portions, and each was sedimented (100*g*, 1 minute) and resuspended in 1.0 ml of 50 m*M* tris-HCl (*p*H 7.5), 50 m*M* NaCl, 2 m*M* $MgCl_2$ with either 50 μ*M* ATP, 50 μ*M* ATPγS, or no additional nucleotide (or urea in some cases). These reaction mixtures were then incubated at 25°C for 10 minutes and centrifuged as before. Supernatants were removed for SDS-PAGE analysis. All samples were precipitated with trichloroacetic acid before the electrophoresis. (Lane 1) Eighty micrograms of bovine brain cytosol; (lane 2) material bound to the peptide A–Affigel resin and eluted with 8*M* urea; (lane 3) ATP elution; (lane 4) material remaining after ATP elution subsequently extracted with 8*M* urea; (lane 5) no nucleotide; (lane 6) ATPγS elution. (**B**) Immunoblot of peptide A–Affigel bound material (*7*). Protein was transferred to nitrocellulose and probed with rabbit antiserum to a peptide containing a heat shock conserved sequence (*7, 41*). (Lane 1) Five micrograms of BiP; (lane 2) cytosolic material bound to peptide A–Affigel resin (comparable to Fig. 6A, lane 2); (lane 3) 80 μg of bovine brain cytosol.

concentration of peptide A (Fig. 7A). This activity co-chromatographs with BiP on Mono Q–FPLC. The kinetic data give a linear double-reciprocal plot (Fig. 7B) yielding a K_m for ATPase of 900 μ*M* for peptide A. The observation of a peptide-dependent ATPase in BiP provides independent evidence of a peptide-binding site. The linearity of the double reciprocal plot suggests that there is only one kind of peptide binding site for ATPase activity.

Peptide sequence specificity. The peptide-dependent ATPase activity provides a rapid assay with which to screen peptides for sequence specificity. Synthetic peptides, ranging in length from 8 to 30 residues stimulated the ATPase activity of BiP with varying efficiencies (Table 1). Most effective was peptide I, having a K_m for ATP hydrolysis of 12 μ*M*. One peptide did not stimulate ATP hydrolysis detectably, even at high concentration (1.5 m*M*). None of the individual amino acids (when tested at up to 10 m*M*) detectably stimulated the ATPase of BiP. Although it is clear that there is considerable sequence or structural specificity (the K_m's of even the limited set of peptides tested differ by at least three orders of magnitude), further studies are needed to elucidate the basis of peptide specificity.

We have performed fewer but similar studies with cytoplasmic hsc70 purified from bovine brain (*13, 26, 27*). We found that hsc70 has a peptide-dependent ATPase similar to that of BiP, also yielding linear double reciprocal plots. The peptide C–dependent ATPase activities of BiP and of hsc70 are compared in Fig. 8. The apparent K_m of hsc70 for peptide A is 770 μ*M* (900 μ*M* with BiP) while that for peptide C is 55 μ*M* (150 μ*M* for BiP) (Table 1). The V_{max} of hsc70 driven by peptide A is 2.9 nmole/min per milligram (1.7 for BiP) and 3.0 (2.0 for BiP) when driven by peptide C. All of these maximum velocities are similar to the maximum rate of ATP hydrolysis by hsc70 (uncoating ATPase) with clathrin cages as substrate (*26*), 2.6 nmole/min per miligram. Clathrin cages do not stimulate the ATPase activity of BiP (tested at 0 mg/ml). The peptide-independent ATPase activity of BiP is about 0.35 nmole/min per milligram. Kassenbrock and Kelly (*27*) have reported this intrinsic ATPase activity, and also that ATP induces a conformational change in BiP.

Implications for protein folding. Pelham (*5*) has suggested that the hsp70 family binds misfolded or aggregated proteins (or both), releasing them in an ATP-dependent fashion. These cycles were proposed to disassemble aggregated structures to allow subsequent, proper assembly. The constitutively expressed hsp70 family members would be adequate under ordinary circumstances, but during stress when proteins denature, individual members with appropriately inducible promoters would accumulate to aid in the process.

