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Thermal activation of the bovine Hsc70 molecular chaperone at physiological temperatures: physical evidence of a molecular thermometer

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Abstract Differential scanning calorimetry was used to monitor the thermal transitions of the 70 kDa heat shock cognate protein (Hsc70). Hsc70 had endothermic transitions with midpoints (Tm) at 59°C and 63°C in the absence and presence of ATP, respectively, and a similar increase in Tm was observed using intrinsic fluorescence of tryptophan. Combined with increased exposure at 60°C of non-polar residues of Hsc70 to which the hydrophobic, fluorescent probe ANS bound, these data indicate that the endotherms represent thermal denaturation and that bound nucleotide stabilizes Hsc70. An exothermic transition (Tm = 66°C) was detected by calorimetry for Hsc70-apocytochrome *c* (apo *c*) complexes. An increase in intrinsic fluorescence with the same Tm and increased turbidity indicated aggregation of the denatured Hsc70-apo *c*. A novel finding was an exothermic transition of Hsc70 beginning at about 30°C (Tm = 41°C). No changes in either intrinsic fluorescence or ANS fluorescence attributable to protein transitions were detected in this temperature range. Examination of samples run on native polyacrylamide gels indicated that this exothermic transition was not due to Hsc70 aggregation or multimer formation. However, Hsc70 was protease-resistant at 20°C, sensitive at 40°C and resistant when returned to 20°C, indicating that this exotherm is associated with a reversible conformational change. As an assay for Hsc70 chaperoning function, complex formation was measured as a function of temperature using a variety of substrates including the model unfolded protein apo *c*, a pigeon cytochrome *c* fragment, a representative hydrophobic-aromatic peptide FYQLALT, and a representative hydrophobic-basic motif NIVRKKK. For all of these substrates, the amount of complex formed increased with increasing temperature over the same range as the 41°C exotherm. It is proposed that a conformational change exposes polar and charged residues in Hsc70 which subsequently become hydrated, resulting in an active chaperone. Hsc70 may be a thermal sensor that matches the supply of chaperoning activity with demand for it over the physiological temperature range of mammalian cells. Thermal activation of Hsc70 may also have a role in acquired thermotolerance.

INTRODUCTION

Mammalian Hsc70 is a constitutively expressed nucleocytoplasmic member of the Hsp70 family. Hsc70 is a peptide/unfolded protein-stimulated ATPase having the ability to distinguish between native and unfolded forms of the same protein (reviewed in Hightower et al 1994). It binds with high affinity both hydrophobic-aromatic sequences like FYQLALT and hydrophobic-basic sequences like NIVRKKK (Fourie et al 1994; Takenaka et al 1995). There is evidence that Hsc70 associates with nascent polypeptide chains in polysomes and chaperones newly synthesized proteins to other chaperones involved in protein folding and to sites of membrane translocation (Baler et al 1992; Nelson et al 1992; Frydman et al 1994). Thus, Hsc70 chaperoning activity appears to be closely coupled to protein synthetic activity. Hsc70 also protects and

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reactivates thermally inactivated enzymes, suggesting a cellular role in acquired thermotolerance and recovery from thermal damage (Ciavarra et al 1994; Ziemienowicz et al 1995).

At the structural level, Hsc70 has at least two functional domains and one regulatory domain. The 44 kDa ATPase domain contains a deep cleft in which nucleotides bind (reviewed in McKay et al 1994). The 18 kDa peptide-binding domain appears to be the only domain sufficiently hydrophobic to bind sequences like FYQLALT. The C-terminal 10 kDa is predicted to be rich in α-helical secondary structure, followed by a G/P rich aperiodic segment, and ending in EEVD. This domain may couple the ATPase and peptide-binding domains (Freeman et al 1995).

Differential scanning calorimetry is a useful method for probing the domain organization, domain interactions and ligand binding of purified proteins. It measures the heat absorbed or released during a thermotropic transition as temperature is increased at a set rate. Integrating the area of a transition gives the total enthalpy change. A temperature midpoint or Tm is determined for each transition. For denaturation as an example, the Tm would represent the temperature at which one-half of the protein is unfolded. For protein solutions, the heat capacity of the buffer solution is subtracted from the heat capacity of the protein solution. The resulting scans of apparent excess specific heat record changes in the rate of heat absorption associated with protein conformational changes, protein–protein interactions and protein–solvent interactions. Positive changes are endothermic transitions most frequently associated with protein unfolding or denaturation. Negative changes or exothermic transitions may be associated with oligomerization, aggregation and hydration of exposed residues following a conformational change. Differential scanning calorimetry has been used to study the thermal unfolding of DnaK, which has three endothermic transitions centered at 45.2°C, 58°C and 73.3°C (Montgomery et al 1993).

Both DnaK and Hsc70 have similar thermal stabilities and denature to intermediates with properties of molten globule states; however, the consequences of denaturation are very different. DnaK can refold from its molten globule state upon cooling; Hsc70 irreversibly aggregates (Palleros and Fink 1992). Irreversible thermal denaturation of Hsc70 limits the thermodynamic analysis of the calorimetric data. However, differential scanning calorimetry can still yield useful information about the domains of Hsc70. We observed thermal transitions of Hsc70 that are substantially different from the calorimetric analysis of DnaK. In the mammalian physiological temperature range, a novel exothermic transition associated with a conformational change in Hsc70 occurred which activated peptide/unfolded protein binding activity.

MATERIALS AND METHODS

Protein purification

Hsc70 was prepared from bovine brain as described previously (Sadis et al 1990a). All procedures were performed at 4°C and samples were stored at −70°C in buffer C (20 mM Hepes-KOH, pH 7.0, 25 mM KCl, 10 mM $(NH_4)_2SO_4$, 0.1 mM EDTA, 2 mM magnesium acetate, and 1 mM dithiothreitol (DTT)). Purified Hsc70 was dialyzed extensively against buffer C to remove nucleotides. Protein concentrations were estimated from the absorbance at 280 nm (ε [1 mg/ml] = 0.54; Sadis et al 1990a). Apo *c* was prepared from horse heart cytochrome *c* (Sigma Chemical Co) by the Fisher method (Fisher et al 1973) with modifications (Morimoto et al 1983). The concentration of apo *c* was determined by the Lowry assay using bovine serum albumin as the standard.

Differential scanning calorimetry

All scans were obtained with a Microcal MC-2 differential scanning calorimeter (DSC) interfaced to a DEC Pro 380 computer. The concentrations of Hsc70 and apo *c* were 1.5 mg/ml each for a molar ratio of Hsc70 to apo *c* of approximately 1:6. All samples were diluted to 2.5 ml with buffer C without DTT, which was also used as the reference solution in the DSC, giving a final DTT concentration of approximately 0.4 mM. The magnesium salt of ATP (Sigma Chemical Company) was added in excess to either 1 mM or 10 mM with the same results. For the control of non-specific effects of saturating concentrations of nucleotide, 10 mM γS-ATP (Sigma Chemical Co) was included in the sample instead of ATP. The samples were incubated for 1 h at room temperature prior to use. Scans were run from 10°C to 100°C at a scan rate of 1°C/min. The samples were cooled again to 10°C and immediately rescanned to 100°C to determine the baseline.

