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Interaction of hsp70 with unfolded proteins: Effects of temperature and nucleotides on the kinetics of binding

(heat shock proteins/thermal stability/nucleotide binding)

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ABSTRACT **Circular dichroism and HPLC gel filtration were used to show that cytosolic hsp70 is thermally stable but undergoes a conformational transition (midpoint, 43°C; 57°C in the presence of ATP or ADP) leading to oligomerization. hsp70 binds to unfolded, but not to folded, proteins in a temperature-dependent manner; complex formation is significant only at physiologically relevant temperatures. hsp70 binds ADP more tightly than ATP to form a binary complex, which binds to the unfolded protein more rapidly than free hsp70. ADP also inhibits the ATP-induced dissociation of the hsp70–protein complex. A regulatory role for the hsp70–nucleotide binary complexes is proposed.**

Recent studies indicate that protein folding and assembly events *in vivo* are mediated by intracellular components now being referred to as molecular chaperones (reviewed in refs. 1–3). One class of these chaperones is the 70-kDa heat shock protein, hsp70. The cytosolic forms of mammalian hsp70 are present in cells as two different gene products (4): a stress-inducible form, hsp72, and a constitutive member, hsp73, also known as hsc70. hsp70 family members have been shown to interact with a number of proteins undergoing maturation in the cell (5–8). In addition to these nascent events, it has been proposed that hsp70 proteins can recognize and bind to mature polypeptides that may have become unfolded in the cell (e.g., following heat shock) and thereby facilitate their refolding (9). To date relatively little experimental evidence has been provided to support this idea. There are ample data demonstrating that nascent polypeptides, unable to properly fold or assemble, remain bound to their particular hsp70 chaperone (5, 10, 11). *In vitro* studies have shown that hsp70 proteins interact with a variety of short synthetic polypeptides (12).

In the present study, we have utilized an *in vitro* system to examine the possible interaction of the mammalian hsp70 with both folded and unfolded polypeptides. We show that the cytosolic hsp73 will form stable complexes with a variety of unfolded protein targets, but not with their properly folded counterparts. Furthermore, we present data regarding the role of temperature and nucleotides in these interactions.

MATERIALS AND METHODS

Materials. Bovine brain hsp73 and human hsp72/73 from HeLa cells were isolated and purified as described (13). Reduced carboxymethylated α-lactalbumin (RCMLA), bovine α-lactalbumin (type I) (α-LA), and horse heart cytochrome *c* (type VI) were obtained from Sigma. Reduced carboxamidomethylated ribonuclease A was prepared as described (14). Staphyloccocal nuclease (SNase) was purified from a cloned system provided by D. Shortle (Johns Hopkins University). ATP (disodium salt) was from Pharmacia. ADP (monosodium salt), adenosine 5′-[γ-thio]triphosphate (ATPγS) (tetralithium salt) were from Calbiochem. [8-^{14}C]ADP (tetrasodium salt; 52 mCi/mmol; 1 Ci = 37 GBq) was from ICN. [α-^{32}P]ATP [tetra(triethylammonium) salt; 3000 Ci/mmol] was from NEN.

Methods. CD spectra were recorded on an Aviv Associates (Lakewood, NJ) model 60DS instrument. The hsp70 concentration was in the range 4–10 μM (as determined by absorbance; ε_{280} = 47,800 $M^{-1}\cdot cm^{-1}$). Molar ellipticity ([θ]) was calculated as described elsewhere (15). Gel-filtration chromatography was performed with a Beckman HPLC instrument using a Bio SEC-250 silica column (600 × 7.5 mm; Bio-Rad) at 22°C; 20 mM sodium phosphate buffer/0.20 M KCl, pH 6.5 (buffer A), was used as the mobile phase; the flow rate was 1 ml/min. Typical elution volumes for hsp73, dimer, trimer, hsp73 soluble aggregates, reduced carboxymethylated α-lactalbumin (RCMLA), and hsp73–RCMLA complex were 17.0, 14.4, 13.4, 10.6, 19.7, and 15.2 ml, respectively. Detection was by absorbance at 215 nm. All samples were centrifuged for 10 sec (14,000 rpm in an Eppendorf microcentrifuge) before injection on the HPLC. All other protein concentrations were determined from molar extinction coefficients. Liquid scintillation counting (LSC) was performed on a Beckman LS-230 instrument.

