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The molecular chaperone Hsc70 from a eurythermal marine goby exhibits temperature insensitivity during luciferase refolding assays

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Abstract

The role and function of molecular chaperones has been widely studied in model systems (e.g. yeast, *Escherichia coli* and cultured mammalian cells), however, comparatively little is known about the function of molecular chaperones in eurythermal ectotherms. To investigate the thermal sensitivity of molecular chaperone function in non-model ectotherms, we examined the in vitro activity of Hsc70, a constitutively expressed member of the 70-kDa heat-shock protein gene family, purified from white muscle of the eurythermal marine goby *Gillichthys mirabilis*. The activity of *G. mirabilis* Hsc70 was assessed with an in vitro refolding assay where the percent refolding of thermally denatured luciferase was monitored using a luminometer. Assays were conducted from 10–40 °C, a range of temperatures that is ecologically relevant for this estuarine species. The results showed that isolated Hsc70 displayed chaperone characteristics in vitro, and was relatively thermally insensitive across the range of experimental temperatures. In addition, the thermal stability of the luciferase refolding capacity of Hsc70 was relatively stable, with refolding activity occurring as high as 50 °C. Overall, Hsc70 from *G. mirabilis* displayed thermal properties in vitro that suggest that the molecular chaperone is capable of binding and chaperoning proteins at temperatures that the goby encounters in nature.

Keywords: *Gillichthys mirabilis*; Heat-shock protein; Hsc70; Luciferase; Molecular chaperone; Protein folding; Temperature; Refolding

1. Introduction

Temperature has pervasive effects on organisms in nature (Hochachka and Somero, 2002); these range from temperature effects on molecular processes to a role in defining species' biogeographical range boundaries. At the molecular level, protein structure and function are greatly influenced by temperature (for a review see Somero, 1995). Although many features of protein structural and functional biology have been examined in the context of temperature adaptation and acclimation, one unexplored area is the effect of temperature on molecular chaperones, proteins that stabilize non-native proteins and maintain proteins on productive folding pathways during protein synthesis (Fink, 1999; Frydman, 2001; Hartl and Hayer-Hartl, 2002). In vivo, individual molecular chaperones identify non-native proteins, bind to them and prevent protein aggregation, an outcome that is highly cytotoxic (e.g. Mogk et al., 1999).

In nature, this activity of molecular chaperones occurs across a broad range of temperatures, literally the temperature range over which life exists and proteins are synthesized—from −1.86 °C in Antarctic species (Eastman, 1993) to over +121 °C in extremophilic microbes (Kashefi and Lovley, 2003). In addition to functional conservation in situ, the proteins exhibit a great deal of sequence similarity amongst species. For example, *Mytilus edulis* hsp70 (partial coding sequence) shares 79% sequence similarity with *Crassostrea gigas* (Pacific oyster) hsp70 (complete coding sequence) (GenBank accession number AF172607 and AF144646, respectively). Taken together, these two observations argue that molecular chaperones are a class of biomolecules that are either very thermally insensitive across taxa, or have undergone significant molecular evolution in order to preserve function at different evolutionary temperatures, a scenario similar to that indicated by LDH sequence analysis from some Antarctic teleost species (Fields and Somero, 1998).

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Thus, the goal of the current study was to explore the thermal sensitivity of molecular chaperones from ectothermic animals, and specifically, to test whether a molecular chaperone, Hsc70, from the 70-kDa heat-shock protein gene family had temperature-sensitive protein folding activity.

Despite a great deal of knowledge about molecular chaperones such as Hsc70 (McKay et al., 1994; Hightower et al., 1994; Gething, 1997; Fink, 1999), relatively little is known about the biochemical function of Hsps as molecular chaperones in non-model systems (for review see Feder and Hofmann, 1999). In particular, there are significant gaps in our knowledge regarding the thermal sensitivities of molecular chaperones in ectothermic organisms, many of whom are eurythermic and therefore fold proteins across a broad range of body temperatures. There is evidence that temperature influences molecular chaperone activity (McCarty and Walker, 1991; Place and Hofmann, 2001). However, since temperature variation may influence the interaction of a molecular chaperone with its target, a non-native or degraded protein, the efficacy of chaperoning activity may be influenced. For example, low temperatures tend to weaken hydrophobic interactions and thus, in a eurythermal animal, the folding activity of a single chaperone might be significantly altered depending on body temperature. Essentially, since most of the data available on molecular chaperone function (in vitro folding activity, interaction with different folding targets, etc.) comes from mammalian cells, there are few studies to offer an explanation of how temperature affects molecular chaperone function in organisms that fold proteins over a wide-range of physiologically-relevant temperatures. Thus, the question of whether molecular chaperones in ectotherms are differentially adapted as compared to mammalian molecular chaperones goes unanswered.