Analysis of DSC data

The scans were first smoothed using a polynomial least squares method. The intrinsic instrumental curvature of the baseline was removed by subtracting the rescan. A correction for the shift in specific heat (ΔC_p) between the native and denatured states was done as previously described (Lepock et al 1990). The main order-disorder transition (i.e. denaturation or unfolding) occurring in the sample appears as an endothermic peak in the curve of apparent excess specific heat (C_p^{ex}).

The exotherm (D) was often superimposed on the main denaturation profiles (B and C) making it impossible to obtain an accurate value for the calorimetric enthalpy (ΔHc) by direct integration. Thus ΔHc was determined by assuming that denaturation could be approximated as a

reversible process (N↔D), and the Freire and Biltonen model (Friere and Biltonen 1978a, 1978b) was used to obtain a best fit profile not using that part of the original curve obviously distorted by exotherm D. Specifically, the equation used for modelling and curve fitting was

$$C_p^{ex} = m\left(\frac{(\Delta H)^2 e^{-(\Delta H(1/T - 1/Tm)/R)}}{RT^2(e^{-(\Delta H(1/T - 1/Tm)/R)} + 1)^2}\right)$$

where m is a scaling factor, ΔH is the Van't Hoff enthalphy, and T_m is the transition temperature defined as the temperature at which the concentrations of the native and denatured species are equal. Curve fitting was done using the simplex algorithm and ΔHc determined from integration of the best fit curve (Lepock et al 1990).

Tryptophan fluorescence

Fluorescence intensities were measured using an SLM 4800S spectrofluorometer interfaced with a DEC Pro 380 computer for data acquisition. Hsc70 was used at a concentration of 0.3 mg/ml in the same buffer used for the DSC studies. Intrinsic protein fluorescence was measured using an excitation wavelength of 290 nm and an emission wavelength of 346 nm. At these wavelengths the fluorescence is dominated by the tryptophans at residues 90 and 580 of Hsc70. For all measurements of fluorescence intensity as a function of temperature, the temperature of the sample was raised at a rate of 1°C/min, the same scan rate used for the DSC studies. The solution was stirred continuously to reduce the effects of aggregation at high temperature. The change in fluorescence was normalized and the linear decrease in fluorescence above and below the T_m was removed by taking the first derivative, subtracting the baseline and integrating this subtracted curve. The two component curve in Figure 5B was deconvoluted by assuming an irreversible transition of the form A→B for each transition as previously described (Lepock et al 1990). The equation used was

$$\frac{dF}{dT} = \frac{m}{V} e^{A - Ea/RT} e^{-\frac{1}{v}\int_{T_0}^{T} e^{A - Ea/RT} dT}$$

where dF/dT is the temperaturederivative of the fluorescence intensity, v is the scan rate, E_a the activation energy, and A the frequency factor.

1-Anilino-naphthalene-8-sulfonate (ANS) fluorescence

Changes in the surface hydrophobicity of Hsc70 were followed by monitoring the fluorescence intensity of ANS. Hsc70 was diluted to 0.15 mg/ml in the same buffer used for the DSC and trp fluorescence measurements. ANS was added to a final concentration of 100 μM from a 10 mM stock solution. To study the effect of nucleotide, 10 mM ATP or 10 mM γS-ATP (nucleotide control) was included in the sample. The ANS fluorescence was measured at excitation and emission wavelengths of 380 nm and 470 nm, respectively, as a function of the temperature at the same rate of heating of 1°C/min. The ANS controls were done under the same conditions but in the absence of Hsc70. The protein control was run in the absence of ANS, and the small change in intensity detected is due exclusively to light scattering.

Limited proteolysis of *hsc70*

Nα-p-tosyl-L-lysine chloromethyl ketone (TLCK)-treated α-chymotrypsin (Sigma Chemical Co) and nucleotide stocks were prepared in buffer C. Nucleotide stocks were adjusted to pH 7.0 using potassium hydroxide. All of the reactions were assembled on ice and the incubation conditions for Hsc70 were similar to those used for DSC. Hsc70 (5 μg) was preincubated with or without 10 mM ATP in a total volume of 38.3 μL in buffer C at 25°C for 1 h followed by a 15 min cooling period at 10°C. Then the samples were incubated at 20°C and 40°C, respectively. After 30 min, 0.83 μg (6.7 μL) of TLCK-treated α-chymotrypsin (the weight ratio of α-chymotrypsin to Hsc70 was 1 : 6) was added to the samples and incubation continued for another 15 min. Then 1 μg (9 μL) of Hsc70 was removed from each sample and the α-chymotrypsin was inactivated by 1 mM phenylmethylsuflonyl fluoride (PMSF) prepared in acetone. The α-chymotrypsin cleavage products were then analyzed using SDS-polyacrylamide gel electrophoresis (PAGE) on 9% gels and the protein bands were visualized by silver staining.

To determine the reversibility of Hsc70 sensitivity to α-chymotrypsin cleavage, samples were prepared and preincubated as above except there was an additional 30 min incubation at 40°C followed by a 15 min cooling period at 10°C before the samples were incubated at 20°C and 40°C. When chymotrypsin activity was determined according to the manufacturer's procedures and was compared at these two temperatures using N-benzoyl-L-tyrosine ethyl ester (BTEE) as a substrate in buffer C, α-chymotrypsin hydrolysed 0.53 μmol BTEE/min at 20°C, compared to 0.47 μmol BTEE/min at 40°C.

Complex formation between Hsc70 and apo *c* or peptide substrates

All of the reactions were assembled on ice in a total volume of 10 μL in buffer C. For Hsc70 and apo *c* complex: Hsc70 (2 μg) was incubated with apo *c* (2 μg) and 10 mM ADP at 0°C, 28°C, 37°C, 42°C, 47°C, 52°C, and 60°C, respectively. For Hsc70 and peptide complexes: Hsc70 (2 μg) was incubated with 35 μM pigeon cytochrome *c* peptide (P*c*, IFAGIKKKAERADLIAYLKQATAK), 25 μM FYQLALT, or 400 μM NIVRKKK and 10 mM ADP at 20°C

and 40°C. After 30 min, 1 μg of Hsc70 was withdrawn from each sample and analyzed in a 6% native polyacrylamide gel. The protein bands were then visualize by silver staining.