Protein binding experiments. Aliquots (20–25 μl) of hsp70 stock solution (7–12 μM) in buffer B (50 mM Tris·HCl/20 mM KCl, pH 7.5) were mixed with 2–4 μl of substrate protein stock solution (150–200 μM). The reaction mixtures were incubated (see figures for details) and injected on the HPLC instrument. RCMLA stock solution was made in HCl (pH 2). Staphylococcal nuclease (SNase) and NCA–SNase [the mutant NCA-S28G of SNase (16)] stock solutions were in 20 mM sodium cacodylate (pH 7.0). When the effects of nucleotides on protein binding were investigated, 1- to 2-μl aliquots of 10 mM nucleotide solutions in buffer A plus 10 mM $MgCl_2$ (pH 7.2) were added to the reaction mixture (see Fig. 5 for incubation times).

Nucleotide binding. The affinities of ATP and ADP for hsp73 were determined by equilibrium dialysis. [α-^{32}P]ATP solutions (10–100 μM; 9–70 nCi/μl in buffer A) were equilibrated with hsp73 solution (7.5 μM in buffer A) for 19 hr at 4°C using 100-μl equilibrium dialysis cells and 12- to 14-kDa cut-off membrane. Four 10-μl aliquots from each compartment were assayed by LSC. The readings were identical to those determined after 6 hr, indicating that equilibrium was attained. To account for ATP hydrolysis, 2-μl aliquots from each compartment were spotted on a polyethyleneimine-cellulose F TLC plate that had been spotted with carrier nucleotides (1 μl each of 10 mM ATP and ADP). The plate

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Abbreviations: ATP[γS], adenosine 5′-[γ-thio]triphosphate; LSC, liquid scintillation counting; RCMLA, reduced carboxymethylated α-lactalbumin; SNase, staphylococcal nuclease.

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was developed in 0.7 M LiCl/1 M HCOOH. ADP and ATP spots were visualized by UV light, scraped off, and assayed by LSC. This allowed the determination of the fraction of ATP and ADP present in each compartment.

ADP binding to hsp73–RCMLA complex. A 4-μl aliquot of [8-^{14}C]ADP solution (52 mCi/mmol; 1.92 mM; 15% ethanol) was mixed with 16 μl of hsp73 solution (6.1 μM; buffer A) and 5 μl of RCMLA [109 μM in HCl (pH 2)] and the solution was incubated for 30 min at 37.7°C. The reaction mixture was injected on the HPLC and aliquots of the eluant were assayed by LSC (see Fig. 6).

Complex dissociation by ATP. hsp73 (22 μl; 6.81 μM in buffer A) was incubated for 30 min (37°C) with RCMLA [3 μl; 105 μM in HCl (pH 2)]; [α-^{32}P]ATP (3 μl; 2 mM; 8.25 mCi/ml in 8 mM Tricine/4 mM sodium phosphate/40 mM KCl/2 mM $MgCl_2$, pH 7.4) was then added and the mixture was incubated for 15 min (37°C) to dissociate the hsp73–RCMLA complex. Upon injection on the HPLC, 22 fractions were collected and analyzed by TLC and LSC.

RESULTS

Gel-filtration HPLC and CD were used to study the thermal stability of cytosolic hsp70. Since nucleotides may be involved in the functions of hsp70, we analyzed the effects of ATP and ADP on hsp70 conformation, stability, and interaction with unfolded proteins. Formation of stable complexes with denatured proteins was investigated for a number of unfolded or thermally unstable proteins.