It seems reasonable to assume (although difficult at present to prove) that the synthetic peptide substrates mimic the interaction of hsc70 and BiP with cellular proteins. It is striking that two gene products, hsc70 and BiP, evolving independently at least since the divergence of yeast and animals, maintain quantitatively similar patterns of interaction with peptides. As these peptide-binding properties are evolutionally conserved and thus likely biologically relevant, it would appear that the hsp70 members can, in principle,

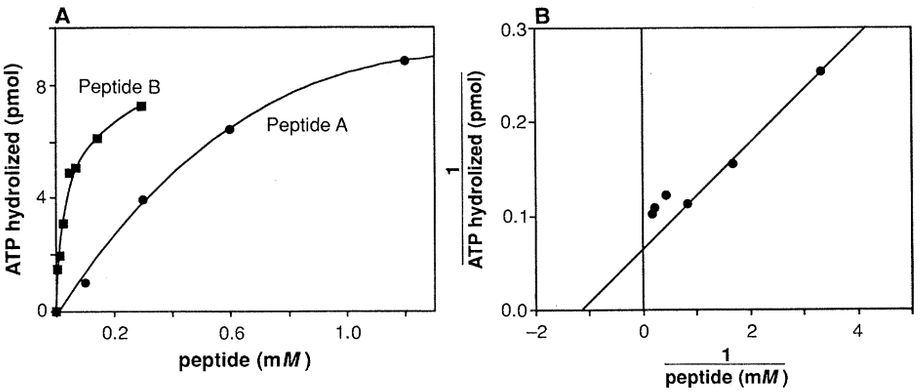

Fig. 7. Peptide-dependent ATPase activity of BiP. Purified BiP (0.8 μg) was incubated in buffer F [20 mM Hepes (pH 7.0), 20 mM NaCl, 2 mM $MgCl_2$, and 1 percent sodium cholate] with 8 μM ATP, 1 μCi of [^{3}H]ATP, and the indicated concentration of peptide A or peptide B for 10 minutes at 37°C in a 20-μl reaction. For these ATPase assays the synthetic peptides were further purified by gel filtration (*42*). The reaction was terminated by applying 1 μl of sample to polyethyleneimine cellulose thin-layer chromatography plates that had been spotted with carrier nucleotides (0.5 μl each of 10 mM ATP, ADP, and AMP). Chromatography was performed in 0.7M LiCl–1M HCOOH, and the plates were dried. The ATP and ADP spots were located with the aid of an ultraviolet light, excised, and counted (*26, 43*). The radioactivity in each spot was expressed as a fraction of the total recovered in each lane. The peptide-independent hydrolysis of ATP (2.6 pmole per 10-minute assay) was subtracted from each curve. (**A**) ATP hydrolysis as a function of peptide concentration. (○) Peptide A, (■) peptide B. (**B**) Double reciprocal plot for peptide A.

interact with many if not most suitably (see below) exposed segments of any protein. These hsp70 members could bind to nascent polypeptide chains prior to folding, or to partially denatured or incompletely assembled proteins in which only a limited internal segment of polypeptide chain is exposed. That hydrophilic and charged peptides are substrates (in contrast to a common view that "heat shock" proteins necessarily interact with hydrophobic domains (*5*)) means that BiP and hsc70 can in principle bind to the kinds of peptide segments that are usually on the outer surfaces of folded proteins.

Binding energy could be used in any of a number of ways, depending on the design of the protein substrate and the placement of sequences that preferentially interact with BiP or hsc70. For example, BiP or hsc70 could bind the polypeptide so as to retain it in an imcompletely folded form competent for translocation across membranes. For a differently structured polypeptide, binding to an hsp70 might occur in a conformer that favors folding or oligomerization on release. Other polypeptide substrates might be designed so that the energy of binding of an exposed segment to an hsc70 is used to unfold the protein as a whole, allowing refolding or disaggregation on subsequent release. An appropriate peptide segment that binds hsp70 might even become exposed in a programmatic fashion when subunits of a structure are polymerized with the binding energy being used for release of protomers. This could explain the use of hsc70 in uncoating clathrin coated vesicles.