To examine the temperature sensitivity of a molecular chaperone in a differentially-thermally adapted organism, this study examined the effect of temperature on the molecular chaperone Hsc70 from *Gillichthys mirabilis*, a eurythermal marine goby that inhabits estuaries, coastal sloughs and lagoons ranging from north of San Francisco, California (Tomales Bay), to Bahía Magdalena (Thomson et al., 2000). *G. mirabilis* was an ideal organism with which to study the function of molecular chaperones because this goby experiences a wide range of ecological temperatures; in summer seawater temperature in the estuaries often exceed 38 °C, and in winter can be as low as 5 °C (Place and Hofmann 2001). Similarly, Hsc70 was an ideal experimental molecule. Hsc70 is a much-studied molecular chaperone in other model study systems, and therefore a great deal is known about its function and structure (Hightower et al., 1994; McKay et al., 1994). A primary function of Hsc70 is to prevent the misfolding of translating polypeptides (Minami et al., 1996; Frydman, 2001; Hartl and Hayer-Hartl, 2002), and this activity has been assessed using in vitro assays in other systems (e.g. Freeman and Morimoto, 1996). In addition, Hsc70 can be purified from tissues using standard low-pressure chromatography methods, resulting in a native protein (i.e. folded in situ and not a recombinant protein). Finally, as a 'generalist' chaperone, Hsc70 is known to interact with a wide range of non-native proteins, whereas other molecular chaperones, such as Hsp90, display greater specificity (for reviews see Fink, 1999; Frydman, 2001). Thus, Hsc70 was an ideal candidate for in vitro assays, since numerous generic proteins (e.g. luciferase, citrate synthase, lactate dehydrogenase, glyceraldehydes-3-phosphate dehydrogenase) could be employed as target molecules in refolding assays.

In order to test the effects of temperature on Hsc70 from *G. mirabilis*, Hsc70 was purified from skeletal white muscle and in vitro thermal sensitivity assays were conducted. Using thermally denatured luciferase as a target molecule, the protein refolding capacity of native Hsc70 was assessed at ecologically relevant temperatures for *G. mirabilis*. The results illustrate for the first time the thermal insensitivity of a molecular chaperone from a non-model, eurythermal ectotherm.

2. Materials and methods

2.1. Collection of study organism

The eurythermal marine goby, *G. mirabilis*, was collected using baited traps in a negative estuary, Estero Morua, near Puerto Peñasco, Sonora, Mexico (31° 9′ N, 113°12′ W) in the Northern Gulf of California (summer 2000). Specimens were euthanized, white muscle dissected and stored at −80 °C (UCSB IACUC Protocol No. 634).

2.2. Protein purification

The molecular chaperone, Hsc70, was purified from *G. mirabilis* using a two-step method previously reported; for full protocol and buffer constituents see Place and Hofmann (2001). Briefly, using white skeletal muscle from *G. mirabilis*, anion exchange chromatography and ATP affinity chromatography were used to purify Hsc70 to ≥95% homogeneity. Hsc70 protein content was determined using a modified Bradford assay (Pierce) and purity was confirmed with silver staining (BioRad Silver Stain Plus Kit).

2.3. Luciferase assay and thermal denaturation

For refolding assays, the protocol was a modification of the methods reported by Lu and Cyr (1998). Recombinant luciferase (Promega, Madison, WI, USA) was diluted to 2.19×10^{-10} M with refolding buffer [25 mM HEPES, 50

mM KCl, 5 mM $MgCl_2$, 2 mM ATP, pH 7.4]. For thermal denaturation, 0.2 μl of diluted luciferase was added to denaturing buffer [25 mM HEPES, 50 mM KCl, 5 mM $MgCl_2$, 5 mM dithiothreitol at pH 7.4] for a total volume of 50 μl and incubated at 38 °C for 2 h. Luciferase activity was measured over the course of 2 h by adding 2 μl of denatured luciferase to 50 μl aliquots of luciferin assay reagent (LAR) (Promega). Reactivation progress was measured in a luminometer (Turner TD 20/20) as relative light units (RLU).