Gel electrophoresis, silver staining, and quantitative analysis

Analytical discontinuous SDS-PAGE was done using a modified procedure of Laemmli (Laemmli 1970). Gel sample buffer was added to samples to a final concentration of 62.5 mM Tris-HCl, pH 6.8, 2.3% (w/v) SDS, 10% (v/v) glycerol, 5% (v/v) β-mercaptoethanol, and 0.002% (w/v) bromophenol blue. Samples were boiled at 100°C for 5 min and then were analyzed in 9% (w/v) acrylamide: 0.27% (w/v) bis-acrylamide mini-slab gels using a Bio-Rad mini-gel apparatus. The proteins were separated at constant voltage (200 V) for 45 min in 25 mM Tris base, 192 mM glycine, and 0.1% (w/v) SDS as run buffer. For analyzing samples in non-reducing, non-denaturing continuous PAGE (native gels), proteins were prepared in sample buffer without SDS, β-mercaptoethanol, and bromophenol blue (native gel sample buffer). Samples were then analyzed at room temperature using 6% continuous gels in precooled run buffer without SDS.

The gels were fixed in 30% (v/v) methanol, 10% (v/v) glacial acetic acid for 25 min and stained using a modification of the procedure for the AG-25 silver staining kit (Sigma Chemical Co, St Louis, MO) which improved the staining of Hsc70. After fixing, gels were rinsed in three changes of distilled water for 5 min each; and incubated in reducer solution for 30 s. Gels were rinsed in distilled water for 1 min and incubated in silver solution for 25 min. The gels were rinsed briefly in distilled water and placed in developer solution for several min until the bands were clearly visible. The gels were then immediately transferred to 1% (v/v) glacial acetic acid to prevent further development, rinsed in distilled water and dried.

The silver stained Hsc70 protein bands were quantified using a Molecular Dynamics computing densitometer model 300 A (Sunnyvale, CA) by measuring integrated volumes of the protein bands. The degree of cleavage of Hsc70 by α-chymotrypsin was expressed as a percentage of undigested Hsc70.

RESULTS

Thermal stability of Hsc70

The thermal profile of Hsc70 in the absence of added nucleotide was obtained by DSC at a scan rate of 1°C/min. The profile of apparent excess C_p corrected for baseline curvature is shown in Figure 1. One exothermic transition labeled A with a Tm of 42°C at the lowest C_p and an endothermic peak labeled B with a peak Tm of 59°C were obtained. Rescans of Hsc70 were flat and showed no evidence of reversibility after scanning to 105°C and to 59°C, midway through the peak. Endotherm B was used to calculate the apparent calorimetric enthalpy (ΔHc, as described in Materials and Methods) which was 0.68×10^5 cal/mol.

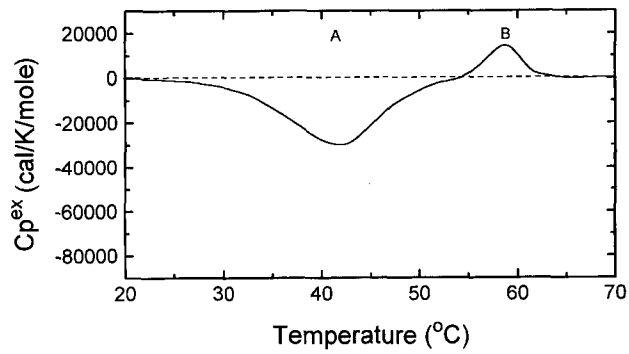

Fig. 1 DSC profile (apparent excess C_p vs temperature) of Hsc70 (1.5 mg/ml) with the baseline corrected (solid line). A major exothermic transition is labeled A and an endothermic transition is labeled B.

Thermal stability of Hsc70 bound to apo *c*

The effect of binding an unfolded protein on the thermal stability of Hsc70 was determined using apo *c* as the substrate. Hsc70 binds to apo *c* resulting in a 3-fold stimulation of ATPase activity (Sadis and Hightower 1992). The DSC scan of Hsc70 in the presence of a six-fold molar excess of apo *c* and in the absence of ATP is shown in Figure 2. The nucleotide analog γS-ATP, which binds weakly to Hsc70 without increasing its thermal stability (see below) and which is only slowly hydrolyzed, was included as a control for non-specific effects of saturating concentrations of nucleotide. The DSC scan of apo *c* alone had no detectable transitions but cytochrome *c* had a sharp, two-state transition with a Tm = 81°C (results not

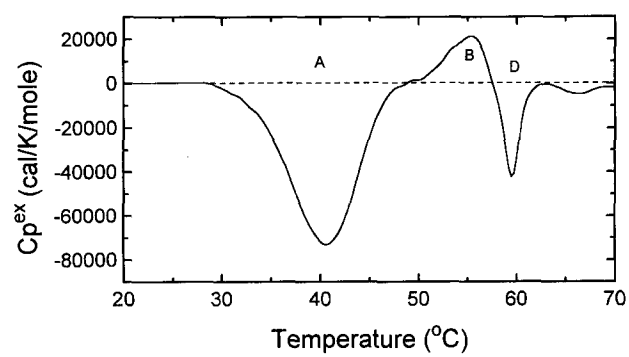

Fig. 2 DSC profile (baseline corrected) of a mixture of Hsc70 (1.5 mg/ml) and unfolded apo *c* (molar ratio 1 : 6) in the presence of 10 mM γS-ATP as a control for non-specific effects of saturating concentrations of nucleotide. A second exothermic transition is labeled D.

shown). Three significant alterations occurred in the thermal profile of Hsc70 when bound to apo *c*: the exotherm centered at 41°C (transition A) increased about 2-fold in magnitude, a new, very sharp exotherm (transition D) was observed with a Tm of approximately 60°C, and the ΔHc of endotherm B increased by approximately 50% to 1.1×10^5 cal/mol. The peak Tm of endotherm B was 56°C. It is likely that this endotherm represents denaturation of Hsc70-apo *c*, which is supported by the fluorescence studies given below. Exotherm D is probably due to aggregation of denatured Hsc70-apo *c* since it was only observed in the presence of apo *c* and corresponds to a major increase in light scattering (see below).

The DSC profile of Hsc70 in the presence of apo *c* and 10 mM ATP is shown in Figure 3. Complexes of Hsc70-ADP-apo *c* are relatively stable whereas Hsc70 undergoes cycles of binding and release of apo *c* in the presence of ATP, stimulated by exchange of bound ADP by ATP. Under these conditions, the concentration of the Hsc70-ADP-apo *c* complex will be lower than in the conditions favoring stable complex formation used for Figure 2. Transitions A, C and D were present in the DSC scan; however, exotherm A was reduced in magnitude compared to that of Hsc70 by itself (Fig. 1) and the single endotherm detected, labeled C, occurred with a Tm of 63°C. It is likely that this endotherm represents the thermal denaturation of the complex Hsc70-ADP/ATP-apo *c*, further stabilized by bound nucleotide. This endotherm is labeled C rather than B since it involves the denaturation of the nucleotide-stabilized conformation. The ΔHc of endotherm C increased more than 3-fold to 2.5×10^5 cal/mol compared to endotherm B (absence of ATP). Exotherm D with a Tm of 66°C may represent the heat released during one step in the aggregation of these thermally denatured complexes containing apo *c*.

Further probing of the thermal stability of Hsc70 using intrinsic trp fluorescence

Endothermic transitions B and C are consistent with a thermally-induced unfolding of Hsc70, but the exothermic transitions A and D required additional analyses by independent methods. Therefore, fluorescence studies using intrinsic trp and ANS fluorescence and protease susceptibility of Hsc70 were used to study these transitions in greater detail.

