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TEMPERATURE INTERACTIONS OF THE MOLECULAR CHAPERONE Hsc70 FROM THE EURYTHERMAL MARINE GOBY *GILLICHTHYS MIRABILIS*

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Summary

Molecular chaperones participate in many aspects of protein biogenesis. Mechanistically, they recognize and bind to non-native proteins, prevent the aggregation of unfolded proteins and also, in some cases, facilitate refolding. Although a great deal is known about the cellular function of molecular chaperones in general, very little is known about the effect of temperature on molecular chaperones in non-model organisms, particularly in ectotherms that fold proteins under variable-temperature conditions in nature. To address this issue, we studied the temperature interactions of a major cytosolic molecular chaperone, Hsc70, from the eurythermal marine goby *Gillichthys mirabilis*. Using *in vitro* assays, we measured the intrinsic activity, unfolded-protein-stimulated activity, temperature sensitivity and heat stability of the ATPase activity of native Hsc70 purified from *G. mirabilis* white muscle. Similar to other chaperones in the 70 kDa heat-shock protein family, *G. mirabilis* Hsc70 exhibited a low intrinsic ATPase activity that was stimulated *in vitro* by the addition of unfolded protein. Across the environmentally relevant temperature range (10–35 °C), the ATPase activity of *G. mirabilis* Hsc70 displayed differential thermal sensitivity, with the greatest sensitivity occurring between 10 and 15 °C and the least sensitivity between 15 and 25 °C. In addition, the activity of Hsc70 was not significantly different between the unstimulated and unfolded-protein-stimulated treatments, suggesting that the ATPase activity and the peptide-binding domain of Hsc70 have similar thermal sensitivities *in vitro*. Finally, the thermal stability of Hsc70 ATPase activity greatly exceeded environmental temperatures for *G. mirabilis*, with activity up to 62.5 °C. Overall, the biochemical characterization of the ATPase activity suggests that, although Hsc70 is not an extraordinarily thermally stable protein, it is capable of protein chaperoning cycles even at the extremes of environmental temperatures encountered by *G. mirabilis* in nature.

Key words: molecular chaperone, Hsc70, ATPase, heat shock, temperature sensitivity, thermal stability, marine goby, *Gillichthys mirabilis*.

Introduction

In ecological physiology, temperature has been recognized as a dominant environmental factor and has been shown to influence biological systems at numerous levels of organization (Cossins and Bowler, 1987; Johnston and Bennett, 1996). The pervasive effects of temperature on living systems range from influencing the biogeographical distribution of ectothermic organisms (Hubbs, 1948; Fields et al., 1993; Marko, 1998; Sanford, 1999) to influencing the structure and function of proteins (for a review, see Somero, 1995). In the latter category, the temperature interactions of certain proteins, particularly enzymes, have received a great deal of attention in comparative biochemistry (e.g. Graves and Somero, 1982; McFall-Ngai and Horwitz, 1990; Dahlhoff and Somero, 1993; Fields and Somero, 1997; Holland et al., 1997; Jaenicke and Bohm, 1998). However, one important class of proteins, the molecular chaperones, has gone largely unstudied. The basic action of molecular chaperones is to recognize and bind selectively to unfolded, non-native proteins in a cell (for reviews, see Fink, 1999; Saibil, 2000). As such, molecular chaperones have a central role in the process of protein biosynthesis, and the temperature interactions of molecular chaperones therefore have significant biological consequences for organisms synthesizing proteins in variable-temperature environments. The larger goal of this study was to begin to characterize the relationship between temperature and molecular chaperone function as it occurs in ectothermic animals under environmentally relevant temperatures.

Although very little is known about the temperature relationships of molecular chaperones in ectotherms in natural populations, a great deal is known about the cellular role and biochemistry of the molecular chaperones in general (for reviews, see Hartl, 1996; Gething, 1997; Morimoto, 1998; Fink, 1999; Feder and Hofmann, 1999; Bukau, 1999). Molecular chaperones are ubiquitous and have been found in virtually every organism examined (Lindquist, 1986). The major classes of molecular chaperones include the six families

of heat-shock proteins (Hsps); the Hsps most involved in protein folding are members of the Hsp40, Hsp60 and Hsp70 families (Gething, 1997; Fink, 1999). Within each Hsp gene family, there are different forms of the protein; for example, most organisms have around 12 isoforms of the 70 kDa Hsp family, with members found in various cellular compartments (Gething, 1997). Molecular chaperones are also characterized as having different expression patterns; some isoforms are constitutively expressed (e.g. Hs*c*70) and others are stress-induced (e.g. Hs*p*70). Despite expression patterns that differ with respect to the physiological status of the cell, all chaperones function *via* a similar mechanism: molecular chaperones recognize exposed, hydrophobic regions of non-native proteins and bind specifically to partially folded proteins, preventing aggregation and misfolding (Hightower, 1991; Parsell and Lindquist, 1993; Hesterkamp and Bukau, 1998; Mogk et al., 1999). As a group, chaperones operate in a functional network in which some chaperones are 'holders' and others are 'folders' (Freeman and Morimoto, 1996: Buchberger et al., 1996; Johnson and Craig, 1997; Lüders et al., 1998; Veinger et al., 1998; Netzer and Hartl, 1998).