2.4. Thermal stability of Hsc70

To assess the thermal stability of Hsc70 from *G. mirabilis*, 1.03 μg/μl of Hsc70 was incubated at temperatures ranging from 35 to 60 °C for 1 h. Following the incubation treatment, the refolding ability of pre-incubated Hsc70 was measured in a refolding assay (100 μl total volume; composed of refolding buffer, 2 mM ATP, and 4 μl of thermally denatured luciferase) at 25 °C. During each assay, 2 μl of the refolding assay cocktail was combined with LAR and luciferase activity was measured in a luminometer.

2.5. Refolding assays

Refolding assays were performed using a protocol modified from Lu and Cyr (1998) and Minami et al. (1996). The assays were composed of refolding buffer, 3.504×10^{-12} M of thermally denatured luciferase with, or without, the inclusion of 1 μM of bovine serum albumin (BSA), or 1 μM of purified *G. mirabilis* Hsc70 for a total volume of 100 μl. The refolding activity of Hsc70 was measured at 5 °C increments across the temperature range of 10–35 °C. Over a 4 h period, 2 μl aliquots were removed at various time intervals, combined with 50 μl of LAR, and assayed for activity at 10 min intervals. For the refolding data, Q_{10} values were calculated using the Van't Hoff equation: $Q_{10}=(K_2/K_1)^{(10/t2-t1)}$.

2.6. Statistical analysis

All nested and one-way analyses of variance (ANOVA) were performed using JMP 5 software (SAS Institute) for Windows. We verified that the ANOVA assumptions of normality were met by testing a fit distribution. Only the luciferase refolding activity across ecologically relevant temperature (10–40 °C) data needed to be log transformed to improve normality. For the thermal stability data, a one-way ANOVA with Tukey HSD was used to test the significant differences between temperatures. All tests had a significant difference of $P<0.05$, except for the data comparing refolding activity within each treatment across the temperature ranges of 10–40 °C (Fig. 3). Unless otherwise indicated, all data are presented as mean ± S.E.M.

3. Results

3.1. Testing of Hsc70 preparations

Prior to experimentation, we screened each purified Hsc70 preparation for chaperone activity. These in vitro assays, often termed protection assays, tested the ability of *G. mirabilis* Hsc70 to 'protect' luciferase during in vitro thermal denaturation. This technique is routinely used to demonstrate protein refolding activity of purified molecular chaperones (e.g. Lee et al., 1995) and has been used to demonstrate chaperone activity of Hsc70 purified from *G. mirabilis* (Place and Hofmann, 2001). In the current study, all preparations of *G. mirabilis* Hsc70 used in the experiments had chaperone activity, where luciferase denatured at 38 °C had activity that was five times greater after 10 min as compared to assays containing equivalent concentrations of a non-chaperone, generalist protein (BSA) or luciferase alone (data not shown).

3.2. Refolding assays

To determine the thermal sensitivity of protein folding by *G. mirabilis* Hsc70, in vitro refolding assays using luciferase as the target protein were conducted across a range of ecologically relevant temperatures (10–30 °C). This approach tested the capability of *G. mirabilis* Hsc70 to stabilize and refold a denatured protein to a functional state in vitro. Time course experiments showed that Hsc70 refolding activity peaked within 30 min and began to decrease over a 4 h period (see Fig. 1).

Fig. 2 shows the refolding activity of Hsc70 after 30 min at 25 °C and includes the activity measured in two accom-

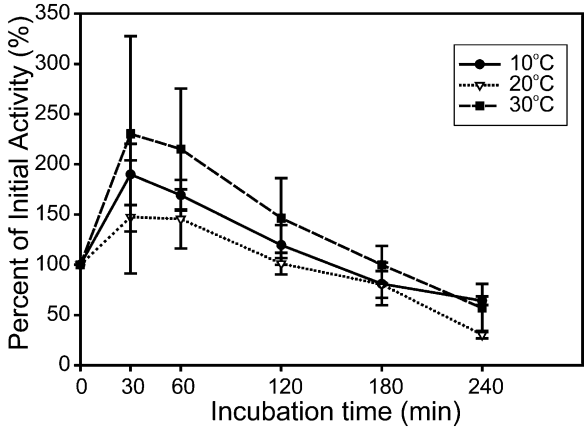

Fig. 1. Time course measurements of luciferase refolding by *G. mirabilis* Hsc70 at 10, 20 and 30 °C. Luciferase was thermally denatured at 38 °C for 2 h and then added to assay tubes containing refolding buffer, and 2 mM ATP, for a final volume of 100 μl. At 30–60 min time intervals, 2 μl aliquots of sample were added to 50 μl of LAR and luciferase activity was measured. At each time point, symbols represent mean ± S.D. of three replicate assays.