A profile of intrinsic trp fluorescence of Hsc70 in the absence of nucleotide is shown in Figure 4A. The same rate of temperature increase (1°C/min) as in the DSC studies was used. Fluorescence intensity gradually decreased with increasing temperature, probably due to increased quenching by water, except for a sharp increase between 55 and 63°C. This profile was converted to a profile of ΔF vs temperature by differentiation, baseline correction, and integration to remove the temperature dependent decrease in fluorescence (Fig. 4B). A baseline correction was done as for the DSC profiles to set the linear region below 50°C to zero. The resulting curve was fitted using the irreversible model described in Experimental Procedures, integrated, and plotted. A better visualization of the relative change in fluorescence intensity through the transition was obtained by removal of the decrease in fluorescence not associated with the transition. No change in fluorescence intensity attributable to a protein transition was detected in the 30–50°C range, indicating that exothermic transition A proceeded without a change in the environments of trp 90 or trp

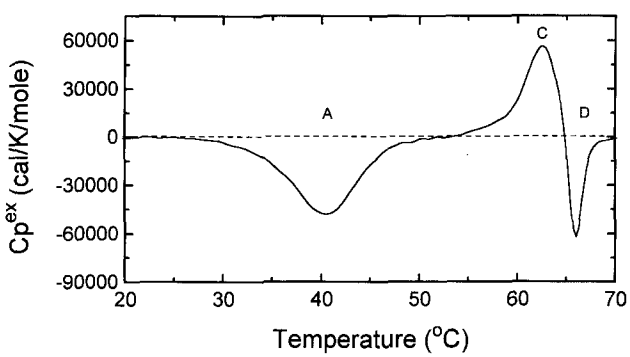

Fig. 3 DSC profile (baseline corrected) of a mixture of Hsc70 (1.5 mg/ml) and unfolded apo c (molar ratio 1 : 6) in the presence of 10 mM ATP. A second endothermic transition is labeled C.

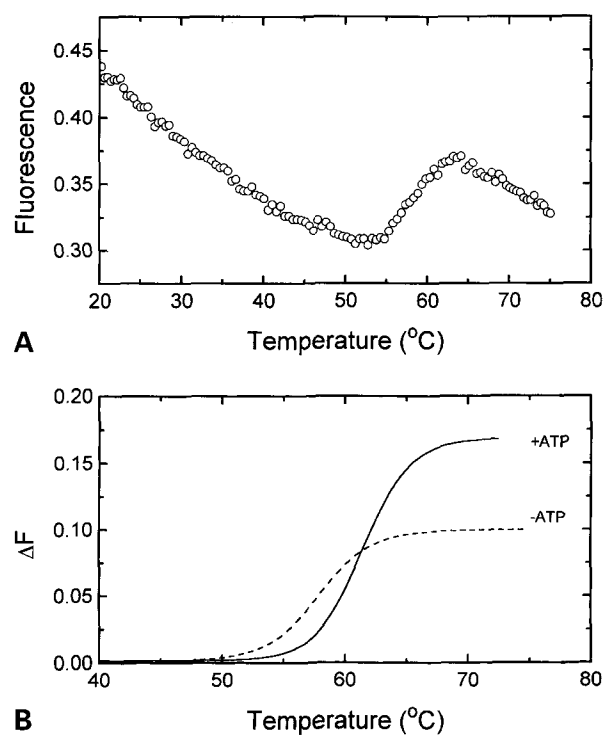

Fig. 4 Fluorescence intensity (λ_{ex} = 290 nm, λ_{em} = 346 nm) of Hsc70 as a function of temperature increased at 1°C/min. Panel A: original values in the absence of ATP. Panel B: best fit, corrected curves in the absence and presence of 10 mM ATP.

580 large enough to result in a detectable change in fluorescence emission. A Tm of 58°C was found for Hsc70 in the absence of nucleotide, and this transition was shifted to 62°C upon addition of ATP. These Tm match closely those determined by DSC for the endothermic transitions B (59°C) and C (63°C). Both methods indicated a change in Hsc70 to a more thermally stable form with bound nucleotide and both the increased fluorescence and the endotherm of transition B are indicative of denaturation of Hsc70. These data are consistent with previous studies using circular dichroism to show that Hsc70 with bound nucleotide is more thermally stable than nucleotide-free Hsc70 (Palleros et al 1991).

The temperature profile of intrinsic fluorescence of Hsc70 in the presence of apo *c* and γS-ATP is shown in Figure 5A. No shift in ΔF was detected upon addition of γS-ATP alone (not shown), indicating that this analog is not able to increase the thermal stability of Hsc70. Again, no transitions were detected in the region of exothermic transition A. A sharp increase, consisting of two resolvable steps, occurred between 55 and 70°C with a sharp decrease above 70°C. The fluorescence intensity of apo *c* alone decreased smoothly with increasing temperature (results not shown). Thus, there were no conformational changes detectable by intrinsic fluorescence in apo *c* alone.

The profile was corrected as described for Figure 4 and fit with two components as shown in Figure 5B. The first component with a Tm of 58°C corresponds to transition B in the DSC scans shown in Figures 1 and 2. The one-half change in fluorescence of the second component occurred at approximately 66°C, corresponding to transition D in the DSC scans. There was noticeable aggregation in this temperature range resulting in increased turbidity. Above approximately 70°C aggregation was so extensive that large clumps where visible with a subsequent decrease in scattering. This correlated with the decrease in fluorescence intensity above 70°C. Thus, transition D was associated with aggregation resulting in increased light scattering followed by decreased scattering as very large clumps formed. No detectable aggregation or increased scattering occurred for Hsc70 in the absence of apo *c* (Fig. 4) and no exotherm D was observed at higher temperatures by DSC (Fig. 1).

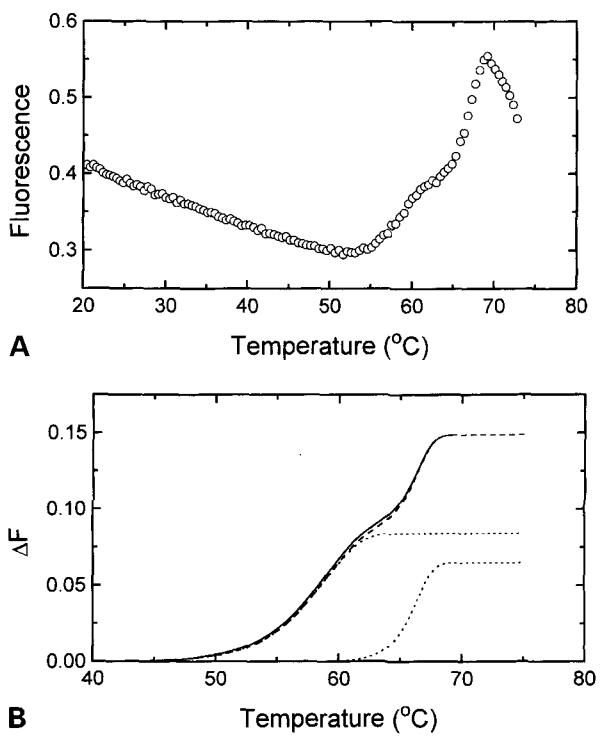

Fig. 5 Fluorescence intensity (λ_{ex} = 290 nm, λ_{em} = 346 nm) of Hsc70. Panel A: in the presence of unfolded apo *c* and 10 mM γS-ATP (nucleotide control). No shift in ΔF was detected upon addition of γS-ATP to Hsc70 (data not shown), indicating that this analog is not able to increase the thermal stability of Hsc70. Panel B: Data in Panel A normalized such that the dotted lines are the best fits assuming two irreversible transitions and the dashed line is the sum of the two fits.