Despite major advances in the field of chaperone biology, our understanding of how Hsp function varies with environmental temperature in organisms in nature is tenuous. Nevertheless, a few points are clear. First, since all living organisms synthesize proteins, molecular chaperones across the phyla participate in protein folding over a wide range of temperatures – literally, the temperatures over which life exists, from approximately −2 °C to +113 °C (Somero, 1995). Second, from studies on model systems, it is clear that temperature affects not only the rate of chaperone activity (e.g. McCarty and Walker, 1991) but also the degree to which proteins will denature and aggregate (e.g. Mogk et al., 1999). Thus, organisms living in environments characterized by variable temperatures will have chaperoning needs that are almost certainly different from those of mammalian cells at 37 °C. Whether molecular chaperones from ectotherms, particularly eurythermic ectotherms, are structurally different so as to compensate for temperature effects is unknown.

This latter point begs the question of how the biochemical function of Hsps as molecular chaperones relates to the environmental temperature range over which an organism must perform physiologically. To this end, we have examined the temperature interactions of a molecular chaperone from a marine goby, *Gillichthys mirabilis*. *G. mirabilis* occurs from the Northern Gulf of California to Tomales Bay, California (Miller and Lea, 1972). In its estuarine habitat in Mexico, *G. mirabilis* encounters annual environmental temperatures ranging from 5 to 30 °C and appears to avoid higher temperatures by burrowing into the estuarine sediment (Barlow, 1961). Because of its extreme eurythermality, *G. mirabilis* was an ideal study organism for our investigation of molecular chaperone function in ectotherms. For biochemical characterization, we purified Hsc70, a cytoplasmic molecular chaperone, from *G. mirabilis* white muscle and tested the thermal sensitivity and stability of the ATPase activity, a weak activity exhibited by members of the 70 kDa Hsp gene family (McKay et al., 1994). Using *in vitro* ^{32}P-based ATPase assays, our results confirmed that purified native Hsc70 from *G. mirabilis* displayed biochemical activity across the range of temperatures encountered by *G. mirabilis* in nature and that the Hsc70 protein itself was thermally stable, displaying ATPase activity at temperatures that greatly exceeded physiologically relevant temperatures.

Materials and methods

Collection of study organism

Long-jawed mudsuckers (*Gillichthys mirabilis* Cooper, Gobiidae) were collected using baited minnow traps deployed in a negative estuary, Estero Morua, near Puerto Peñasco, Sonora, Mexico (31°9′N, 113°12′W) in the Northern Gulf of California during the winter of 1999. Specimens were stored at −70 °C prior to use in protein purification. Water temperature in the estuary was measured using Stowaway data loggers (Onset Instruments Inc.). The data loggers were secured to rebar at a height that allowed for constant submersion during tidal cycles.

Protein purification

Hsc70 was purified from *G. mirabilis* white skeletal muscle according to a modified two-step purification protocol described previously (Welch and Feramisco, 1985). For each purification procedure, 50–60 g of white muscle was homogenized in 5 volumes of homogenization buffer (10 mmol l^{-1} Tris-HCl, pH 7.5, 10 mmol l^{-1} NaCl, 0.1 mmol l^{-1} EDTA, 1.0 mmol l^{-1} phenylmethylsulfonyl fluoride, PMSF) in a Waring industrial blender. The homogenate was centrifuged at 10 700 ***g*** for 20 min at 4 °C. The supernatant was then applied to a DEAE anion-exchange column (2.5 cm×20 cm; DEAE Sephacel, Pharmacia Biotech) in DEAE column buffer (DEAE-CB; 10 mmol l^{-1} Tris-HCl, pH 7.5, 10 mmol l^{-1} NaCl, 0.1 mmol l^{-1} EDTA). The DEAE column was then washed with 200 ml of DEAE-CB containing 50 mmol l^{-1} NaCl and 15 mmol l^{-1} 2-mercaptoethanol. Elution of Hsc70 from the DEAE column was performed by washing the column with 150 ml of DEAE-CB containing 200 mmol l^{-1} NaCl and 15 mmol l^{-1} 2-mercaptoethanol. Fractions were pooled and then applied to an ATP–agarose affinity column (approximately 5 ml; Sigma). The ATP affinity column was washed with 45 ml of affinity column buffer (A-CB; 10 mmol l^{-1} Tris-HCl, pH 7.5, 10 mmol l^{-1} NaCl, 0.1 mmol l^{-1} EDTA, 15 mmol l^{-1} 2-mercaptoethanol, 3 mmol l^{-1} $MgCl_2$) followed by 50 ml of A-CB containing 0.5 mol l^{-1} NaCl and then with 45 ml of A-CB to remove the high concentration of salts. Low-molecular-mass chaperones were removed by washing the affinity column with 1 mmol l^{-1} GTP in A-CB. Hsc70 was eluted with 3 mmol l^{-1} ATP in A-CB, and the resulting Hsc70-containing fractions were pooled. The pooled fractions were concentrated using Centricon-30 centrifugal concentrators (Millipore) at 5000 ***g*** for 30 min. Protein content was determined using a modified Bradford assay (Coomassie