Thermal Stability of hsp70. Far-UV CD studies revealed hsp70 to be thermally stable and to retain a substantially native-like spectrum at temperatures as high as 75°C. Fig. 1*A* shows the far-UV CD spectra of purified bovine brain hsp73 at low and high temperatures (pH 7.5) and denatured by 6 M guanidinium hydrochloride (pH 2). However, at temperatures between 37°C and 48°C, and in the absence of nucleotides, the protein undergoes a cooperative transition corresponding to a loss of ≈20% of the signal (θ_{222}) of the native state [midpoint (t_m), 43°C; Fig. 1*B*]. Similar behavior was observed with an equimolar mixture of human hsp72/73 from HeLa cells (data not shown). When the thermal stability was studied in the presence of 0.9 mM dithiothreitol the t_m was essentially unchanged (44°C). This transition is irreversible since decreasing the temperature from 50°C to room temperature does not restore the CD signal to its original value. In the presence of excess Mg^{2+}/ATP or Mg^{2+}/ADP, the midpoint of this transition was shifted to 57°C (Fig. 1*B*).

When the thermal stability was monitored by gel-filtration HPLC it was found that between 20°C and 40°C, and in the absence of nucleotide, hsp73 is primarily in the monomeric form with a small amount of the protein existing as dimer and trimer, as has already been shown (17). Dimer and trimer were dissociated by incubation at 37°C with Mg^{2+}/ATP but not with Mg^{2+}/ADP or Mg^{2+}/ATP[γS] (a nonhydrolyzable ATP analog). At temperatures above 41°C, the presence of larger oligomeric species (≥300 kDa) was observed. Above 50°C, only these soluble oligomers were detected. The midpoint of this transition (t_m, 45°C) coincided with that observed by CD (Fig. 1*C*). When the same experiment was repeated in the presence of excess Mg^{2+}/ATP or Mg^{2+}/ADP (1 mM) the midpoint was 59°C (Fig. 1*C*). Oligomers were formed at all hsp73 concentrations tested (1–20 μM). In contrast to the behavior of dimers and trimers, Mg^{2+}/ATP did not dissociate the large oligomer(s). Furthermore, incubation of hsp73 with excess Mg^{2+}/[8-^{14}C]ATP under conditions that favor the formation of oligomers (63°C; 10 min; see Fig. 1*C*) revealed that ATP was not associated with the oligomers as determined by the absence of radioactivity in the HPLC oligomeric fraction. These oligomers could not be dissociated by incubation (37°C; 0.25–2 hr) with exogenous hsp73 in the presence of ATP. The implications of these findings are discussed below.