Further probing of Hsc70 thermal stability using ANS fluorescence

Additional information about the nature of endothermic transition C was obtained from the curve of ANS fluorescence in the presence of Hsc70. This profile is shown in Figure 6 for Hsc70, 10 mM ATP, and 100 μM ANS. The fluorescence intensity was relatively constant from 20–50°C after which a sharp increase occurred with the 50% rise at approximately 60°C. Increased exposure of hydrophobic groups, due to unfolding, results in an increase in the fluorescence intensity of ANS which has been used as a hydrophobic probe for the study of conformational changes in proteins (Candamone and Puri, 1992). This supports the identification of the endothermic transitions with the unfolding of Hsc70. There

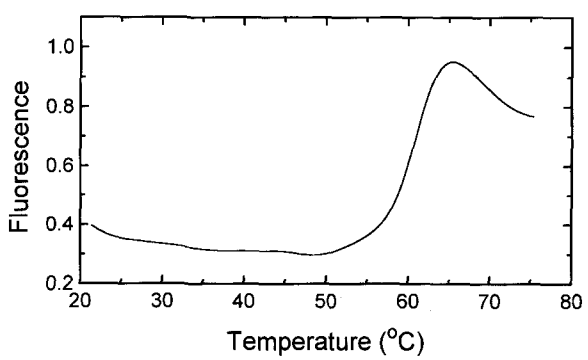

Fig. 6 ANS (100 μM) fluorescence intensity (λ_{ex} = 380 nm, λ_{em} = 470 nm) in the presence of Hsc70 and 10 mM ATP as a function of temperature increased at 1°C/min.

was no change in the fluorescence intensity near exothermic transition A. Thus, in addition to a lack of effect on the fluorescence intensity of trp 90 and trp 580, this transition did not result in detectable exposure of nonpolar residues.

A temperature-dependent conformational change in Hsc70 corresponding to exotherm A

We next probed for conformational changes in Hsc70 in the temperature range of transition A by limited proteolysis with α-chymotrypsin. The results shown in Figure 7 indicated that Hsc70 was more sensitive to α-chymotrypsin cleavage at 40°C (midpoint of transition A) than at 20°C (below transition A) both in the absence and presence of ATP (lanes 3 and 5). Using a computing densitometer to quantify relative amounts of Hsc70 remaining after limited α-chymotrypsin cleavage, 61% of Hsc70 was cleaved by α-chymotrypsin within 15 min at 40°C (lane 3) compared to only 11% at 20°C (lane 2). Similar results were found when Hsc70 was incubated in 10 mM ATP with 15% of Hsc70 cleaved at 20°C (lane 4) and 58% cleaved at 40°C (lane 5). The resistance of Hsc70 to α-chymotrypsin cleavage at 20°C was not due to a lack of chymotrypsin activity since the protease was more active at 20°C than at 40°C (see Experimental Procedures). We concluded that Hsc70 is in a protease-resistant conformation at 20°C. The increased sensitivity of Hsc70 to α-chymotrypsin cleavage at 40°C indicated that a temperature-dependent conformational change occurred in Hsc70 detectable as an exothermic process, i.e. transition A in the DSC scans.

In order to determine whether or not the change in Hsc70 to a protease sensitive conformation at 40°C was the result of thermal denaturation, the reversibility of this change was measured. As shown in Figure 8, Hsc70 returned to its protease resistant conformation when the incubation temperature was shifted from 40°C (lane 2) back to 20°C (lane 3). Similar results were found when the incubation was performed in the absence of ATP (data not shown). Therefore, the change in Hsc70 to an α-chymotrypsin sensitive conformation was reversible and independent of added ATP.

Temperature-dependent activation of unfolded protein binding activity

Next we investigated whether the chaperoning function of Hsc70 was temperature-dependent. The binding of Hsc70 to a unfolded protein, apo *c*, was used as a functional assay. The assay was preformed in the presence of 10 mM ADP to facilitate trapping Hsc70-substrate complexes. The monomeric and dimeric Hsc70 protein bands in native gels were identified as 70-kDa and 140-kDa proteins, respectively using Superose-12 gel filtration (data not shown). The Hsc70-apo *c* complexes were identified by analyzing the complexes in SDS-PAGE after native gel electrophoresis. The protein band which corresponded to the complexes in native gel was cut out of the native gel and analyzed in SDS-PAGE. Two proteins were

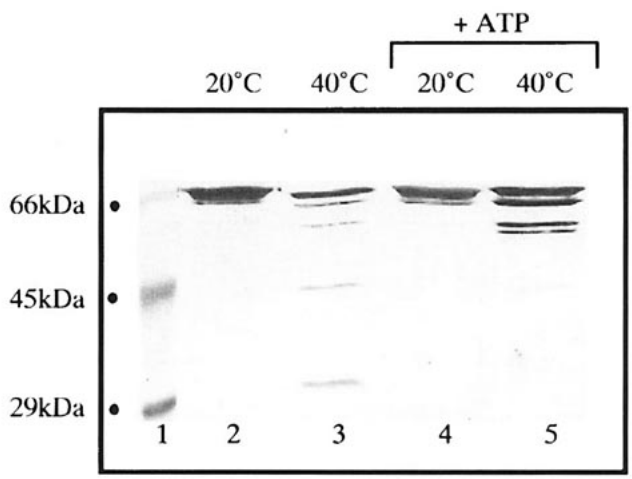

Fig. 7 Sensitivity of Hsc70 to limited α-chymotrypsin cleavage at 20°C and 40°C analyzed by SDS-PAGE. Hsc70 was preincubated with or without 10 mM ATP at 25°C for 1 h followed by a 15 min cooling period at 10°C. After the preincubation, the samples were kept at 20°C and 40°C for 30 min and then were incubated with chymotrypsin at the indicated temperatures for 15 min. Lane 1 contains marker proteins with molecular masses indicated. Lanes 2–3 contain cleavage products of Hsc70 generated at 20°C and 40°C without ATP, and lanes 4–5 with ATP.