Plus; Pierce), and the purity of each preparation was determined by two-dimensional SDS–PAGE separation followed by silver staining (BioRad Silver Stain Plus kit). Western blotting (as described in Hofmann and Somero, 1995), using a monoclonal anti-Hsp70/Hsc70 primary antibody (MA3–001; Affinity Bioreagents), was performed to identify the purified protein.

Luciferase protection assays

Luciferase protection assays were performed using a modified protocol from Lu and Cyr (Lu and Cyr, 1998). Immediately prior to the assay, luciferase was diluted to 0.2 nmol l^{-1} in 100 µl of refolding buffer (10 mmol l^{-1} Mops, pH 7.2, 50 mmol l^{-1} KCl, 5 mmol l^{-1} $MgCl_2$, 1 mmol l^{-1} ATP) and then 1 µmol l^{-1} Hsc70, 0.2 µmol l^{-1} mammalian Hsp40 and 50 µmol l^{-1} ATP were added. These mixtures were incubated at 38 °C for 40 min. Activity was measured at 5 min intervals by combining 10 µl samples with 50 µl of luciferase assay reagent (Promega). Relative light units were then immediately measured using a luminometer (Turner Designs).

ATPase assays

ATP hydrolysis was assayed by measuring the formation of [α-^{32}P]ADP from [α-^{32}P]ATP. *In vitro* activity assays were assembled on ice and consisted of 2 µg of Hsc70, 50 µl of assay buffer (AB; 20 mmol l^{-1} Hepes-KOH, pH 7.0, 25 mmol l^{-1} potassium chloride, 1.0 mmol l^{-1} $(NH_4)_2SO_4$, 2 mmol l^{-1} magnesium acetate, 0.1 mmol l^{-1} EDTA, 1.0 mmol l^{-1} dithiothreitol and 50 µmol l^{-1} ATP) and 3.7×10^5 Bq of [α-^{32}P]ATP. In some assays, reduced carboxymethylated α-lactalbumin (RCMLA; Sigma), an unfolded stable protein, was used as a target to test the stimulation of the intrinsic ATPase activity of Hsc70. Duplicate or triplicate samples were incubated with 80 µmol l^{-1} RCMLA for selected times and temperatures, and 1 µl samples were removed for nucleotide separation. Nucleotides were separated by thin-layer chromatography (TLC) on Baker·flex PEI-cellulose plates (J. T. Baker) in 0.5 mol l^{-1} LiCl and 1 mol l^{-1} formic acid (O'Brien and McKay, 1993). Levels of product, [α-^{32}P]ADP, were quantified using a Storm PhosPhor Imager system (Molecular Dynamics), and densitometric analysis was performed using ImageQuant software. For all figures in the Results section, the resulting densitometric data are expressed as absorbance volume, the mean pixel intensity of a standard area defined and analyzed by the ImageQuant software.

The effect of temperature on Hsc70 ATPase activity was expressed as a Q_{10} value; Q_{10} was calculated using the van't Hoff equation as follows:

$$Q_{10} = (k_2/k_1)^{10/(t_2-t_1)},$$

where k_1 and k_2 are rate processes and t_1 and t_2 are temperatures. A Q_{10} value is the ratio of the rate of a reaction at a given temperature to its rate at a temperature 10 °C lower (see Randall et al., 1997).

Results

Environmental temperature data

To confirm the environmental temperature range for *Gillichthys mirabilis*, submersible data loggers were deployed in channels of Estero Morua, where the fish for this study were collected. Fig. 1 shows a sampling of the water temperature data collected in 1999 and 2000. On average, water temperature in the estuary ranged from 5 to 30 °C during tidal cycles in the winter months and from 18 to 36 °C during tidal cycles in the summer months (Fig. 1A–C).

Hsc70 purification

The results of the purification of Hsc70 from *G. mirabilis* white muscle are shown in Fig. 2. Following DEAE anion-exchange and ATP affinity column chromatography, the purity of each preparation was determined by SDS–PAGE separation followed by silver staining (Fig. 2A). Although the purity of each preparation varied slightly, with the occasional presence of low-molecular-mass contaminants, Hsc70 was routinely purified to greater than 95% homogeneity. Prior to any functional analyses, the identity of the chaperone was verified using western blotting with an antibody specific to 70 kDa Hsp gene family members (Fig. 2B). In addition, the number of isoforms obtained during the purification process was assessed by two-dimensional SDS–polyacrylamide gel electrophoresis followed by western blotting (as described by Carpenter and Hofmann, 2000). The purification process routinely yielded a single isoform of 70 kDa (data not shown). Typically, the

