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Unfolded Proteins Stimulate Molecular Chaperone Hsc70 ATPase by Accelerating ADP/ATP Exchange†

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ABSTRACT: The mammalian 70-kilodalton heat shock cognate protein (Hsc70) is an abundant, cytosolic molecular chaperone whose interactions with protein substrates are regulated by ATP hydrolysis. In vitro, purified Hsc70 was found to have a slow, intrinsic ATPase activity in the absence of protein substrates. The addition of an unfolded protein such as apocytochrome *c* stimulated ATP hydrolysis 2–3-fold. In contrast, the native holoprotein, cytochrome *c*, did not stimulate the ATPase rate, in accord with recent observations that 70-kilodalton heat shock proteins interact selectively with unfolded proteins. Stimulation of ATP hydrolysis by apocytochrome *c* was due to an increase in the V_{max}, with no effect on the K_m for ATP. Following hydrolysis of [^{3}H]ATP, a relatively stable [^{3}H]ADP·Hsc70 complex was formed. Release of [^{3}H]ADP from Hsc70 was most efficient in the presence of other nucleotides such as ADP or ATP, suggesting that ADP release occurs as an ADP/ATP exchange reaction. The loss of radiolabeled ADP from Hsc70 in the presence of exogenous nucleotides followed first-order kinetics. In the presence of nucleotides, apocytochrome *c* induced a 2-fold increase in the rate of ADP release from Hsc70. Moreover, rate constants of the nucleotide exchange reaction measured in the absence and presence of apocytochrome *c* (0.16 and 0.34 min^{-1}, respectively) closely matched the k_{cat} values derived from ATP hydrolysis measurements (0.15 and 0.38 min^{-1}, respectively). The results suggest that ADP release is a rate-limiting step in the Hsc70 ATPase reaction and that unfolded proteins stimulate ATP hydrolysis by accelerating the rate of ADP/ATP exchange.

Exposure of cells to hyperthermia, ethanol, heavy metals, amino acid analogues, or a variety of other perturbants typically elicits a cellular stress response characterized by an increase in the synthesis of several evolutionarily conserved polypeptides known as the heat shock proteins [reviewed in Morimoto et al. (1990) and Nover (1991)]. Heat shock protein induction occurs when stressors cause cells to accumulate unfolded or abnormal proteins (Kozutsumi et al., 1988; Edington et al., 1989; Parsell & Sauer, 1989), damage that has been termed "proteotoxicity" (Hightower, 1991). In eukaryotes, inducible heat shock proteins are members of protein families that also contain constitutively expressed homologues known as cognates (Hightower & White, 1981; Ingolia & Craig, 1982).

Heat shock proteins and their cognates, under normal conditions as well as during stress, function as molecular chaperones to increase the efficiency of protein folding, assembly, and disassembly [reviewed by Ellis and Hemmingsen (1989), Ang et al. (1991), and Gething and Sambrook (1992)]. A family of 70-kilodalton (kDa)[1] heat shock proteins is among the most prominent and well-characterized molecular chaperones. This protein family includes DnaK in *Escherichia coli* and several eukaryotic proteins that differ in their regulation and intracellular locale; Hsc70 and Hsp70 are nuclear/cytosolic, Grp78/BiP resides in the lumen of the endoplasmic reticulum, and mitochondrial Hsp70 resides in the mitochondrial matrix. Members of the DnaK/Hsc70 family, such as the constitutively expressed Hsc70 protein, display a weak ATPase activity and associate with a variety of unfolded protein substrates. Hsc70 binds transiently to nascent polypeptide chains (Beckmann et al., 1990) and facilitates translocation of certain proteins across organellar membranes (Deshaies et al., 1988; Chirico et al., 1988). Hsc70 also binds to diverse, synthetic peptides (Flynn et al., 1989; Vanbuskirk et al., 1989; DeLuca-Flaherty et al., 1990), to unfolded proteins such as apocytochrome *c* (Sadis et al., 1990a) and reduced, carboxymethylated α-lactalbumin (Palleros et al., 1991), and to mutant p53 proteins (Hinds et al., 1987; Clarke et al., 1988). Members of the DnaK/Hsc70 family catalyze the dissociation of protein complexes in a Mg-ATP-dependent manner. For example, Hsc70 dissociates clathrin triskelions from clathrin-coated vesicles (Rothman & Schmid, 1986), and DnaK dissociates phage P1 repA·DnaJ complexes (Wickner et al., 1991) and λP protein from the protein complex assembled at ori λ (McMacken et al., 1987; Zylicz et al., 1989). DnaK also solubilizes and reactivates denatured aggregates of RNA polymerase (Skowyra et al., 1990) and DnaA protein (Hwang et al., 1990). In addition to catalyzing protein dissociation, Hsc70 promotes the assembly of the progesterone receptor in an ATP-dependent manner (Smith et al., 1992).

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[1] Abbreviations: apo *c*, apocytochrome *c*; ADP, adenosine 5′-diphosphate; AMP, adenosine 5′-monophosphate; ATP, adenosine 5′-triphosphate; BSA, bovine serum albumin; DEAE, diethylaminoethyl; DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid; Grp78/BiP, 78-kilodalton glucose-regulated protein/immunoglobulin heavy-chain binding protein; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; Hsc70, 70-kilodalton heat shock cognate protein; Hsp70, 70-kilodalton heat shock protein; k_{cat}, catalytic constant or molecular turnover number; K_m, Michaelis constant; PEI, poly(ethylenimine); TLC, thin-layer chromatography; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride; UV, ultraviolet, V_{max}, maximum velocity; kDa, kilodalton(s).