The strikingly slow turnover time of the ATPase of hsp70 members (about 0.2 per minute) may be important in tuning the activity of these proteins to the rates of corresponding cellular processes. For example, in an animal cell it takes about a minute or so for a protein to be synthesized or to be cotranslationally translocated. If cycles of binding and release were to occur much faster, the hsp70 members would be ineffective; if associations were more permanent, such processes would be impeded.

In that catalysts of protein assembly like BiP and hsc70 must somehow distinguish correctly folded from improperly or incompletely assembled proteins, they would seem to embody a relevant definition of what successful folding actually consists of. Appreciation of what such proteins recognize would thus help in understanding protein folding in cells. Interaction of the many randomly chosen peptides with BiP suggests that steric accessibility of peptide segments is likely to be the limiting factor in preventing interactions in folded proteins. Peturbations in structure resulting in sufficient (even if transient) exposure of peptide segments to allow binding could constitute the cellular definition of an "unfolded" state, triggering the action of an hsp70 family member. A non-native state

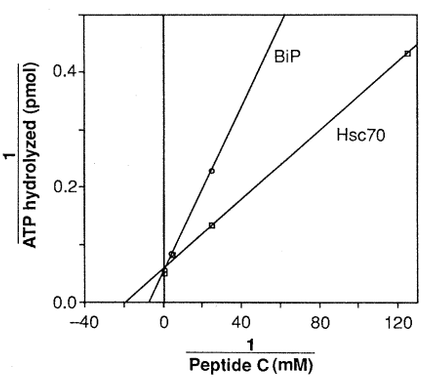

Fig. 8. Comparison of peptide C–dependent ATPase activity of BiP and hsc70. The reaction was performed as described in the legend to Fig. 7, except that 0.6 μg of hsc70 (□) and BiP (○) was used. No cholate was present in either sample. The hsc-70 was purified according to Scholossman *et al.* (*13*).

would then be defined as that in which one or more potentially interacting peptide segments are accessible, and whose stability is exceeded by the energy made available by interaction with BiP or similar proteins (*23*). In this regard, the free energy stabilizing the entire structure of many folded proteins (5 to 15 kcal/mole) is in the range of that stored in a single molecule of ATP (*29*). Despite the promiscuity of their peptide binding sites, BiP and hsc70 exhibit marked sequence preferences; for example, the K_m's of BiP for various peptides differ by a factor of at least 10^3.

How do the K_m values at hand compare with the concentrations of possible protein substrates in cells? A lower limit on sequence specificity could reduce constraints on protein structure. Certain loops or ends could consist of sequences that do not interact well to prevent spurious binding to surfaces of folded proteins; particularly effective binding sequences could serve as "signals" to guide interactions with hsp70 members, determining the manner and location in which the binding energy will be used. The steady-state concentration of growing polypeptide chains in the cytoplasm (one potential substrate or source of substrates for hsc70) can be estimated as follows. With a doubling time of 10 hours, about 0.2 percent of cellular protein is synthesized per minute. If it takes about 1 minute to make a protein, then about 0.2 percent of cytoplasmic protein in nascent. For a 30 percent solution of protein (an average size of 50 kD) in the cytoplasm (that is, a total protein concentration of about 5 mM) this corresponds to about 10 μM nascent protein. The steady-state concentration of freshly synthesized secretory protein in the lumen of the rough ER (substrates or potential substrates for BiP) can be crudely estimated as follows: The average number of ribosomes bound to the rough endoplasmic reticulum (RER) for an individual hepatocyte has been measured to be 13×10^6 (*30*). Since each bound ribosome bears a nascent polypeptide chain being

inserted through the membrane, the concentration of nascent chains can be estimated by dividing the moles of bound ribosomes by the volume of RER per hepatocyte. Using 470×10^{-15} liter as the calculated volume of the RER (*30*), we obtain 50 μM nascent polypeptide. These represent minimum values for potential BiP and hsc70 substrates since these calculations assume only a single binding site per polypeptide and do not include the pool of not yet assembled but already synthesized chains.