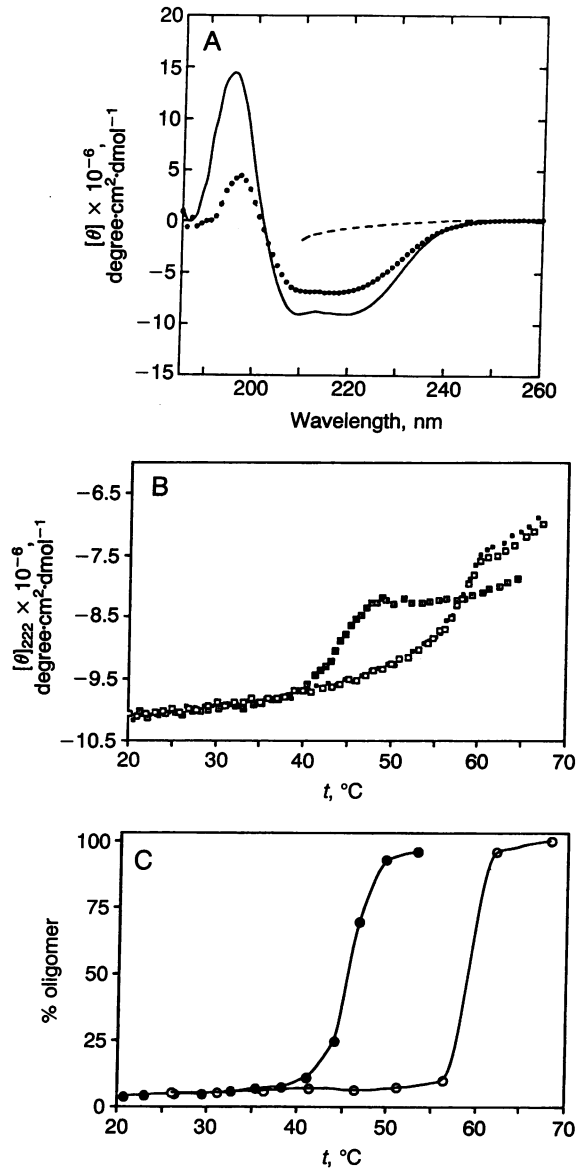


FIG. 1. Thermal stability of bovine brain hsp73. Effects of nucleotides. (*A*) Far UV CD spectra of hsp73 in buffer B at 20.0°C (solid line) or at 50.3°C (circles) and in 6 M guanidinium hydrochloride (pH 2) at 20.0°C (dashed line). The spectra (recorded at 1-nm intervals; 5-sec time constant) are the average of 10 scans. (*B*) Molar ellipticity (222 nm) of hsp73 as a function of temperature. Protein solutions were made in buffer A with or without the addition of nucleotide; □, no nucleotide; ■, with 1.0 mM Mg^{2+}/ATP; □, with 1.0 mM Mg^{2+}/ADP. In all cases, temperature was increased from 20°C at a rate of 0.33°C/min; data were collected every 60 sec with a time constant of 5 sec. (*C*) Thermal oligomerization of hsp73 followed by gel-filtration HPLC. Protein solutions in buffer A, with or without nucleotide, were incubated at the desired temperature for 10 min and analyzed; ●, [hsp73] = 10 μM, no nucleotide; ○, [hsp73] = 5 μM, [Mg^{2+}/ATP] = 5 mM.

Association with Denatured Proteins. Reductive alkylation of α-lactalbumin (α-LA) provides a stable unfolded form of the protein, RCMLA, which can be used to study its interaction with hsp70 under nondenaturing conditions. The interaction of RCMLA with hsp73 was studied by gel-filtration HPLC. A stable complex between the two proteins was detected, as a function of time, as a well-resolved peak with an elution volume distinct from those for hsp73 and its dimer (Fig. 2). The complex between hsp73 and RCMLA was

isolated by collecting the eluant from the HPLC and analyzed by SDS/PAGE; characteristic bands for RCMLA and hsp73 were detected with an approximate 1:1 stoichiometry (data not shown). No interaction was observed when native α-LA was incubated with hsp73 under similar conditions.

The kinetics of binding to hsp73 at 37.1°C were followed by HPLC (Fig. 2, traces B–E) and CD (θ_{222}; data not shown) using RCMLA as a substrate protein; the second-order rate constants were 7.4 $sec^{-1}\cdot M^{-1}$ and 9.1 $sec^{-1}\cdot M^{-1}$, respectively. Complex formation led to a time-dependent change in the CD spectrum ($\Delta[\theta]_{222} = 5.6 \times 10^5$ degree·cm^2·$dmol^{-1}$ at 37°C) consistent with a conformational change. These results indicate that under our *in vitro* conditions complex formation is a slow process. The half-life of the reaction is ≈40 min with starting concentrations of both proteins at 50 μM.

The interaction of hsp73 with SNase and its mutant NCA-S28G (16) was investigated. This mutant (NCA–SNase) is an intrinsically unstable protein that is predominantly denatured at physiological temperatures (t_m, 30°C; A.L.F. and L. Antonino, unpublished results). When hsp73 was incubated at 37°C with excess native SNase, no formation of complex was observed (Fig. 3, trace A). However, incubation under similar conditions with denatured NCA–SNase resulted in the appearance of a well-resolved peak between those for the monomer and dimer of hsp73 (Fig. 3, trace B). Complex formation between NCA–SNase and hsp73 was also confirmed by native PAGE. A distinctive band with mobility lower than that of hsp73 was detected.