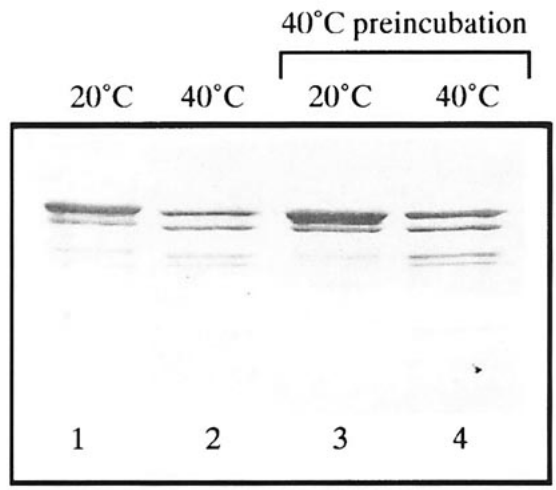

Fig. 8 Reversibility of Hsc70 sensitivity to limited α-chymotrypsin cleavage at 40°C in the presence of 10 mM ATP. Samples were prepared and preincubated as in Figure 7, except there was an additional 30 min incubation at 40°C follow by a 15 min cooling period at 10°C before incubating the samples at 20°C and 40°C for 30 min. Then Hsc70 was incubated with α-chymotrypsin at the indicated temperatures for 15 min. Lanes 1–2 contain cleavage products of Hsc70 generated at 20°C and 40°C without preincubation, and lanes 3–4 with a 40°C preincubation.

resolved with molecular masses of about 70-kDa and 12-kDa, respectively (data not shown). As shown by analyses using native gel electrophoresis (Fig. 9), Hsc70-apo *c* complexes were first detected at 28°C. The relative amounts of these complexes increased as incubation temperatures increased up to 47°C. At 52°C, substantial thermal denaturation as expected from the DSC scan, was evidenced by loss of Hsc70 monomers, dimers and Hsc70-apo *c* complexes, accompanied by increased amounts of protein at the top of the gel. At 60°C, virtually all of the denatured proteins formed large aggregates which were unable to migrate into the gel. We concluded that the binding of Hsc70 to apo *c* increased with temperature over the same range as a conformational change in Hsc70 to a protease-sensitive form, and over the same range as exotherm A. Furthermore, transition A was not due to aggregation or association of Hsc70 into multimers since these changes were not observed using native gel electrophoresis (Sadis et al 1990b). However, exothermic transition D corresponded to the accumulation of large aggregates detected on native gels (Fig. 9, lane 7).

Three peptides were also tested for temperature-dependent binding to Hsc70. The mobilities of these Hsc70-peptide complexes were very different in native gel electrophoresis. Both Hsc70-P*c* and Hsc70-NIVRKKK (indicated by dot and dart in Fig. 10, lanes 2 and 6) migrated slower compared to Hsc70 monomer (lane 8). While Hsc70-FYQLALT (indicated by arrowhead in lane 4) migrated slightly faster than Hsc70 monomer. At 20°C, only small amounts of P*c*, FYQLALT and NIVRKKK bound to Hsc70 (compare lanes 1, 3, and 5 to Hsc70 alone in lane 7) whereas the amounts bound to Hsc70 increased dramatically for all three peptides incubated at 40°C (compare lanes 2, 4, and 6 to Hsc70 alone in lane 8).

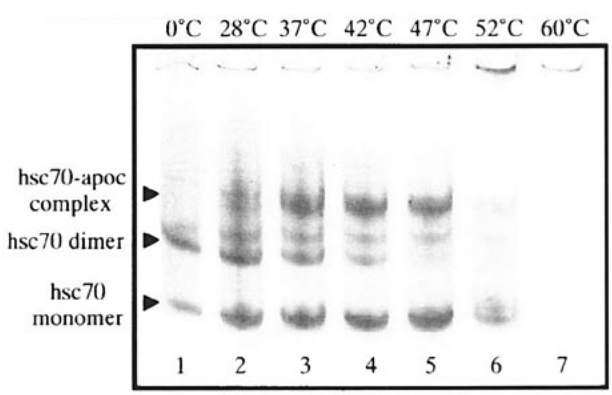

Fig. 9 Temperature-dependent formation of Hsc70-apo *c* complexes analyzed on native gels. Hsc70 (2 μg) was incubated with apo *c* (2 μg) and 10 mM ADP at different temperatures for 30 min. Lanes 1–7 show complexes formed at the indicated temperatures. Positions of Hsc70 monomers, dimers, and Hsc70-apo *c* complexes are indicated on the left.

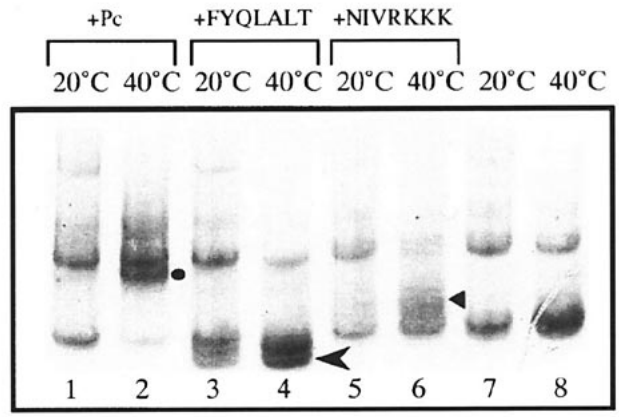

Fig. 10 Temperature-dependent formation of Hsc70-peptide complexes analyzed on native gels. Hsc70 (2 μg) was incubated with either 35 μM of Pc, 25 μM FYQLALT, or 400 μM NIVRKKK and 10 mM ADP at 20°C and 40°C for 30 min. After incubation, samples containing 1 μg Hsc70 were analyzed on a native gel. Lanes 2, 4, and 6 contain Hsc70-peptide complexes which are marked with a dot, arrowhead, and dart, respectively. Hsc70-Pc (dot) and Hsc70-NIVRKKK (dart) migrated slower than Hsc70 monomer while Hsc70-FYQLALT (arrowhead) migrated slightly faster than Hsc70 monomer. Lanes 7 and 8 are control lanes containing Hsc70 incubated without peptides.