Further and detailed analysis of the interactions of members of the hsc70 family with a variety of peptide substrates and nucleotides can be expected to offer insights into the mechanism of action of these proteins. These studies should now be possible with the kind of simple enzyme system that we have described. Productive interaction of hsp70's with short peptides of variable length already suggests that these act in folding reactions as "single strand" polypeptide chain binding proteins as envisioned (*23*). The approach we have taken in using simple peptides as substrate analogs might also be useful in elucidating the mechanism and specificity of other catalysts of assembly, such as the groEL family of proteins.

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24. Salt-washed microsomes containing 5 g of protein were solubilized by the addition of equal volumes of buffer C [100 m*M* tris-HCl (*p*H 7.5), 100 m*M* NaCl, 10 m*M* Na_2EDTA, and 2 percent Triton X-100] and passed over a 250-ml DEAE-cellulose (Whatman) column that was equilibrated with buffer D [50 m*M* tris-HCl (*p*H 7.5), 50 m*M* NaCl, 5 m*M* Na_2EDTA, and 1 percent Triton X-100]. After being loaded with detergent-extracted microsomes, the column was first washed with five column volumes of buffer F and then with buffer D lacking Triton X-100 until the absorbance at 280 nm (A_{280}) was that of background. The BiP-containing fraction was then eluted with buffer E [50 m*M* tris-HCl (*p*H 7.5), 250 m*M* NaCl, and 5 m*M* Na_2EDTA]. The major peak, as determined by A_{280}, was concentrated in an Amicon pressure concentrator and loaded on a 10-ml ATP-agarose column (Sigma A 2767) equilibrated in buffer F [20 m*M* Hepes (*p*H 7.0), 25 m*M* KCl, 2 m*M* $MgCl_2$, 10 m*M* $(NH_4)_2SO_4$, and 1 percent sodium cholate]. The column was washed with buffer F, then buffer F containing 1*M* NaCl, each until the A_{280} approached background. The column was again equilibrated in buffer F, and BiP was eluted with 1 m*M* ATP in buffer F. The major protein peak was concentrated as before, then desalted over a Sephadex G-25 gel filtration column (Pharmacia) in buffer F to remove ATP. The purified protein was frozen and stored at −80°C.
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35. The postmitochondrial supernatant was initially centrifuged without a sucrose cushion, and the ribosomes were extracted with Na_2EDTA in the presence of 0.5*M* NaCl. Microsomes containing approximately 5 g of total protein were obtained from 500 g of bovine liver.
36. Amino acid divinylbenzene resins and *t* butyloxycarbonyl chloride L-amino acids were purchased from either Vega Biochemicals or Pennisula Laboratories. Peptides were synthesized by standard solid-phase techniques and released from the synthetic resin by treatment with hydrogen fluoride. Peptides were resuspended in 5 percent acetic acid and lyophilized. The resultant peptide acetate salts were used in further experiments. High-performance liquid chromatogrpahy (HPLC) analysis revealed purities of >90 percent. For binding studies, the peptide A was attached to Affigel 10 (Bio-Rad) (4 hours at 4°C in 100 m*M* Hepes, *p*H 7.5) with enough peptide to give a final concentration of 5 m*M* in the packed resin. Excess *N*-hydroxysuccinimide ester groups were neutralized with 50 m*M* tris-HCl, *p*H 7.5.
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40. After incubation with ATP, the equal portions of resin were incubated with 8*M* urea for 10 minutes at 25°C. Extracted material was precipitated with trichloroacetic acid and subjected to electrophoresis on 7.5 percent SDS-polyacrylamide gels. Coomassie-stained bands representing BiP were quantified by integration scanning (Bio-Rad laser densitometer). The fraction released was calculated as the ratio of BiP eluted with nucleotide to the BiP eluted with urea plus the BiP eluted with nucleotide.
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42. The crude peptide acetates (peptides A through C) were purified by gel permeation chromatography over a G-10 column in 10 percent acetic acid. The purified peptides were lyophilized, redissolved in water, and lyophilized again. After dissolving the purified peptide acetates in buffer F, the *p*H was readjusted (with NaOH) and the solution was clarified (15,000*g*, 15 minutes). The purified peptides stimulated the ATPase activity identically to their corresponding crude acetates.
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