The effect of temperature on the binding of hsp73 to substrate proteins is illustrated with NCA–SNase. Reaction mixtures of NCA–SNase and hsp73 were analyzed by HPLC after 10 min of incubation at temperatures in the range

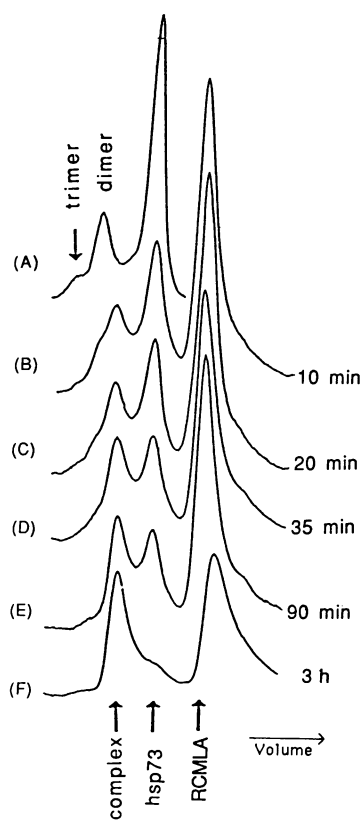


FIG. 2. Binding of RCMLA to hsp73 followed by HPLC. Traces: A, hsp73 profile showing dimer and trimer; B–F, hsp73 was incubated with RCMLA at 37°C for the time periods indicated and the mixtures were analyzed by HPLC. Traces B–E, [hsp73] = 2.9 μM and [RCMLA] = 14.8 μM; trace F, [hsp73] = 5.2 μM and [RCMLA] = 19.4 μM. See *Materials and Methods* for buffers.

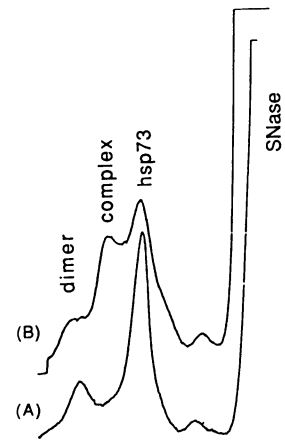


FIG. 3. Comparison of the interactions of NCA–SNase and SNase with hsp73. hsp73 (3.5 μM) was incubated at 37°C for 10 min with SNase (63 μM) (trace A) or NCA–SNase (64.3 μM) (trace B) and the mixtures were analyzed by HPLC.

20°C–40°C. In the range 20°C–30°C, only the peaks for hsp73, its dimer, and the substrate protein were prominent. However, as the temperature was increased in the range 30°C–40°C, the ratio [complex]/[hsp73] increased accordingly (Fig. 4). This effect of temperature on protein binding is indicative of a high activation energy requirement for the process. As in the case of RCMLA, the amount of complex increased with longer incubation times. Preformed hsp73–protein complexes did not dissociate on lowering the temperature to 20°C.

Effects of Nucleotides on Complex Formation. Members of the hsp70 family of heat shock proteins bind ATP and ADP (13, 18) and display ATPase activity (17, 19, 20). Nucleotides bind to a highly conserved N-terminal 44-kDa fragment of hsp73 known as the ATPase domain (18). The more variable C-terminal domain has been proposed to be responsible for binding to target proteins (21). It has been suggested that ADP binds to hsp73 with even greater affinity than ATP (19). Since ADP may play an inhibitory role in the ATPase activity of hsp70, a knowledge of the nucleotide binding constants is important. We used equilibrium dialysis to determine the binding constants of ATP and ADP. The dissociation constants for ATP and ADP were determined to be 9.5 ± 3.9 and 1.6 ± 0.5 μM, respectively. Thus, binding of ADP is ≈6 times stronger than that of ATP.

The effects of ATP, ADP, and ATP[γS] on the binding of RCMLA to hsp73 were examined. When hsp73 was incubated with RCMLA in the presence of excess Mg^{2+}/ATP in buffer A for 10–20 min at 37°C and the mixture was analyzed by HPLC, no hsp73–RCMLA complex was detected. As expected, the addition of Mg^{2+}/ATP to preformed hsp73–RCMLA complex resulted in its dissociation (Fig. 5, trace A; compare to Fig. 2, traces D and E).