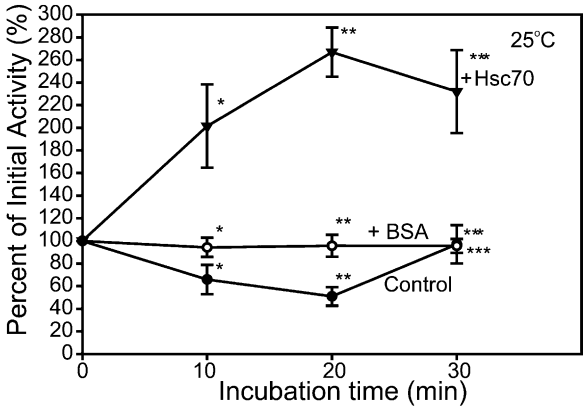

Fig. 2. Comparison of luciferase refolding activity of *G. mirabilis* Hsc70 with control treatments at 25 °C. For the refolding assays, luciferase was thermally denatured at 38 °C for 2 h and then added to assay tubes containing refolding buffer, and 2 mM ATP, for a final volume of 100 µl. At 10 min time intervals, 2 µl aliquots of sample were added to 50 µl of LAR and measured in a luminometer. The percentage of initial activity was measured against each incubation time shown on the *X*-axis. An increase in percent activity is shown with a value greater than 100% and a decrease in activity is shown with a value less than 100%. Assay conditions are as follows: filled triangles (▼)=+ Hsc70, open circles (○)=+ BSA, filled circles (●)='no addition' control. Symbols represent the mean ± S.E.M.; for each time point, asterisks are used to indicate significance in a Nested ANOVA as follows: * for 10 min, ** for 20 min and *** for 30 min.

panying control assays: BSA added as a control for the addition of small amounts of protein to the dilute assay medium, and a 'no addition' control that lacked BSA and Hsc70. At 25 °C, a common environmental temperature for

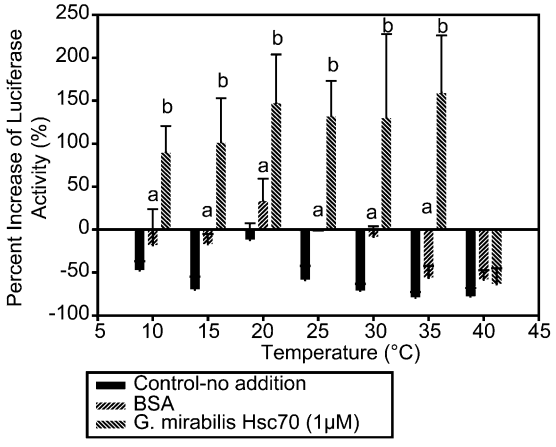

Fig. 3. Effects of temperature on the luciferase refolding activity of *G. mirabilis* Hsc70 across the ecological relevant temperature range of 10–40 °C. The *x*-axis shows the refolding temperature of each assay series; all assays were conducted for 30 min. Percent of initial activity was the remaining activity after 30 min at each specific refolding temperature. One-way ANOVA confirmed no significant difference within each treatment ($P=0.0679$), however, amongst control, BSA-containing, and Hsc70-containing assays, nested ANOVA revealed significant differences ($P=0.0002$). Bars represent mean ± S.E.M.; values with different letters differ significantly from one another (nested ANOVA).

Table 1
Q_{10} values for the temperature effects on luciferase refolding by *G. mirabilis* Hsc70 at 30 min

Temperature (°C)	Q_{10}*
10–20	1.13
20–30	1.08
20–35	1.14
10–35	1.13

* Calculated using the Van't Hoff equation (see Section 2).

the estuarine goby, the presence of *G. mirabilis* Hsc70 increased luciferase activity significantly as compared to a no addition assay (Nested ANOVA, $P=0.0008$), or an assay containing BSA (Nested ANOVA, $P=0.0007$). In addition, there was a statistical difference between the control assay and the BSA assay (Nested ANOVA, $P=0.0078$). These control assays were both conducted at all three temperatures shown in Fig. 1 and displayed a similar pattern (data not shown).