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Biochemical characterization of the 70-kDa heat shock proteins suggests that one role of ATP hydrolysis may be to regulate cycles of polypeptide binding and release. Protein complexes containing 70-kDa heat shock proteins rapidly dissociate in the presence of ATP [reviewed by Pelham (1990)]. Recently, Palleros et al. (1991) monitored complex formation between Hsc70 and reduced, carboxymethylated α-lactalbumin by gel filtration high-pressure liquid chromatography and report that the presence of ADP promotes complex formation whereas ATP disrupts this interaction. Palleros et al. (1991) also tentatively identified a ternary complex of Hsc70, ADP, and reduced, carboxymethylated α-lactalbumin. Liberek et al. (1991b) have demonstrated that hydrolysis of nucleotide triphosphates causes a conformational change in DnaK as evidenced by an altered sensitivity to trypsin digestion. The ability of various nucleotides to serve as hydrolytic substrates correlates with those that induce the release of unfolded bovine pancreatic trypsin inhibitor, suggesting that a conformational change triggered by nucleotide hydrolysis releases the bound protein substrate. These results suggest that 70-kDa heat shock proteins may form stable complexes with unfolded proteins when they contain bound ADP. The exchange of ADP for ATP, and its subsequent hydrolysis, induces a conformational change in the heat shock protein that displaces the bound protein substrate.

Hsc70 is typically purified as a dimer (our unpublished observations; Guidon & Hightower, 1986; Kelley & Schlesinger, 1982) or as a mixture of monomers and dimers (Palleros et al., 1991; Schmid et al., 1985). ATP hydrolysis has also been reported to dissociate Hsc70 dimers to monomers (Schmid et al., 1985), although the role of this reaction in Hsc70 function is unclear.

In order to determine the rate-limiting step in this cycle, we have analyzed the Hsc70 ATPase in the absence and presence of apocytochrome *c* (apo *c*), an unfolded protein substrate. Hsc70 had an intrinsic ATPase activity that was stimulated 2–3-fold in the presence of apo *c*. The presence of the native holoprotein, cytochrome *c*, did not stimulate the Hsc70 ATPase. Apo *c* specifically increased the V_{max} of the ATPase reaction, with no effect on the K_m for ATP. Release of ADP from Hsc70 occurred through a nucleotide exchange reaction that was stimulated by apo *c*. The rate constants for the dissociation of ADP in the absence and presence of apo *c* matched the k_{cat} values of the ATP hydrolysis reaction, suggesting that ADP/ATP exchange is the rate-limiting step in the overall hydrolysis reaction.

MATERIALS AND METHODS

Proteins. Apo *c* was prepared from horse heart cytochrome *c* (Sigma Chemical Co.) based on the method of Fisher et al. (1973) as modified by Morimoto et al. (1983). Apo *c* concentration was measured with the Lowry assay using bovine serum albumin (BSA) as the standard. Hsc70 was purified from bovine brain as previously described (Sadis et al., 1990b) using DEAE-cellulose chromatography, ATP–agarose affinity chromatography, and FPLC anion-exchange chromatography on Mono Q. Hsc70 preparations were greater than 95% pure as judged by laser scanning densitometry of one-dimensional polyacrylamide gels stained with silver. Hsc70 concentrations were determined by the absorbance at 280 nm ($\Sigma_{1.0mg/mL}$ = 0.54; Sadis et al., 1990b).

ATPase Assays. ATP hydrolysis catalyzed by Hsc70 was assayed by measuring the release of [^{32}P]P_i from [γ-^{32}P]ATP (Bais, 1975; Seals et al., 1978). Assays (50 μL in volume) were assembled on ice and typically contained 2.5 μg of Hsc70 (0.36 μM dimer), 50 μM ATP, 20 μg of protein substrate (34.3 μM apo *c*), and 2 μCi of [γ-^{32}P]ATP (>3000 Ci/mM) in buffer C [20 mM Hepes–KOH, pH 7.0, 25 mM KCl, 10 mM $(NH_4)SO_4$, 2 mM magnesium acetate, 0.1 mM EDTA, and 1 mM DTT]. To determine the background release of radioactivity into the supernatant prior to incubation, an 8-μL aliquot was taken from each assay and added to 0.5 mL of a suspension containing 50 mM HCl, 5 mM H_3PO_4, and 7% (w/v) activated charcoal. Control experiments showed that, once the sample was added to the acidified charcoal suspension, no significant hydrolysis of the [γ-^{32}P]ATP occurred during a subsequent 1-h incubation at room temperature. After the background sample was taken, the samples were immediately transferred from ice to a heating block filled with H_2O adjusted to 37 °C. Four additional 8-μL aliquots were removed at timed intervals and quenched with the acidified charcoal suspension. The samples were mixed and subjected to centrifugation in a swinging-bucket rotor in an IEC centrifuge at 4000 rpm for 20 min at 20 °C. Two 100-μL aliquots were withdrawn and transferred to scintillation vials, and the radioactivity present was determined by liquid scintillation counting. The cpm values were converted to picomoles of P_i released into the supernatant at each time point, and the data were analyzed with the Cricket Graph software package on a Macintosh personal computer. A least-squares analysis produced a best-fit straight line of the data along with the slope, which represents the rate of the reaction in picomoles per minute. Rate data were corrected for the micrograms of enzyme present to give the specific ATPase activity value of picomoles of ATP hydrolyzed per minute per micrograms of Hsc70.