DISCUSSION

In a previous DSC study (Montgomery et al 1993), the lowest thermal transition (Tm = 45.2°C) of DnaK was ascribed to unfolding of the ATPase domain, which was stabilized in the presence of ADP as indicated by a 9.2°C increase in its Tm. The middle transition (Tm = 58°C) was assigned to the peptide-binding domain and the transition with the highest Tm = 73.3°C contained contributions from both domains. The peptide-binding domain was considered to be essentially intact when the ATPase unfolding transition was completed. It was concluded that the unfolding of DnaK resembles other multidomain proteins in which intermediate states consisting of molecules with folded and unfolded domains exist. It is difficult to compare the calorimetric study of DnaK with the results reported herein for Hsc70 because the buffers used were qualitatively different. And more importantly, the denaturation of DnaK is reversible while for Hsc70 denaturation is irreversible. For DnaK, a glycine buffer (pH 9.0) was used and Mg^{+2} was included only when nucleotides were used. Our Hepes buffer (pH 7.0) was more complex and included Mg^{+2} and K^{+} which is required for both DnaK and Hsc70 function (Palleros et al 1993; Wilbanks and McKay 1995). This difference might explain why an exothermic transition was not detected in the 30–40°C range for DnaK and why multiple endothermic transitions were obtained. It is possible that the DnaK domains were uncoupled and behaved independently in the buffer used. Alternatively, the DnaK domains may not be as tightly coupled through the C-terminal

regulatory domain as in Hsc70. DnaK does not end in EEVD, which is required for the functional coupling of the ATPase and peptide-binding domains of Hsc70 (Freeman et al 1995). Interestingly, Palleros and co-workers (1992) observed a biphasic transition using circular dichroism spectroscopy to monitor DnaK thermal unfolding, but they argued against its interpretation as independent domain unfolding. Thermal unfolding of both DnaK and Hsc70 has been followed by intrinsic fluorescence, but comparisons are complicated by the presence of a single tryptophan in DnaK (W102) in the ATPase domain and the presence of an additional tryptophan (W580) in the C-terminal region of Hsc70 (Palleros and Fink 1992).

In our fluorescence studies and in the previous ones of Hsc70, a single transition corresponding to thermal unfolding was observed. Furthermore, the Tm and their shift to higher temperature due to the thermal stabilizing effect of a bound nucleotide were similar for both fluorescence measurements and scanning calorimetry. Only a single endothermic transition was detected by calorimetry, and minimally, this must include the ATPase domain. We cannot distinguish between further interpretations that the ATPase and peptide-binding domains are so closely coupled that this transition represents concerted denaturation of the entire molecule or that the domains denature independently with virtually identical transitions.

There is ample evidence of close coupling of the ATPase and peptide-binding domains of both DnaK and Hsc70, starting with the initial observations that they are peptide/unfolded protein-stimulated ATPases. For DnaK, binding of ATP induces conformational changes in both the ATPase and peptide-binding domains as measured by changes in trypsin sensitivity. In addition, the ATP-induced change in the fluorescence emission spectrum requires the presence of the peptide-binding domain, providing more evidence of domain interactions (Buchberger et al 1995). A conformational change in Hsc70 induced by peptide-binding has been detected by circular dichroism (Park et al 1993), and conformational changes in the ATPase domain upon ATP binding have been detected by tryptophan fluorescence and solution small-angle X-ray scattering (Ha and McKay 1995; Wilbank et al 1995). Exchange of ADP for ATP reduces the affinity of Hsc70 for unfolded proteins (Palleros et al 1993) and binding of apo *c* stimulates nucleotide exchange (Sadis and Hightower 1992). The EEVD domain of Hsc70 is needed to couple these two domains functionally, and by inference to transmit the effects of a conformational change in one domain to the other.

Interestingly, binding of the unfolded substrate reduced, carboxymethylated α-lactalbumin to DnaK is temperature-dependent. It increases from 20°C to a maximum at 40°C followed by a sharp decrease as temperature increases to 50°C. The calculated activation energy of 12.4 kcal/mol is relatively high (Palleros et al 1992). Few studies have addressed the temperature dependence of Hsc70 binding to substrates. Using a thermolabile mutant of *Staphylococcus* nuclease with a t_m of 30°C, Paleros and co-workers (1991) found that binding was activated between 25 and 30°C and increased up to 40°C. Interpretation of this experiment is complicated by the fact that both thermal unfolding of the substrate and thermal activation of Hsc70 probably occurred simultaneously. Others have noticed that activation of steroid hormone receptors by Hsc70 does not occur below 30°C. Formation of activated steroid hormone receptor requires bound steroid, a temperature of at least 25°C, ATP and Hsc70 (Smith et al 1992; Bohen et al 1995). In light of our findings, it is possible that this minimum temperature requirement is for conformational activation of Hsc70.

Herein, we have shown that binding of peptides and unfolded proteins to Hsc70 increased substantially above 30°C. Binding of both the hydrophobic peptide FYQLALT and the basic peptide NIVRKKK, which is likely to bind at a different site, increased with increasing temperature, indicating that more than the hydrophobic peptide-binding domain of Hsc70 changed conformation. Our model unfolded protein, apo *c*, contains sequences similar to both of these peptides, so apo *c* may bind to Hsc70 at more than one site. A temperature-dependent conformational change in Hsc70 was demonstrated by increased sensitivity to chymotryptic cleavage with increasing temperature, and this change was reversible. Previous studies have shown that several sensitive chymotryptic cleavage sites are located in the C-terminal region of Hsc70. The 60 kDa fragment detected in our study, is generated from a cleavage of an approximately 10 kDa segment from the C-terminus. Further degradation of the 60 kDa fragment to a 44 kDa fragment results in loss of substrate binding activity but not ATPase activity (Chappell et al 1987), indicating that this fragment represents the N-terminal domain. Based on the chymotryptic fragment patterns of Hsc70 incubated at 40°C and the activation of peptide-binding activity, we conclude that at least the C-terminal part of Hsc70 undergoes a temperature-dependent conformational change.

An exothermic transition was associated with the conformational change in Hsc70 that activated peptide binding. We hypothesize that this negative change in excess heat capacity may be due to hydration of polar and charged residues in the C-terminus. Other potential explanations such as oligomerization and aggregation of Hsc70 were ruled out. The most likely sequences to be involved in hydration are the α-helix (aa 512–536) identified by NMR (Morshauser et al 1995), and two predicted α-helices (aa 542–573, aa 587–605) that are shown in Figure 11.

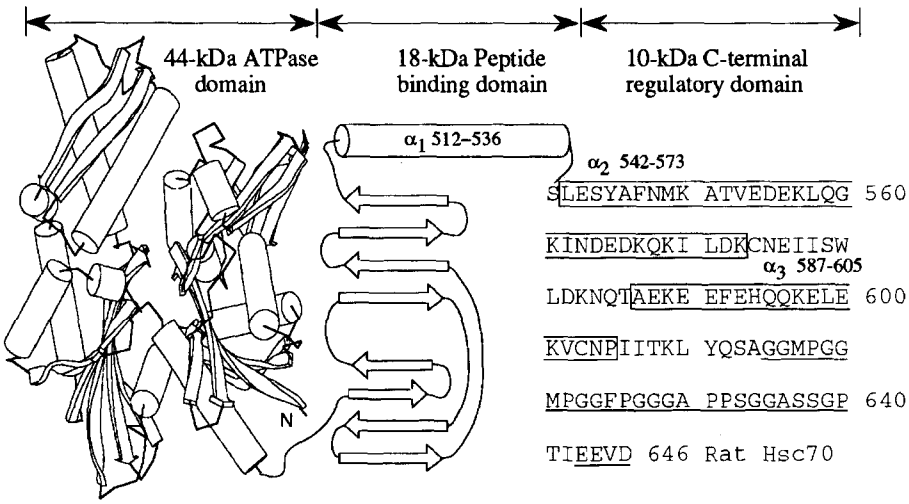

Fig. 11 Molecular structure of Hsc70. The 44-kDa ATPase domain (residues 1–386) was determined by X-ray crystallography and consists of four domains forming two lobes with a deep cleft between them in which nucleotide binds (McKay et al 1994). The 18-kDa peptide binding domain (residues 384–543) consists of two four-stranded antiparallel β-sheets and a single α-helix, determined using multidimensional NMR (Morshauser et al 1995). The C-terminal 10-kDa (residues 542–646) is predicted using the Garnier algorithm to be primarily α-helix followed by a gly/pro rich aperiodic segment next to the highly conserved EEVD terminal sequence (Hightower et al 1994). The two predicted α-helices are enclosed in the boxes. The gly/pro region and EEVD sequence are underlined.