In contrast to the results with ATP, complex formation occurred in the presence of Mg^{2+}/ADP as shown in Fig. 5

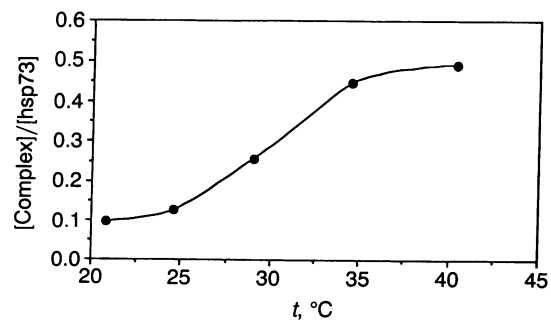


FIG. 4. Effect of temperature on the binding of hsp73 to NCA–SNase. Reaction mixtures of hsp73 (3.5 μM) and NCA–SNase (20.6 μM) were incubated at the temperatures indicated for 10 min and analyzed by HPLC. The ratio [complex]/[hsp73] was calculated as the ratio of heights of the hsp73–NCA–RNase and hsp73 peaks.

(trace B). Furthermore, when Mg^{2+}/ATP (0.88 mM) was added to hsp73–RCMLA complex preincubated with Mg^{2+}/ADP (0.88 mM), there was no significant change in the ratio [complex]/[hsp73] (Fig. 5, trace C), indicating an inhibitory effect of ADP on ATP-induced complex dissociation. Similarly, incubation in the presence of Mg^{2+}/ATP[γS] resulted in complex formation (Fig. 5, trace D).

As discussed later, these results indicate that ATP hydrolysis is necessary to dissociate the complex since ADP and ATP[γS] do not preclude complex formation, nor do they cause dissociation. This is also supported by the observation that hsp73 ATPase activity is elicited by RCMLA. When hsp73 (1.56 μM) was incubated with RCMLA (70.4 μM) and Mg^{2+}/ATP (24 μM) (10 mM Tris·HCl/12 mM sodium phosphate/0.13 M KCl, pH 7) for 15 min at 37°C, 27% of the ATP was converted to ADP according to HPLC analysis. Under similar conditions in the absence of substrate protein, <2% of ATP was hydrolyzed. The hsp73 ATPase activity induced by RCMLA does not follow a simple Michaelis–Menten mechanism; rather, the kinetics were indicative of strong product inhibition.

In addition, it was demonstrated that ADP stays bound to hsp73 after release of RCMLA from the hsp73–RCMLA complex by ATP. The complex between hsp73 and RCMLA was formed by incubation at 37°C for 30 min, Mg^{2+}/[α-^{32}P]ATP was added, and the mixture was incubated at 37°C to dissociate the complex (see *Materials and Methods* for details). It was found that ≈3% ATP had been hydrolyzed based on the free ADP/ATP ratio. ADP and ATP were found associated with hsp73 with relative amounts of bound ADP and ATP close to 2:3. This remarkably high proportion of bound ADP is another indication that ADP binds tighter than ATP. The results indicate that the rapid initial hydrolysis of ATP corresponded to 1 molar equivalent of hsp73.