ADP Release Assays. One hundred micrograms of Hsc70 was combined with 5 μCi of [^{3}H]ATP (20 μM) in 100 μL of buffer C and incubated on ice for 60 min. The sample was applied to a small, disposable desalting column containing Sephadex G-50 (Pharmacia NICK columns) equilibrated in buffer C, and ovalbumin (0.1 mg/mL) was added to reduce nonspecific absorption of Hsc70. The sample was applied to the glass frit at the top of the gel bed and allowed to enter the column. Two milliliters of buffer C containing ovalbumin (0.1 mg/mL) immediately followed the sample, and the first 10 fractions (90–100 μL each) were collected into scintillation vials. The leading peak of radioactivity, representing mainly [^{3}H]ADP bound to Hsc70, was identified by liquid scintillation counting of 2-μL aliquots from each column fraction. The pooled fractions were held on ice. Aliquots of the pooled fractions were combined with nucleotides and/or apo *c* and then immediately incubated at 37 °C. Portions of this sample were removed at timed intervals and fractionated by rapid gel filtration through a desalting column. Ten fractions (90–100 μL) were collected, and the radioactivity that remained bound to Hsc70 was determined by liquid scintillation counting. The cpm data were converted to picomoles of [^{3}H]ADP using a standard curve relating cpm to microcuries which was prepared by liquid scintillation counting of known quantities of [^{3}H]-ATP. Best-fit straight lines of the data plotted on a semilog graph were calculated by the exponential function of the Cricket Graph software graphics program. The slopes of the straight lines were used to calculate the half-life of the Hsc70–[^{3}H]ADP complex, $t/2 = (0.3010)(\text{slope}^{-1})$. The half-life measurements were then used to calculate the rate constants, $k = (0.693)(t/2)^{-1}$.

Thin-Layer Chromatography. Thin-layer chromatography (TLC) on poly(ethylenimine)(PEI)–cellulose was performed

to evaluate the phosphorylation state of the ^{3}H-nucleotide bound to Hsc70. Ten micrograms of Hsc70 was combined with 2 μCi of [^{3}H]ATP (20 μM) in 100 μL of buffer C and incubated on ice for 60 min. A 3-μL aliquot was removed and held on ice. The remaining sample was applied to a desalting column as described above, and 10 2-drop fractions were collected and placed on ice. The first major peak of radioactivity, present in fractions 5–7, was pooled. A 3-μL aliquot of the column-purified sample was removed. This aliquot and the aliquot removed earlier, along with 0.5 μL of [^{3}H]ATP (0.1 μCi/μL), were each separately combined with 2 μL of a solution containing 3.33 mM each of ATP, ADP, and AMP. These three samples were spotted onto a TLC plate which was then developed in 0.75 M Tris-HCl, pH 8.0. The plate was dried and visualized under UV to determine the positions of the adenine nucleotide standards. One-centimeter sections of each lane were removed, and the radioactivity present in each section was determined by liquid scintillation counting.

RESULTS

We initially tested the ability of Hsc70 to activate ATP hydrolysis in response to a variety of proteins, such as BSA and ovalbumin, that were denatured by heating to 95 °C. Proteins denatured in this way did not stimulate the Hsc70 ATPase. We reasoned that the hydrophobic aggregates produced under such extreme conditions could not serve as substrates for Hsc70. We sought instead a natural polypeptide ligand that existed in solution as an unfolded protein under isotonic salt and neutral pH conditions. A candidate protein having these properties is apo *c*, the cytosolic precursor to the heme-containing mitochondrial protein cytochrome *c*. Apo *c*, unlike other cytosolic precursors of imported mitochondrial proteins, does not contain a cleavable presequence (Matsuura et al., 1981). Removal of the heme ligand from cytochrome *c* regenerates the translocation-competent, apo form of the protein (Veloso et al., 1981). Circular dichroism spectra obtained for cytochrome *c* clearly indicate a large fraction of α-helix in the native protein, whereas the spectra obtained for apo *c* indicate an aperiodic, random-coil-like structure (Fisher et al., 1973). Therefore, apo *c* seemed like an ideal protein substrate to serve as a model for Hsc-70 interactions with unfolded proteins.