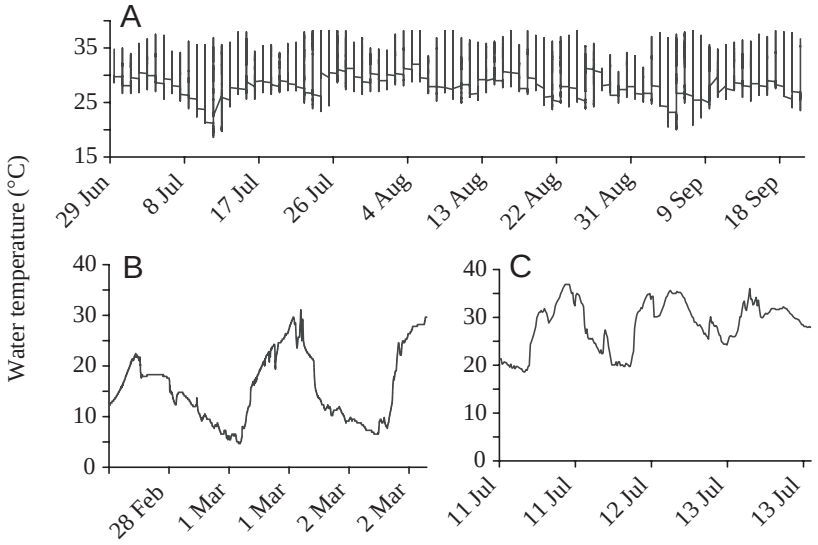

Fig. 1. Water temperature for the estuary inhabited by *Gillichthys mirabilis*. (A) Seawater temperature readings taken during the summer from 29 June to 19 September 2000 and (B) during the winter from 28 February to 3 March 1999. (C) Seawater temperature during tidal cycles in the estuary between 11 July and 13 July 2000. Temperature data were collected using programmable Stowaway data loggers (Onset Instruments Inc.).

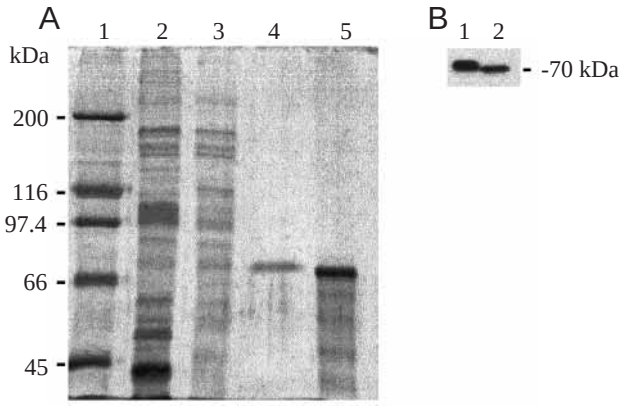

Fig. 2. Analysis of sequential steps in the purification of *Gillichthys mirabilis* Hsc70. (A) Silver-stained gel of various stages in the purification process. For silver staining, 5 µg of total protein was applied to lanes 1–4 and separated by electrophoresis on a 7.5 % SDS–polyacrylamide gel. Lanes are as follows: (1) silver-stained protein molecular mass standards; (2) 10 700 ***g*** supernatant from *G. mirabilis* white muscle; (3) pooled eluant from the DEAE anion-exchange column; (4) pooled eluant from the ATP affinity column (purified Hsc70 preparation); (5) 0.1 µg of an Hsc70 standard purified from bovine brain. (B) Western blot detection of purified 70 kDa heat-shock proteins using a rat monoclonal anti-Hsp70 antibody. Lane 1, 0.1 µg of bovine brain Hsc70 standard; lane 2, 5 µg of Hsc70 purified from *G. mirabilis*.

amount of Hsc70 purified was less than 0.1 % of the total protein content of the 10 700 ***g*** supernatant.

Luciferase protection assay

To demonstrate that *G. mirabilis* Hsc70 possessed chaperone activity *in vitro*, we tested the ability of Hsc70 to protect luciferase from thermal denaturation (Fig. 3). The results of the luciferase protection assay indicate that *G. mirabilis* Hsc70 was able to effectively maintain luciferase in a competent form and to considerably extend the half-life of the enzyme's activity at 38 °C. During the assay, luciferase incubated alone, or in the presence of bovine serum albumin, lost 90 % of its measurable activity after 10 min of incubation at 38 °C (Fig. 3). However, in the presence of Hsc70 and the co-chaperone Hsp40, loss of 90 % activity did not occur until 30 min into the 38 °C incubation period.