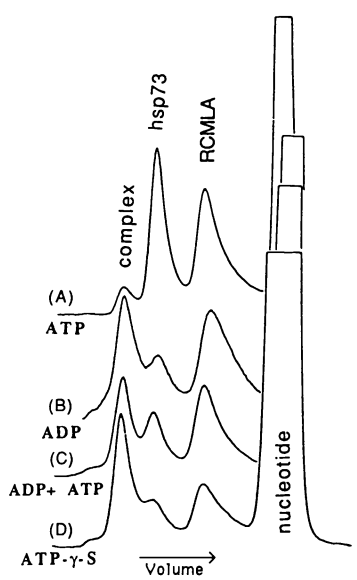


FIG. 5. Effects of nucleotides on the binding of hsp73 to RCMLA. Trace A, effect of ATP on binding: hsp73 and RCMLA were incubated for 65 min; Mg^{2+}/ATP was added and the mixture was incubated for 13 min and analyzed. Trace B, effect of ADP on complex formation: hsp73, RCMLA, and Mg^{2+}/ADP were incubated for 20 min and analyzed. Trace C, inhibitory effect of ADP on ATP-induced complex dissociation: hsp73 and RCMLA were incubated for 65 min; Mg^{2+}/ADP was added and the mixture was incubated for 5 min; then Mg^{2+}/ATP was incorporated into the reaction vessel and the mixture was incubated for 13 min and analyzed. Trace D, effect of a nonhydrolyzable ATP analog on binding: hsp73, RCMLA, and Mg^{2+}/ATP[γS] were incubated for 13 min and analyzed. In all cases, incubation temperature was 37°C. Final concentrations: hsp73, 5.2–4.3 μM; RCMLA, 12.1–19.4 μM; Mg^{2+}/ATP, 0.88–1.0 mM; Mg^{2+}/ADP, 0.88–1.0 mM; Mg^{2+}/ATP[γS], 1.0 mM.

When the kinetics of hsp73–RCMLA complex formation were followed at 37°C by CD in the presence of excess Mg^{2+}/ADP, it was found that the second-order rate constant was 29 $sec^{-1} \cdot M^{-1}$ (data not shown), which represents a 3.5-fold increase in the rate of binding as compared to the same rate constant measured in the absence of nucleotide. The data suggest that the binary complex hsp73–ADP binds the unfolded protein more rapidly than hsp73 alone. The existence of a hsp73–ADP–RCMLA ternary complex was proven by incubating hsp73 and RCMLA with [8-^{14}C]ADP. The reaction mixture was separated by HPLC; fractions were collected and analyzed by LSC. A distinctive peak of radioactivity was found associated with the hsp73–RCMLA fraction (Fig. 6).

DISCUSSION

Although considerable evidence suggests that binding to unfolded proteins is a key feature of the physiological function of hsp73, details of the molecular mechanisms have been substantially lacking.

The data presented here indicate that cytosolic hsp70 is a rather thermally stable protein. The formation of oligomers may account for this unusual behavior. The presence of nucleotides significantly increases its thermal stability in the range of temperatures known to induce stress response in mammalian cells. These thermal studies coupled with the nucleotide binding experiments indicate that hsp70–nucleotide complexes, rather than free hsp70, are physiologically relevant.

The most likely interpretation of the thermally induced oligomerization of hsp73 in the temperature range 40°C–47°C is that a conformational change causes the protein to self-associate. Our data indicate that upon oligomerization the nucleotide binding sites are no longer functional or exposed to the medium. Evidence to support this is provided by the facts that (*i*) nucleotide molecules are excluded from hsp70 upon oligomerization and (*ii*) incubation of the oligomers with Mg^{2+}/ATP does not result in dissociation. The dimers and trimers observed in the temperature range 20°C–37°C are not intermediates in the self-association process, but rather represent a different type of protein–protein interaction since, contrary to oligomerization, dimer formation is decreased by increasing temperatures and dimers and trimers can be dissociated by addition of excess Mg^{2+}/ATP.

Our results show that hsp73 binds to mature, unfolded proteins, but not to their native counterparts, to afford stable complexes that can be isolated by gel-filtration HPLC. In addition to RCMLA and NCA–SNase, reduced carboxamidomethylated ribonuclease A and staphylococcal β-lacta-

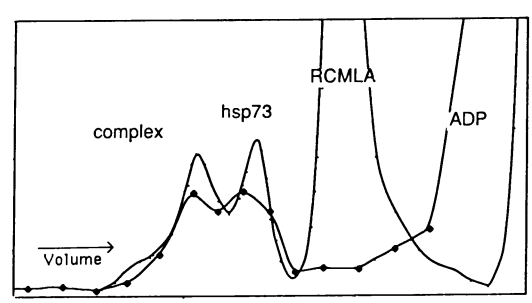


FIG. 6. ADP binding to hsp73–RCMLA complex. hsp73 (3.9 μM), RCMLA (21.8 μM), and [8-^{14}C]ADP (307 μM; 52 mCi/mmol) were incubated at 37°C for 30 min and the mixture was separated by HPLC (upper trace). Aliquots of the HPLC eluant were assayed by LSC (●).