In a previous report, we observed binding of apo *c* to Hsc70 using native gel assays (Sadis et al., 1990a). To test whether apo *c* was also capable of stimulating the Hsc70 ATPase, we measured ATP hydrolysis catalyzed by Hsc70 in the absence and presence of this polypeptide. Results from a typical experiment that measured the release of [^{32}P]P_i from [γ-^{32}P]-ATP at 37 °C in buffer C (see Materials and Methods) are shown in Figure 1. Hsc70 catalyzed ATP hydrolysis at a slow but linear rate in the absence of any other polypeptide substrate. The sensitivity of our assay was such that this intrinsic rate of hydrolysis could be easily measured during a 10-min incubation. The specific activity of the intrinsic Hsc70 ATPase measured in this particular experiment was 1.26 pmol min^{-1} μg^{-1}. This value varied from 0.9 to 1.3 pmol min^{-1} μg^{-1} with different Hsc70 preparations. The addition of apo *c* at a concentration of 400 μg/mL (34.3 μM) to the assay containing Hsc70 increased the specific ATPase activity to 2.23 pmol min^{-1} μg^{-1}. In contrast, the addition of an equivalent amount of cytochrome *c* had no effect on the rate of ATP hydrolysis catalyzed by Hsc70. Control experiments were performed to measure the background rate of ATP hydrolysis occurring in the absence of Hsc70 and in the presence of the polypeptide

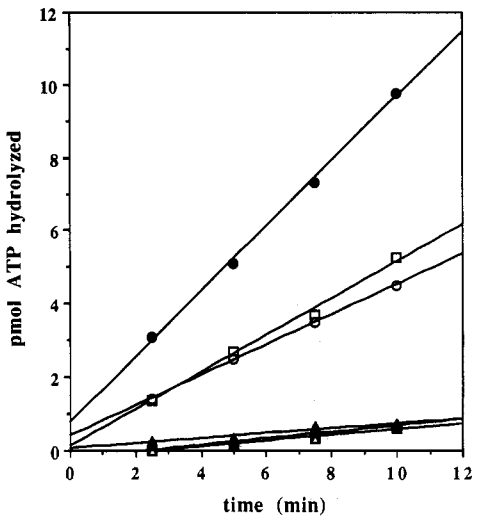

FIGURE 1: Stimulation of an intrinsic ATPase activity of Hsc70 by unfolded proteins. ATPase activity was measured in reactions containing 50 μM ATP, 2 μCi of [γ-^{32}P]ATP, and 2.5 μg of Hsc70 in buffer C and was performed at 37 °C. Polypeptide substrates [cytochrome *c* (open circles) or apo *c* (solid circles)] were added to a final concentration of 400 μg/mL or were omitted to measure the intrinsic rate of ATPase activity by Hsc70 alone (open squares). Spontaneous ATP hydrolysis in the absence of Hsc70 was measured without polypeptide substrates (solid squares) and in the presence of cytochrome *c* (open triangles, 400 μg/mL) or apo *c* (solid triangles, 400 μg/mL). Aliquots of the reaction were removed at the times indicated and quenched in an acidified suspension of activated charcoal, which served to bind unhydrolyzed nucleotide. The amount of [^{32}P]P_i released into the supernatant was determined by liquid scintillation counting. The cpm data were converted to picomoles after correction for background release of radioactivity into the supernatant, counting efficiency, and the specific activity of [γ-^{32}P]-ATP. Best-fit straight lines were drawn by the Cricket Graph software package. Additional experimental details are provided under Materials and Methods. All other [γ-^{32}P]ATP hydrolysis experiments presented herein were performed similarly.

substrates. The results, also shown in Figure 1, revealed that spontaneous ATP hydrolysis under these conditions was extremely slow. It was clear that the apo *c* preparation was not contaminated by an artifactual ATPase activity that could explain our results. Therefore, the evidence suggests that purified Hsc70 has an intrinsic ATPase activity that is specifically stimulated by unfolded proteins.

We tested the concentration dependence of apo *c* stimulation of the Hsc70 ATPase. The results are shown in Figure 2. Significant stimulation over the intrinsic rate of ATP hydrolysis was observed at apo *c* concentrations as low as 50–100 μg/mL. Increasing amounts of apo *c* provided greater stimulation of the Hsc70 ATPase until near-maximal levels of ATP hydrolysis were observed between 400 and 600 μg/mL. This concentration of apo *c* represents a 100–150-fold molar excess to the concentration of Hsc70 dimer in the reaction. Over the course of several experiments, we noted some variability in the potential of different apo *c* preparations to stimulate the Hsc70 ATPase. This may be related to oxidative damage or perhaps the tendency of apo *c* to self-aggregate when incubated for extended periods in solution or after multiple freeze–thaw cycles. The data discussed in this work were obtained with freshly prepared apo *c* and represent a minimal estimate of the ability of this polypeptide to stimulate the Hsc70 ATPase.

The data in Figure 2 were analyzed by inverse plots to determine whether stimulation of the Hsc70 ATPase by apo *c* followed Michaelis–Menten kinetics. These plots were slightly nonlinear, especially at low apo *c* concentrations (data not shown).

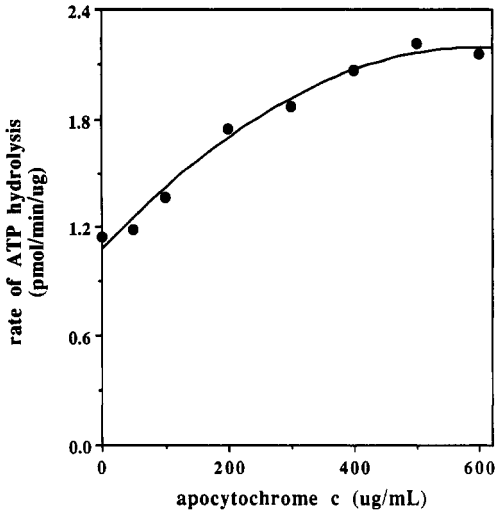

FIGURE 2: Concentration dependence of apo *c*'s effect on the Hsc70 ATPase. The rate of ATP hydrolysis (picomoles per minute) at varying apo *c* concentrations was determined and corrected for Hsc70 concentration to give the specific ATPase activity in picomoles per minute per microgram. See the legend to Figure 1 and Materials and Methods for experimental details.