Fig. 3 expands the data set and shows the results of refolding assays across the range of ecologically relevant temperatures of 10–35 °C. The in vitro chaperone activity of Hsc70 was relatively temperature insensitive, with refolding levels varying from 75 to 160% at 10 °C and 35 °C, respectively, in a 30 min assay (Fig. 3). Statistical analysis confirmed (one-way ANOVA) that refolding levels were not significantly different within each treatment across the temperature ranges of 10–35 °C ($P=0.0679$). However, in a comparison between control, BSA and Hsc70 treatments there was a significant difference across all temperatures (Nested ANOVA, $P=0.0002$ with arc-sine transformation of the data). Refolding activity of Hsc70 decreased dramatically at 40 °C, most likely due to the loss of luciferase activity itself at this elevated temperature (Fig. 3). Calculations of Q_{10} values for luciferase refolding by Hsc70 indicated that refolding was relatively thermally insensitive (Table 1). Most values deviated from a physiological average or norm of 2. Calculated Q_{10} values for luciferase refolding by *G. mirabilis* Hsc70 were all near 1.0, with a Q_{10} of 1.13 for the entire range of 10–35 °C (Table 1).

3.3. Thermal stability

In order to test the thermal stability of Hsc70, luciferase refolding assays were conducted. Here, Hsc70 was pre-incubated at elevated temperatures prior to running the refolding assay at 25 °C. Fig. 4 shows the refolding capabilities of *G. mirabilis* Hsc70 at 25 °C after pre-incubation at temperatures ranging from 35 to 60 °C. The refolding capacity of Hsc70 was affected by temperature and decreased from 217% at 35 °C to 19% at 60 °C, as determined by refolding assays conducted at 25 °C for 1 h. Across this temperature range in a step-wise fashion, a one-way ANOVA with Tukey HSD revealed significant differ-

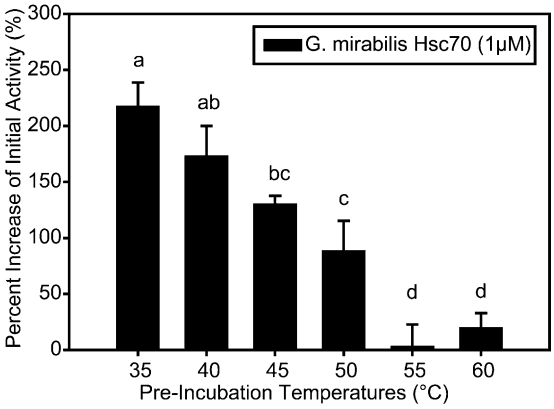

Fig. 4. Thermal stability of in vitro chaperone activity of *G. mirabilis* Hsc70. 2 μM of *G. mirabilis* Hsc70 was pre-incubated for 1 h at varying temperatures (*x*-axis) and refolding was tested in a luciferase assay at 25 °C for 1 h. Means ($n=3$) ± S.E.M. are shown. One-way ANOVA with Tukey HSD was used for statistical analysis ($P<0.05$); bars with different letters differ significantly from one another (ANOVA).

ences between 35 and 45 °C, and 50 and 55 °C (Fig. 4; $P<0.05$).

4. Discussion

In this study, we investigated the thermal sensitivity of the molecular chaperone Hsc70 from a eurythermal estuarine fish, *G. mirabilis*. Since this goby synthesizes and folds protein over an approximately 25 °C range in nature, there must be some presumed flexibility in the capacity of the molecular chaperone system in these animals. One potential explanation for Hsc70's conserved function across a broad temperature range may be thermal insensitivity of the folding cycle, or chaperone function, of the protein. Data from this study provide evidence that this may indeed be the case.

Three salient findings in this study addressed the 'thermal insensitivity' hypothesis and examined the refolding capability and thermal sensitivity of Hsc70 in the eurythermal goby, *G. mirabilis*. First, purified *G. mirabilis* Hsc70 displayed 2.5-fold greater luciferase refolding activity than the known non-chaperone protein, BSA (Fig. 2). Second, in luciferase refolding assays conducted from 10 to 35 °C, *G. mirabilis* Hsc70 displayed refolding capacity across the range of temperatures that are typical of the annual environmental temperatures encountered by this eurythermal goby (Fig. 3). Finally, thermal stability assays revealed that the in vitro chaperone activity of Hsc70, as measured by luciferase refolding, started to decline at temperatures above 35 °C (Fig. 4).