Measurement of Hsc70 ATPase activity

The *in vitro* measurement of Hsc70 ATPase activity is shown in Fig. 4. For all ATPase assays, background ATP hydrolysis was determined in assays containing only [^{32}P]ATP, and these background values were subtracted from values measured in assays containing Hsc70. Overall, the intrinsic ATPase activity of Hsc70 was generally low (Fig. 4) and reached maximal values after 30 min (data not shown). Assays were conducted for longer periods, and the activity reached maximal levels by 30 min and remained unchanged up to 2.5 h (data not shown). The intrinsic ATPase activity was stimulated by the addition of RCMLA, a stable unfolded protein (Fig. 4). After incubation at 23 °C for 30 min, the activity of Hsc70 in the presence of 80 µmol l^{-1} RCMLA increased by an average of 28 % above the intrinsic activity (27.99±4.7 %, mean ± S.E.M., N=3 assays). Although the absolute activity of the Hsc70 ATPase varied among purifications, each preparation displayed similar relative changes in ATPase activity in response to RCMLA addition (Fig. 4).

Effects of temperature on Hsc70 ATPase activity

The effects of temperature on both the unstimulated and substrate-stimulated forms of Hsc70 measured over the ecological temperature range of *G. mirabilis* are shown in Fig. 5. Similar to the results shown in Fig. 4, the presence of RCMLA stimulated ATP hydrolysis by Hsc70. However, there was no marked difference in temperature effects on the rates of hydrolysis for unstimulated and unfolded-protein-stimulated Hsc70 (Fig. 5). Q_{10} values calculated for the unstimulated and RCMLA-stimulated rates of ATPase activity (as measured by ATP hydrolysis) showed little deviation from each other, as shown in Table 1. Although the treatments did not differ, there was a difference in overall Hsc70 activity as a function of temperature (Fig. 5). Hsc70 ATPase activity displayed a high temperature sensitivity between 10 and 15 °C, with Q_{10} values of 12.07 and 16.8 for the unstimulated and RCMLA-stimulated treatments, respectively (Table 1). In contrast, Hsc70 ATPase activity displayed thermal insensitivity between the temperatures 15 and 25 °C, with Q_{10} values of 1.0 and 1.09 for the unstimulated and RCMLA-stimulated treatments,

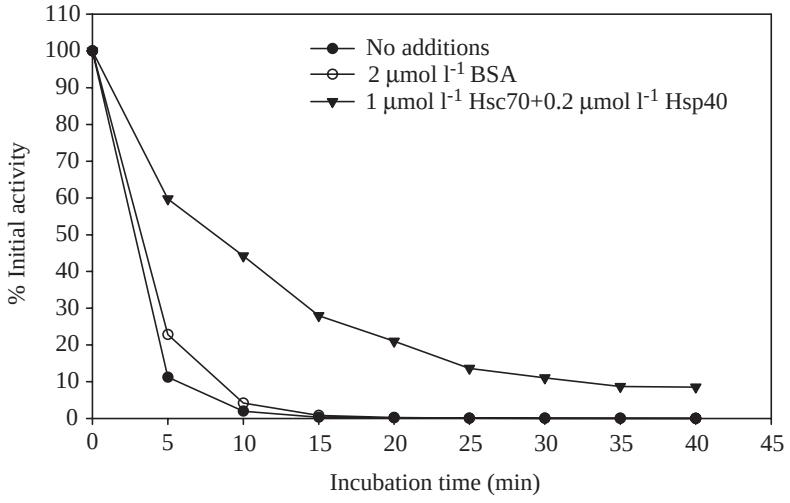

Fig. 3. Prevention of thermal denaturization of luciferase at 38 °C by *Gillichthys mirabilis* Hsc70. Samples of luciferase (0.2 nmol l^{-1}) were incubated at 38 °C for 40 min, and 10 µl aliquots were removed and placed in 50 µl of LAR (Promega; relative light units were measured in a luminometer). The percentage of initial activity remaining after the indicated incubation period is given on the *y*-axis. BSA, bovine serum albumin.

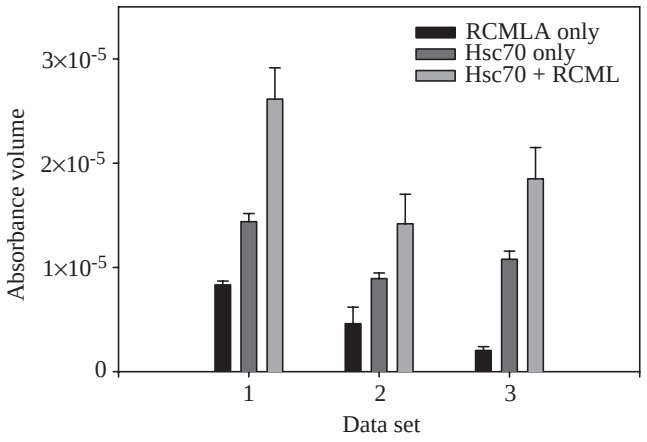

Fig. 4. Effects of substrate on the ATPase activity of Hsc70 purified from *Gillichthys mirabilis*. On the *x*-axis, data sets 1–3 represent ATPase activity measurements for three independent Hsc70 preparations. Duplicate samples were incubated with 3.7×10^5 Bq of [α-^{32}P]ATP at 23 °C, and the amount of ATP hydrolysis was measured after 30 min by thin-layer chromatography. Values are the mean pixel absorbance volume (background-corrected) + variance (*N*=2). Black, dark grey and light grey columns represent assays composed of 80 μmol l^{-1} RCMLA in assay buffer (AB), 2 μg of Hsc70 in AB and 2 μg of Hsc70 plus 80 μmol l^{-1} RCMLA in AB, respectively. See Materials and methods for a full description of the assay buffer components.