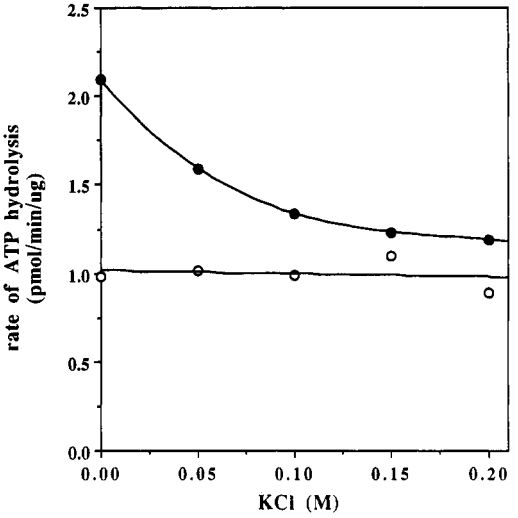

FIGURE 3: Effect of KCl concentration on intrinsic and stimulated ATP hydrolysis. The intrinsic ATPase rate was measured in the absence of polypeptide substrate (open circles); the stimulated ATPase rate was measured in the presence of apo *c* (solid circles, 400 μg/mL). Additional KCl at the concentrations indicated was added to assays that already contained 25 mM KCl as part of buffer C. See the legend to Figure 1 and Materials and Methods for experimental details.

Next we characterized the effects of pH and ionic concentration on the Hsc70 ATPase. Similar to results obtained in the presence of clathrin cages (Braell et al., 1984), we found that the intrinsic Hsc70 ATPase was most active at pH 5.5–7.0 (data not shown). The presence of apo *c* simply raised the ATPase activity throughout the pH range where the Hsc70 ATPase was active. In Figure 3, the effect of increasing KCl concentration on ATPase activity is shown. We found that relatively modest increases in KCl concentration progressively inhibited apo *c*'s ability to stimulate the Hsc70 ATPase. At 0.05 M added KCl, only about half the normal stimulation of ATPase activity was obtained. At 0.2 M added KCl, the ATPase activity was reduced to about 25% of the control activity. However, there was no effect of added KCl (0–0.2 M) on the intrinsic ATPase activity of Hsc70. Similar concentrations of KCl also inhibit the ability of clathrin cages to stimulate the Hsc70 ATPase (Braell et al., 1984). The

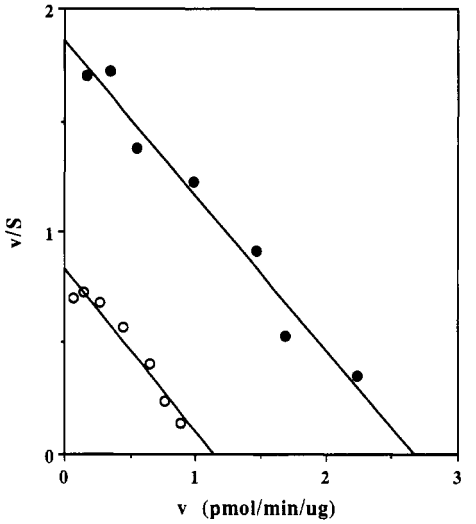

FIGURE 4: Effect of ATP concentration on the intrinsic and stimulated ATPase rates. The specific ATPase activity of Hsc70 was determined at varying ATP concentrations in the absence of protein substrate (open circles) or in the presence of apo *c* (solid circles, 400 μg/mL). The data were transformed according to the Eadie–Hofstee method where the rate (picomoles, per minute per microgram) divided by ATP concentration was plotted against the rate. See the legend to Figure 1 and Materials and Methods for experimental details.

ability of increasing ionic strength to discriminate between the intrinsic and the stimulated ATPase activities of Hsc70, along with other observations discussed later, suggested to us that the intrinsic ATPase activity we have characterized was not due to a small amount of denatured protein in our Hsc70 preparation that was activating a normally "silent" enzyme. If this were the case, we could reasonably expect KCl to inhibit this route of stimulation as well.

We investigated the mechanism by which polypeptide substrates stimulate the Hsc70 ATPase. First, the dependence of the ATP hydrolysis rate on ATP concentration was evaluated. Increasing ATP concentration up to 1–2 μM caused a steady increase in the rate of ATP hydrolysis obtained both in the absence and in the presence of apo *c*. There was a marked reduction in further increase for both the intrinsic and stimulated ATPase rates above 1–2 μM ATP. It was apparent from the rate versus [substrate] curves that the main effect of apo *c* was on the V_{max} of the ATP hydrolysis reaction. This interpretation was confirmed by plotting the data according to the Eadie–Hofstee transformation (Figure 4). The results indicated that the ATPase reaction followed Michaelis–Menten kinetics when ATP concentrations were varied, as shown previously (Braell et al., 1984). The V_{max} values, obtained from the *x*-intercept, were 1.14 pmol min^{-1} μg^{-1} for the intrinsic ATPase rate and 2.67 pmol min^{-1} μg^{-1} in the presence of apo *c* (400 μg/mL). The molecular turnover numbers (k_{cat}), derived from the V_{max} values, were 0.15 and 0.38 molecules of ATP min^{-1} (Hsc70 dimer)$^{-1}$ for the intrinsic and stimulated rates, respectively. In contrast to the differences in V_{max}, the K_m values ATP obtained in the absence and presence of apo *c* were 1.37 and 1.44 μM, respectively. As the K_m value for the intrinsic ATP hydrolysis rate is more than a thousandfold lower than the cytosolic ATP concentration (≈5 mM), substrate availability is not likely to be rate-limiting in the Hsc70 ATPase reaction in vivo.