These are the most hydrophilic sequences in the C-terminus, and hydration tendencies of amino acid side chains are approximated by hydrophilicity scales. In this region, seven charge motifs (E/D-X-E/D-K/R/H) occur within a 90-amino acid segment beginning at amino acid 514 (Sadis et al 1990a). Increased binding of peptide NIVRKKK supports our hypothesis that a conformational change exposes acidic residues. Interestingly, the second tryptophan residue (W580) occurs in a short hydrophobic segment of predicted random coil or loop secondary structure (IISWL) between the two predicted helices. Since no detectable change in intrinsic fluorescence occurred over the temperature range of the conformational change, this segment was probably not involved. The most likely participant may be hydrophilic helix 512–536 which immediately follows eight β-strands that include the most hydrophobic segments in the C-terminal region, the most likely binding site for peptide FYQLALT. Movement of this helix may expose its polar and charged residues along with those of the molecular surface to which it was salt-bridged, perhaps an adjacent hydrophilic helix (Takenaka et al 1995). This may either allow access to the hydrophobic peptide binding site or accompany a conformational change in the hydrophobic β-strand domain which makes it a higher affinity binding site for FYQLALT. Interestingly, ANS fluorescence did not detect increased accessibility to a hydrophobic environment in this temperature range. It is possible that this FYQLALT-binding site is not sufficiently hydrophobic to bind significant amounts of ANS at the concentrations normally used. In support of this possibility, millimolar concentrations of individual hydrophobic and aromatic amino acids, which are roughly similar in size to ANS, are required to stimulate the ATPase activity of DnaK (Richarme and Kohiyama 1993).

In addition to exothermic transition A, an endothermic transition was associated with unfolding of the ATPase domain, based upon its shift to a higher Tm in the presence of ATP. This transition was labeled B when nucleotide was not included and C when it was present. Since this was the only endothermic transition detected, we assume it also included unfolding of the peptide-binding domain. In the absence of ATP and apo *c*, denaturation of Hsc70 (endotherm B) had an apparent calorimetric enthalpy (ΔHc) of 0.68×10^5 cal/mol. The endothermic transition corresponding to denaturation of Hsc70-apo *c* had a modestly higher ΔHc (1.1×10^5 cal/mol) than denaturation of Hsc70 alone. Although the Tm of the transition was shifted down from 59°C to 56°C, this shift may simply be due to the superposition of exotherm D shifting the apparent mid-point of the transition. Hsc70 with bound apo *c* in the absence of added ATP had a larger exothermic transition suggesting that apo *c* binding increased the proportion of the higher affinity conformation.

Addition of ATP and apo *c* to Hsc70 shifted the Tm up to 63°C, the expected increase in thermal stability due to ATP binding and perhaps with a contribution by apo *c* as

well. The ΔHc for the endothermic transition was substantially increased to 2.5×10^5 cal/mol. The higher ΔHc for Hsc70 in the presence of ATP and apo *c* indicates a greater extent of denaturation, suggesting a more ordered native state for the complex. Interestingly, this change may be related to the decrease in the size of the exothermic transition, which may be due to the conversion of Hsc70 to a more ordered conformation in the presence of ATP with a lower affinity for apo *c*.

It has been hypothesized that a homeostatic mechanism based upon cellular levels of free Hsc70 and DnaK constitutes a molecular thermometer for sensing temperature changes (DiDomenico et al 1982; Craig and Gross 1991). In this model, these proteins are negative regulators of the heat shock response that bind heat shock transcription factors (Baler et al 1995; Nunes and Calderwood 1995). Higher temperatures would result in larger amounts of denatured proteins present in cells to bind free Hsc70 and DnaK, leaving more of the transcription factors in an unbound, activated state. McCarty and Walker (1991) showed that DnaK autophosphorylation and ATPase activity are stimulated in the 30–50°C range and proposed that these changes in DnaK activity may serve as a thermometer sensing temperature changes and activating the heat shock response of *Escherichia coli*. Our studies suggest that the concept of Hsc70 as a thermal sensor, an analogy which we prefer over molecular thermometer, may be usefully applied to a much broader temperature range in mammalian cells than previously considered. The model described above would apply to temperatures above the mammalian core body temperature of 37°C. However, if body extremities are considered, the normal temperature of mammalian tissues is more accurately described as a range of approximately 30–37°C. We propose that over this range, unfolded protein binding activity of Hsc70 is matched to demand for chaperoning. As tissue temperatures increase or decrease, demand including amounts of nascent polypeptides, amounts of partially folded proteins in folding pathways, participating in off-pathway interactions, or in transit to cellular compartments and the levels of active Hsc70 chaperones may change roughly in parallel. The proportion of inactive and active Hsc70 in free pools would be determined by the reversible, temperature-dependent conformational change demonstrated herein, i.e. the Hsc70 thermal sensor. Above 37°C, a new chaperoning demand, the accumulation of denatured proteins, is placed upon Hsc70 in addition to further increases in production of nascent polypeptides. It may be more than a coincidence that the maximum rate of conversion of inactive to active forms of Hsc70 in vitro occurred at 41°C, based on the Tm of the exothermic transition, and close to the upper limit for a survivable heat shock by mammalian cells.

The discovery of inactive and active chaperoning states of Hsc70 may bear on the altered cellular state known as acquired thermotolerance. Two states of thermotolerance have been described in mammalian cells, yeast and snails (Hall 1983; Boon-Niermeijer et al 1986; Laszlo 1988). One of these states depends upon the synthesis of heat shock proteins whereas the other can be established in the presence of cycloheximide. We suggest the following explanation for the latter type of thermotolerance. The inhibition of protein synthesis by cycloheximide blocks the temperature-dependent increase in one of the targets of Hsc70 activity, nascent polypeptide chains. Simultaneously, a rise in temperature conformationally activates more Hsc70, and the combined effect of this activation and the cycloheximide block is to rapidly increase the pools of active Hsc70, i.e. rapidly increase chaperoning capacity. More active Hsc70 would then be available to bind increasing amounts of thermally denatured cellular proteins as the temperature increased. In the absence of cycloheximide, increasing temperature would result in increased rates of nascent polypeptide production and accumulation of denatured protein, rapidly exhausting active pools of Hsc70 and hastening the induction of the heat shock response.

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