Table 1. *Calculated Q_{10} values for unstimulated and RCMLA-stimulated rates of ATP hydrolysis by* Gillichthys mirabilis *Hsc70*

Temperature range (°C)	Q_{10} for (unstimulated Hsc70)	Q_{10} for (RCMLA-stimulated Hsc70)
10–15	12.07	16.8
15–25	1.0	1.09
25–35	1.92	2.15
35–45	1.5	1.31

respectively (Table 1). The temperature sensitivity of Hsc70 ATPase activity increased over the range 25–35 °C with a Q_{10} of 1.92 and 2.15, before thermal sensitivity again declined over the temperature range 35–45 °C, with Q_{10} values of 1.5 and 1.31 for stimulated and RCMLA-stimulated treatments, respectively (Table 1).

In addition to assessing the thermal sensitivity of Hsc70 ATPase activity at ecological temperatures, we also determined the intrinsic thermal stability of the protein as measured by the heat stability of the *in vitro* ATPase activity (Fig. 6). Following 60 min incubations at each test temperature, Hsc70 displayed nearly unchanged ATPase activity between 50 and 62.5 °C (Fig. 6). However, ATPase activity declined precipitously at 65 °C and showed a similar rapid loss of activity at the higher temperature of 80 °C (Fig. 6).

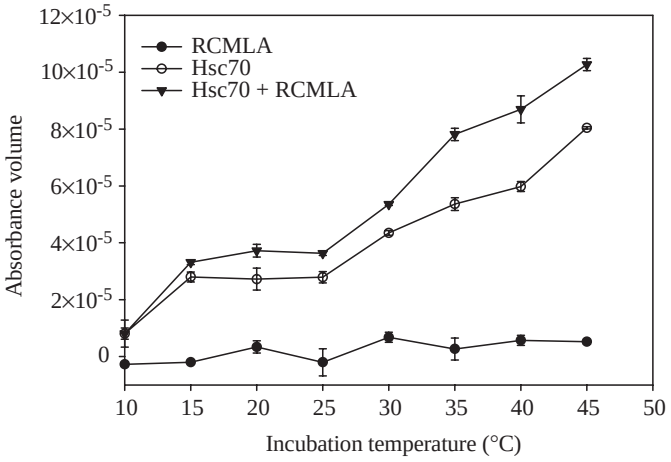

Fig. 5. Effects of temperature on the ATPase activity of unstimulated and RCMLA-stimulated Hsc70 purified from *Gillichthys mirabilis* white muscle. Assays, containing 2 μg of Hsc70 plus 3.7×10^5 Bq of [α-^{32}P]ATP in assay buffer, were incubated in the absence (open circles) or presence (triangles) of 80 μmol l^{-1} RCMLA at 5 °C increments in a temperature gradient that ranged from 10 to 45 °C. Following a 60 min incubation, triplicate 1 μl samples were removed from each assay tube and analyzed for ATP hydrolysis by thin-layer chromatography. The black circles represent a control assay containing only 80 μmol l^{-1} RCMLA. Each data point represents the mean pixel absorbance volume (background-corrected) ± S.D. (*N*=3). See Materials and methods for a full description of the assay buffer components.

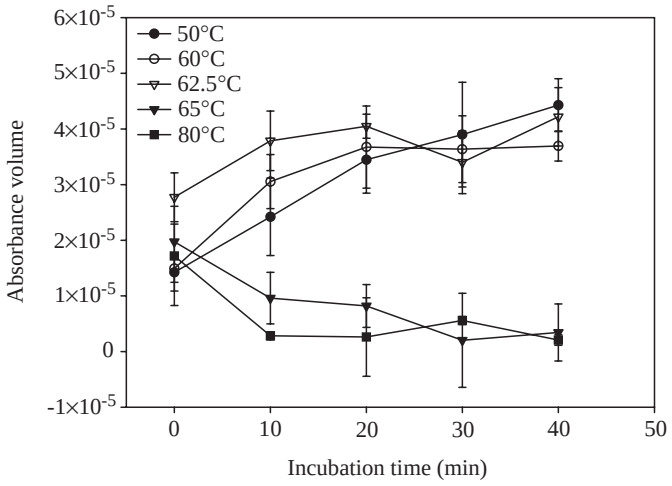

Fig. 6. Thermal stability of the ATPase activity of *Gillichthys mirabilis* Hsc70. Native Hsc70 was incubated in assay buffer at 50, 60, 62.5, 65 and 80 °C for 40 min. During the 40 min incubation period, 2 μg samples were taken in triplicate every 10 min, combined with 3.7×10^5 Bq of [α-^{32}P]ATP in 50 μl of assay buffer and placed in a water bath at 23 °C. The amount of ATP hydrolysis was measured after 15 min by thin-layer chromatography. Values are mean pixel absorbance volume (background-corrected) ± S.D. for *N*=3 samples for each time point. See Materials and methods for full a description of the assay buffer components.