We tested whether a stable complex of Hsc70 and ADP formed after ATP hydrolysis. [^{3}H]ATP was incubated with Hsc70 on ice and then passed over a small desalting column containing Sephadex G-50 to separate ^{3}H-nucleotide bound to Hsc70 from free ^{3}H-nucleotide. [^{3}H]ATP and aliquots of

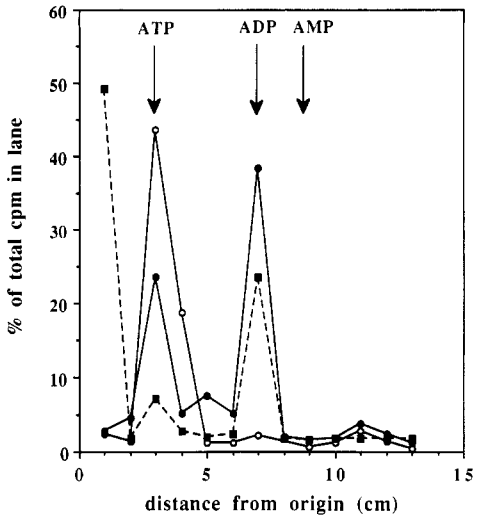

FIGURE 5: Thin-layer chromatography analysis of [^{3}H]ATP binding to Hsc70. Hsc70 was combined with 2 μCi of [^{3}H]ATP, incubated on ice for 60 min, and fractionated with a rapid desalting column. Aliquots were taken before fractionation (solid circles, solid line) and from the column void volume (solid squares, dashed line) and were combined with 2 μL of a solution containing 3.33 mM each of AMP, ADP, and ATP. A 0.5-μL aliquot of 0.1 μCi/μL [^{3}H]ATP was also combined with the AMP, ADP, and ATP mix (open circles, solid line). The three samples were spotted onto a PEI–cellulose plate and developed in 0.75 M Tris-HCl, pH 8.0. Adenine nucleotide standards present in each lane were visualized under UV light. Each lane was divided into 1-cm sections that were processed by liquid scintillation counting. The amount of radioactivity in each section is expressed as a percentage of the total radioactivity in each lane.

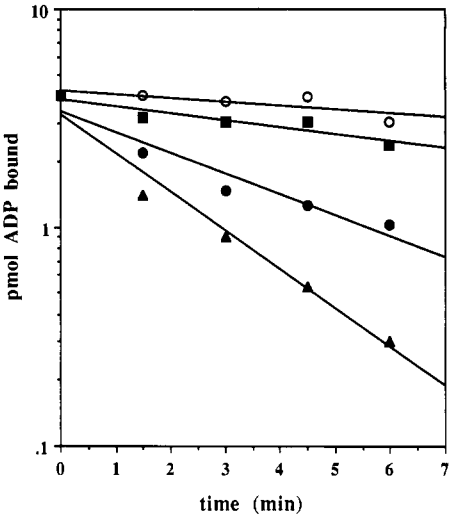

FIGURE 6: Effect of apocytochrome *c* and ATP on the rate of ADP release from Hsc70. Hsc70 was combined with [^{3}H]ATP and incubated on ice for 60 min. The sample was passed over a desalting column, and the void volume, containing [^{3}H]ADP bound to Hsc70, was collected. The solution of Hsc70·[^{3}H]ADP complex was divided into four aliquots, which received no addition (open circles), apo *c* (closed squares, 400 μg/mL), 100 μM ATP (closed circles), or both apo *c* and ATP (closed triangles). The samples were incubated at 37 °C, and aliquots were removed at 1.5-min intervals and applied to rapid desalting columns to measure the amount of [^{3}H]ADP remaining bound to Hsc70.

Hsc70 combined with [^{3}H]ATP taken before and after application to the desalting column were analyzed by thin-layer chromatography on poly(ethylenimine)–cellulose. The results, depicted in Figure 5, show the distribution of radioactivity in each lane of a PEI–cellulose plate relative to ATP, ADP, and AMP standards whose positions were determined by UV illumination. As expected, the radioactivity present in the [^{3}H]ATP sample incubated in the absence of Hsc70 comigrated almost exclusively with the ATP standard, indicating that little spontaneous hydrolysis of ATP occurs under these conditions. However, incubation of [^{3}H]ATP in the presence of Hsc70 led to the conversion of a major fraction of [^{3}H]ATP to [^{3}H]ADP. Analysis of the reaction after passage through the desalting column showed that most of the ^{3}H-nucleotide associated with Hsc70 had been converted to ADP. We concluded that Hsc70 bound and slowly hydrolyzed [^{3}H]ATP to [^{3}H]ADP during prolonged incubation on ice or during passage through the desalting column at room temperature. Furthermore, the [^{3}H]ADP was stably bound to Hsc70 as the radioactive nucleotide remained associated with Hsc70 after gel filtration. Therefore, the release of bound ADP may be a slow, possibly rate-limiting step in the Hsc70 ATPase reaction cycle.