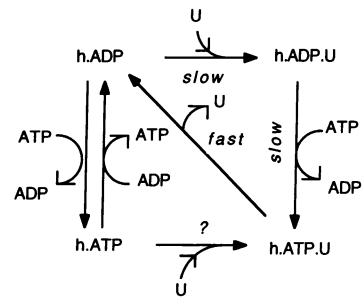
mase (a relatively thermally unstable protein; t_m, 44.7°C) were found to form stable complexes with cytosolic hsp70. We conclude, therefore, that proteins that cannot refold because of structural modification (e.g., RCMLA and carboxamidomethylated ribonuclease A) and proteins that are thermally unstable in the vicinity of 37°C (e.g., NCA–SNase and staphylococcal β-lactamase) are targets for cytosolic hsp70.

The kinetics of binding are much slower than expected for a diffusion-controlled reaction, suggesting that complex formation entails a conformational change. This is supported by the facts that (*i*) the process has a high energy of activation, occurring only at physiologically relevant temperatures and (*ii*) binding is accompanied by a change in the CD spectrum. A consequence of the slow rates of complex formation is that under our *in vitro* conditions the binding of hsp73 to unfolded proteins occurs only when the unfolded state of the protein is long-lived. This was confirmed in several experiments. When acid-denatured cytochrome *c* or apomyoglobin (pH 2) was mixed with hsp73 (under native-like conditions), no stable complex was detected after incubation at 37°C for 5 min. We attribute this to the fact that upon return to native-like conditions, refolding of the denatured substrate proteins was faster than their binding to hsp73. The slow rate of binding observed under our *in vitro* conditions with purified hsp73 and unfolded proteins may indicate that a second (protein?) cofactor present in the cell is necessary for rapid binding of hsp70 to unfolded proteins.

In all cases examined, hsp70–protein complex formation was inhibited by ATP and accelerated by ADP. The existence of hsp73–ADP–protein ternary complexes was proven for the case of RCMLA. It was also shown that the ATP-induced release of the target protein is accompanied by ATP hydrolysis and strongly inhibited by ADP. As discussed below, these findings may have important implications in the regulation of the association/dissociation process *in vivo*.

The presence of physiological concentrations of ATP and ADP may modulate the process of binding as follows. Our data suggest that *in vivo* hsp73 exists predominantly as a binary complex with nucleotides. Assuming the cytosolic concentration of free ATP and ADP to be 5 and 1 mM (22), respectively, at least 99% of hsp73 would be in the nucleotide-bound form with roughly equivalent amounts of hsp73–ATP and hsp73–ADP binary complexes. The binary hsp70–ATP complexes may play an important role in processes such as the uncoating of clathrin-coated vesicles, as has already been shown (17, 19), but are unlikely to participate in the formation of stable complexes with denatured proteins (or nascent polypeptide chains) as our results indicate. On the other hand, the binary hsp70–ADP complexes will bind unfolded proteins to form hsp70–ADP–substrate protein ternary complexes, which are long-lived, at higher rates than free hsp70. As shown above, the existence of such complexes was proven for RCMLA as a substrate protein (Fig. 6). The release of the substrate protein would be triggered by the displacement of the ADP by ATP and its concurrent or subsequent hydrolysis. As indicated in the following scheme our data suggest that a three-state cycle involving the relatively long-lived ADP binary and ternary complexes and the short-lived ATP ternary complex would operate under physiological conditions. In the scheme, h and U represent hsp73 and target protein, respectively.

Our results show that hsp73 discriminates between the folded and unfolded state of target proteins. This would be consistent with the idea that hsp73 can interact with nascent polypeptides that have yet to reach their final folded state. Moreover, the idea that *in vivo* mature proteins that become denatured as a consequence of a stress event become targets for hsp73 appears plausible. Indeed, earlier studies demonstrated high levels of hsp70 within the nucleolus after heat



Scheme I

shock and, in particular, within those areas of the nucleolus where there occurred an accumulation of denatured/aggregated preribosomes. Thus, if hsp70 is capable of recognizing and binding to denatured proteins *in vivo*, the question that remains open concerns the fate of the bound target. Specifically, is the denatured protein bound to hsp70 eventually repaired (i.e., refolded), or alternatively might the binding merely suffice to maintain the solubility of the denatured target until its subsequent presentation to an appropriate proteolytic system?

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