Discussion

To determine the temperature interactions of a molecular chaperone from a eurythermal ectotherm, we purified native Hsc70 from white skeletal muscle of the eurythermal goby *Gillichthys mirabilis* and tested the thermal sensitivity of the ATPase activity. It should be noted that we opted to work on a native chaperone, rather than a recombinant form, because comparisons of other chaperones have shown a great deal of difference in the ATPase activity between native and recombinant proteins (e.g. Blond-Elguindi et al., 1993). Prior to testing the thermal dependence of Hsc70 function, we first demonstrated that Hsc70 had *in vitro* chaperoning activity in a luciferase protection assay in which Hsc70 was able to slow the thermal denaturation of luciferase *in vitro* (Fig. 3). Protection assays are routinely used as a means to verify the ability of a presumptive molecular chaperone to bind a target protein and slow the rate of thermal inactivation of the target protein (e.g. Lee et al., 1995). In addition, we confirmed that *G. mirabilis* Hsc70 was an unfolded-protein-stimulated ATPase (Hightower et al., 1994). As observed for other members of the Hsp70 family, *G. mirabilis* Hsc70 was stimulated by the unfolded protein substrate RCMLA (Fig. 4). Once we had demonstrated that our Hsc70 preparations had chaperone activity, we proceeded to examine the profile of temperature dependence of Hsc70 function. For this aspect of the investigation, the ATPase assay was a good biochemical indicator of thermal effects on the function of Hsc70 because the ATPase activity has been shown to regulate the binding and release of protein substrates (Liberek et al., 1991). Our results showed that *G. mirabilis* Hsc70 possesses an ATPase activity that was functional across a range of environmentally relevant temperatures and displayed an approximately fourfold increase in activity between 10 and 35 °C (Fig. 5). Overall, the temperature sensitivity of Hsc70 function was generally within the expected range of physiological rate changes (i.e. Q_{10} between 2 and 4; except for the range 10–20 °C). Furthermore, as a protein that possesses two domains, an ATPase domain and a peptide-binding domain (Hightower et al., 1994), the temperature sensitivity of the intrinsic ATPase activity was not significantly different from the unfolded-protein-stimulated ATPase activity (Fig. 5). Finally, we found that Hsc70 from *G. mirabilis* was a relatively thermally stable protein and lost ATPase activity only at 65 °C (Fig. 6).

Overall, Hsc70 from *G. mirabilis* displayed biochemical characteristics that are consistent with those reported for other Hsc70 homologs. Similar to Hsp70-class chaperones from *Escherichia coli*, *Thermus thermophilus* and homeothermic vertebrates (McCarty et al., 1995; Motohashi et al., 1990; Motohashi et al., 1996; Russell et al., 1998; Zylicz et al., 1983), Hsc70 from *G. mirabilis* had a low intrinsic ATPase activity in the absence of its substrate, unfolded proteins (Fig. 4). However, the ATPase activity of the *G. mirabilis* Hsc70 was stimulated by 28 % by the addition of reduced carboxymethylated α-lactalbumin (RCMLA), a stable unfolded form of α-lactalbumin that acts as a substrate for Hsc70 *in vitro* (Palleros et al., 1991; Fig. 4). Other studies of Hsp70-class chaperones have demonstrated unfolded-protein-dependent activation of the ATPase activity *in vitro*. Human Hsp70 was activated by 40 % by the addition of RCMLA (Freeman et al., 1995), and bovine brain Hsc70 exhibited a twofold increase in ATPase activity in response to apocytochrome *c* (Sadis and Hightower, 1992). A general explanation for the stimulatory effect of unfolded proteins on Hsc70 ATPase activity can be found in the kinetics of the chaperoning cycle. Although the precise nature of Hsc70 activity is still a subject of debate, the ATPase cycle is closely coupled to the binding and release of unfolded peptides during which the hydrolysis of ATP to ADP fosters a more stable complex of Hsc70 and substrate peptides (for a review, see Hightower et al., 1994). This interaction creates the driving force for increased hydrolysis in the presence of unfolded peptides (Flynn et al., 1989; Jordan and McMacken, 1995).