To test whether apo *c* might stimulate the rate of ADP release, Hsc70 was combined with [^{3}H]ATP and incubated on ice to allow for binding, and the resulting [^{3}H]ADP·Hsc70 complex was isolated from the void volume of a desalting column. This complex was incubated at 37 °C in the absence and presence of ATP and/or apo *c*. Aliquots were removed during the course of the incubation, and the [^{3}H]ADP that remained bound to Hsc70 was determined by subjecting the aliquot to another around of rapid desalting. Liquid scintillation counting of the second void volume was performed to determine the amount of [^{3}H]ADP that remained bound to Hsc70. The results are shown in Figure 6. In each case, the half-life and the first-order rate constant of the decay of the [^{3}H]ADP·Hsc70 complex were derived from the slope of data plotted on a semi-log graph. In the absence of added polypeptide substrate or nucleotide, the [^{3}H]ADP bound to Hsc70 had a half-life of approximately 13 min at 37 °C (k = 0.053 min^{-1}). The addition of apo *c* in the absence of exogenous nucleotide increased the rate of ADP release only slightly, resulting in a half-life of 12 min (k = 0.058 min^{-1}). The decay of the [^{3}H]ADP·Hsc70 complex was substantially increased by the addition of 100 μM ATP, which generated a half-life of 4.2 min (k = 0.16 min^{-1}). ADP (100 μM) was also effective at stimulating the release of [^{3}H]ADP from Hsc70 (data not shown). Thus, ADP release from Hsc70 occurs most efficiently in the presence of other nucleotides, indicating that release normally occurs as part of a nucleotide exchange reaction. A synergistic effect was observed in the presence of both ATP and apo *c* as the [^{3}H]ADP·Hsc70 complex decayed with a half-life of 2.0 min (k = 0.34 min^{-1}). Thus, apo *c* induced a 2-fold increase in the rate of the nucleotide exchange reaction carried out by Hsc70, similar to its effect on the overall ATPase rate.

We noted that the rate constants calculated from the decay of the [^{3}H]ADP·Hsc70 complex in the presence of ATP were similar to the turnover numbers calculated from the ATP hydrolysis measurements. The close similarity of these two sets of independently derived rate constants suggests that, under these conditions, it is primarily the rate of nucleotide exchange that limits ATP hydrolysis catalyzed by Hsc70. Binding of protein substrates activates this rate-limiting step, thereby increasing the overall ATP hydrolysis rate. A summary of the kinetic constants and other parameters determined in this study is provided in Table I.

DISCUSSION

Weak ATPase activities have been observed for two prominent families of molecular chaperones, the DnaK/Hsc70 family and the GroEL/Hsp60 family. In both cases, ATP

Table I: Summary of Kinetic Constants Determined for Hsc70

kinetic constant	addition to Hsc70			
	no addition	apo c^a	ATP^b	apo c + ATP^c
K_m (μM)			1.37	1.44
V_{max} (pmol min^{-1} μg^{-1})			1.14	2.67
k_{cat} (min^{-1})			0.15	0.38
$t/2$ of ADP release (min)	13	12	4.2	2.0
k of ADP release (min^{-1})	0.053	0.058	0.16	0.34

[a] Apo c concentration was 400 μg/mL. [b] ATP concentration was 50 μM in the ATP hydrolysis experiments performed to determine K_m, V_{max}, and k_{cat} and 100 μM in the ADP release experiments. [c] Apo c concentration was 400 μg/mL. ATP concentration was the same as listed for footnote b.

hydrolysis appears to regulate the binding and release of protein substrates. Our results show that Hsc70's ATPase activity is regulated to specifically respond to interactions with unfolded protein substrates by accelerating the rate-limiting step in the ATPase cycle, ADP/ATP exchange.

The stimulated ATPase rate we have measured in the presence of apo c (approximately 2.7 nmol min^{-1} mg^{-1}) is similar to the specific ATPase activities measured previously for Hsc70 in the presence of other protein substrates. Rothman and colleagues report specific ATPase activities for Hsc70 of 2.6 nmol min^{-1} mg^{-1} in the presence of clathrin cages and 2.9–3.0 nmol min^{-1} mg^{-1} in the presence of two synthetic peptides (Braell et al., 1984; Flynn et al., 1989). In addition, we calculate turnover numbers of 0.15 and 0.38 molecules of ATP min^{-1} (Hsc70 dimer)$^{-1}$ for the intrinsic and stimulated rates, respectively. The value for the stimulated rate is within the range [0.2–1.0 molecule of ATP min^{-1} (Hsc70 dimer)$^{-1}$] reported previously for Hsc70 in the presence of clathrin cages, clathrin light chains, and peptides (Braell et al., 1984; DeLuca-Flaherty et al., 1990; Flynn et al., 1989). The concentration of apo c required for this level of stimulation is low (34 μM) relative to previously studied substrates, suggesting that this polypeptide is more avidly bound by Hsc70. Overall, the results suggest that apo c is an appropriate model for further studies of Hsc70–protein substrate interactions.