A second significant finding of this study was that *G. mirabilis* Hsc70 displayed an ATPase activity that was moderately temperature sensitive and that Hsc70 was functional over the ecological temperature range of this species of goby (Fig. 5). Hsc70 ATPase activity increased approximately fourfold between 10 and 35 °C, with the maximal rate occurring at 45 °C (Fig. 5). Interestingly, the preferred temperature for *G. mirabilis* is reported to be 23 °C (Barlow, 1961), and the ATPase activity of Hsc70 displayed very low thermal sensitivity between 15 and 25 °C (Fig. 5). The activity of Hsc70 ATPase increased between 25 and 45 °C (Fig. 5), although it should be noted that 40 °C and above is a thermal extreme that we believe *G. mirabilis* is unlikely to experience in the field (see Fig. 1A). Finally, the ATPase activity of Hsc70 was measured under two conditions, an intrinsic rate that was measured in the absence of substrate and a substrate-stimulated rate in the presence of RCMLA (Fig. 5). The relative similarities of the intrinsic and the RCMLA-stimulated ATPase activity of Hsc70 indicate that the ATPase domain and the peptide-binding domain of *G. mirabilis* Hsc70 are not different. Thus, there was no measurable differential thermal sensitivity of Hsc70 when both the ATP- and substrate-binding domains were taken into consideration.

Although there are few data sets with which to compare, members of the 70 kDa Hsp gene family have been shown to display thermal sensitivity. In *E. coli*, the ATPase activity of DnaK, the bacterial Hsp70 homolog, varied over the range of physiologically relevant temperatures for growth of the bacterium. However, the ATPase activity of DnaK exhibited a significantly stronger temperature dependence than Hsc70 from *G. mirabilis* and increased 80-fold across the temperature range 20–53 °C (McCarty and Walker, 1991). Other studies of DnaK from another species, the thermophilic bacterium *Thermus thermophilus*, have not found such extreme thermal sensitivity and reported a modest threefold increase in ATPase activity between 25 and 75 °C (Klostermeier et al., 1998).

In addition to displaying a degree of thermal sensitivity, Hsc70 from *G. mirabilis* was a relatively thermally stable protein (Fig. 6). During incubations across the temperature range 50–80 °C, the ATPase activity of Hsc70 was functional

up to 62.5 °C (Fig. 6). However, activity declined abruptly at 65 °C, and the protein was completely non-functional at higher temperatures (Fig. 6). Similar results have been recorded for DnaK from *E. coli*, in which ATPase activity declined abruptly between 47.5 and 53 °C (McCarty and Walker, 1991). These data indicate that the molecular chaperone under study here was not extraordinarily thermally stable compared with other cellular proteins. For comparison, Fields and Somero (Fields and Somero, 1997) have shown that *G. mirabilis* A_4-lactate dehydrogenase rapidly lost activity between 50 and 55 °C.

Ultimately, the biological consequences of folding proteins in a variable-temperature environment will depend on the interactions between the principal components in the chaperone cycle: the non-native protein and the chaperone. From the perspective of the non-native protein, higher relative temperatures will tend to increase the 'breathing' of proteins and result in the destabilization of pre-existing proteins in the cell. In addition, since hydrophobic interactions are strengthened as temperature increases, nascent polypeptides will increasingly aggregate at elevated temperatures. Consequently, thermal stress can be viewed as a 'double-jeopardy' situation for the cell in which the levels of non-native proteins and the likelihood of protein aggregation both increase as temperature rises. From the perspective of the molecular chaperone, temperature will interact with both the rate of the chaperone cycle and the nature of substrate binding. The efficacy of a molecular chaperone could be influenced directly by temperature *via* the thermal sensitivity of the ATPase activity. Alternatively, temperature could alter the way in which molecular chaperones bind target molecules. Since molecular chaperones interact with unfolded proteins *via* hydrophobic interactions (e.g. Rüdiger et al., 1997), elevated temperature will strengthen target binding and lower temperatures will weaken hydrophobic interactions.

Taken together, the above observations suggest that there are numerous functional properties of a molecular chaperone that could be modulated by environmental temperature. The results presented here provide data regarding the interaction between temperature and the biochemical function of Hsc70 from a non-model organism, the eurythermal goby *Gillichthys mirabilis*. Specifically, the ATPase activity of Hsc70 was temperature sensitive, with increasing activity over the environmentally relevant temperature range for this species, indicating that the molecular chaperone cycle would accelerate as temperature-driven protein denaturation increased. Whether this increase is sufficient to accommodate the increasing number of non-native, and potentially aggregating, proteins is currently unknown.

Our data, however, do not address another major functional aspect of a molecular chaperone cycle. Namely, how does Hsc70 interact and bind non-native proteins as a function of temperature? Freeman and Morimoto (Freeman and Morimoto, 1996) have shown that the refolding activity of human Hsp70 was inhibited at temperatures above 41 °C, a threshold only 4 °C above human body temperature. Whether target binding and protein refolding by molecular chaperones from ectothermic animals are similarly affected by temperature is not known. If there are temperature-dependent differences in the protein-refolding ability of ectothermic chaperones, an important question is whether function varies in a manner that correlates with species' evolutionary thermal history. In future experiments, we hope to address this and other unanswered questions regarding the 'holding' and 'folding' of proteins in nature.

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