Earlier studies discounted the existence of a protein substrate-independent ATPase activity associated with Hsc70, and, therefore, we considered whether the intrinsic ATPase activity we observed was due to a contaminant. This possibility is unlikely for the following reasons. First of all, protein substrate-independent ATPase activities have been previously characterized for other members of the DnaK/Hsc70 family, including Grp78/BiP and *E. coli* DnaK (Kassenbrock & Kelly, 1989; Zylicz & Georgopolous, 1984). In addition, the K_m values for ATP measured in the absence and presence of polypeptide substrate were nearly identical, consistent with the premise that Hsc70 catalyzes both the intrinsic and stimulated ATPase activities. Another possibility was that a low level of denatured protein in our Hsc70 preparations activated ATP hydrolysis. However, increasing ionic strength reduced the stimulated ATPase activity observed in the presence of apo c down to the level of the intrinsic rate. Interestingly, the ability of clathrin cages to stimulate the Hsc70 ATPase is similarly sensitive to ionic strength (Braell et al., 1984), suggesting that this effect may be general. In contrast, increasing ionic strength had no effect on the ATP hydrolysis rate in the absence of apo c, suggesting that protein substrates were not responsible for this activity. These observations suggest that the intrinsic ATPase activity has distinct regulatory properties and is not due to low levels of contaminating protein substrates.

We considered that the Hsc70 ATPase reaction was limited by the rate of catalysis or product release. It was possible to study the effect of protein substrates on product release since complexes of [^{3}H]ADP·Hsc70 were readily obtainable. When ligands were added separately, ADP release was significantly enhanced in the presence of ATP, but not in the presence of apo c, which suggested that ADP release occurs most efficiently as a nucleotide exchange reaction. However, in the presence of nucleotides, the addition of apo c provided a further 2-fold increase in the rate of ADP release. First-order rate constants for [^{3}H]ADP release (in the presence of ATP) in the absence and presence of apo c were similar to the K_{cat} values obtained from [γ-^{32}P]ATP hydrolysis measurements, suggesting that it is primarily the rate of ADP release that limits the Hsc70 ATPase reaction. Although we do not discount an effect of protein substrates on the catalysis step, our results suggest that the primary effect of protein substrates is on product release.

We investigated the limiting step in the Hsc70 ATPase reaction to identify a part of the reaction cycle that may be subject to regulation by factors other than protein substrates in vivo. A precedent for the existence of such regulatory factors comes from studies of DnaK, the Hsc70 homologue in *E. coli*. Genetic evidence indicates that DnaK functionally cooperates with two other proteins, DnaJ and GrpE [reviewed by Gross et al. (1990) and Georgopoulos et al. (1990)]. Recent biochemical evidence indicates that DnaJ and GrpE stimulate the ATPase activity of DnaK by individually affecting catalysis and ADP release, respectively (Liberek et al., 1991a). A family of proteins related to DnaJ has been identified in *Saccharomyces cerevisiae* (Caplan & Douglas, 1991; Luke et al., 1991), suggesting that regulators of the eukaryotic Hsc70 ATPase also exist in higher origanisms. Our finding that ADP release is primarily rate-limiting in the Hsc70 ATPase reaction suggests that functional homologues of GrpE may exist in eukaryotes as well and may be particularly important regulators of Hsc70 in eukaryotes.

Our present understanding of the role of ATP hydrolysis in the function of the DnaK/Hsc70 family suggests similarities with the well-characterized proteins of the GTPase superfamily, such as the heterotrimeric G proteins, EF-Tu, and p21^{ras}, which use nucleotide hydrolysis to regulate cycles of polypeptide binding and release and have regulatory proteins that accelerate the catalysis and product release steps [reviewed by Bourne et al. (1990, 1991)]. An interesting difference is that GTPases are activated to bind effector proteins when they contain bound nucleotide *tri*phosphate, whereas Hsc70 forms stable complexes with protein substrates when nucleotide *di*phosphate is bound (Pelham, 1990; Palleros et al., 1991). In addition to the GTPases, another enzyme with similar properties is the ATP-dependent protease La from *E. coli*. Protease La is a member of the *E. coli* heat shock regulon and has specificity for abnormal proteins. Interestingly, protein substrates for La activate ATP hydrolysis 2-4 fold, similar to our findings with Hsc70. In addition, activation occurs by increasing the V_{max}, not by reducing the K_m (Waxman & Goldberg, 1985). Finally, ADP is a potent inhibitor of La's proteolytic activity, and protein substrates induce the exchange of bound ADP for ATP (Menon & Goldberg, 1987).

The molecular mechanism by which polypeptide binding to Hsc70 stimulates nucleotide exchange is not understood. McKay and colleagues note that the topological similarity between hexokinase and the Hsc70 ATPase domain suggests the possibility that the ATPase domain may undergo substrate-induced conformational changes similar to what is observed

with hexokinase (Flaherty et al., 1990). Thus, according to this model, polypeptide binding at the C-terminal domain of Hsc70 may alter the conformation of the N-terminal ATPase domain. This change in conformation may induce accelerated ADP/ATP exchange on Hsc70, resulting in an overall increase in ATP hydrolysis.

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