To demonstrate that purified native Hsc70 from *G. mirabilis* white muscle displayed in vitro molecular chaperone activity, we conducted protection assays using heat-denatured luciferase as a refolding target (Herbst et al., 1998; Place and Hofmann, 2001). Firefly luciferase (*Phontinus pyralis*) when bound to its substrate, luciferin, catalyzes a light-emitting reaction that can be detected using a luminometer (Herbst et al., 1998). Although luciferase is not an enzyme found in our study animal, the extensive use of the luciferase assay in other studies where assay conditions ranged from 20 to 30 °C (Nimmesgern and Hartl, 1993; Zolkiewski, 1999; Lee and Vierling, 2000; Abdulle et al., 2002) suggested it would be a suitable assay for our research on the eurythermal goby. *G. mirabilis* Hsc70 prevented rapid thermal inactivation of luciferase at 38 °C, in vitro, by maintaining 52% of initial activity after the first 5 min as compared to the luciferase control which lost ~85% activity over the same time period (data not shown). This degree of stabilization of luciferase in vitro was similar to that observed for other preparations of Hsc70 purified from *G. mirabilis* white muscle (Place and Hofmann, 2001). These results are similar to those obtained in studies of Hsp90 from porcine brain (Minami and Minami, 1999), although the mammalian Hsp90 displayed a greater total protective activity with ~50% of luciferase activity remaining after 1 h. Furthermore, in one study where Hsp18.1/luciferase complexes were thermally denatured, Hsc70/Ydj1 (yeast) and Hsc70/Hdj1 (human) were added independently to the reaction resulting in significant recovery of luciferase activity with yields of 50% and 64%, respectively (Lee and Vierling, 2000). Although our luciferase refolding assays did not include co-chaperones, such as Hsp40, other studies have obtained prevention of protein aggregation (Minami et al., 1996; Strickland et al., 1997) or refolding activity by Hsc/Hsp70 alone (Minami and Minami, 1999). In this situation, it is thought while Hsc70 alone has the ability to bind and prevent the aggregation of luciferase (thus some stabilization and refolding is noted), this rate is increased with the addition of Hsp40 (Freeman and Morimoto, 1996; Minami and Minami, 1999).

The ultimate goal of this study was to determine how *G. mirabilis* Hsc70 folded the target protein luciferase over an ecologically relevant range of temperatures. Towards this end, luciferase refolding assays were performed at 10–35 °C (Fig. 3; data not shown for all time periods). In general, we found that the in vitro refolding activity of Hsc70 peaked at 30 min into a refolding assay (Fig. 1). In addition, under standardized in vitro conditions at different experimental temperatures (10–35 °C), Hsc70 had a greater refolding ability than a non-molecular chaperone protein, BSA (Figs. 2 and 3); the BSA assay was included to control for the possible stabilization that would be conferred by the addition of a small amount of protein in the dilute milieu of the refolding assay cocktail. As shown in Fig. 3, *G. mirabilis* Hsc70 was able to refold thermally denatured luciferase to over 100% the initial activity at temperatures from 10 to 35 °C. In addition, the rate of refolding was relatively thermally insensitive as measured by Q_{10} values (Table 1), with a Q_{10} of 1.13 for

the 10–35 °C interval. Overall, these in vitro data show that Hsc70 purified from the eurythermal goby *G. mirabilis* was capable of binding to and temporarily stabilizing the activity of a thermally denatured target protein, luciferase. Translated into an ecological context, these data suggest that Hsc70 may also have a similar thermally insensitive action in vivo and thus chaperones proteins across the range of ecologically relevant temperatures for this species.

Other biochemical studies have tested the effects of temperature on aspects of molecular chaperone activity, the most common example being the assessment of the thermal sensitivity of the ATPase activity in chaperones from model study organisms. For example, DnaK (the bacterial Hsp70 homologue) from *Thermus thermophilus* had a two- to three-fold increase in ATPase activity between 25 and 75 °C (Klostermeier et al., 1998), and, in *Escherichia coli*, the ATPase activity of DnaK increased by 70-fold from 20 to 53 °C (McCarty and Walker, 1991). In addition, while numerous other studies have demonstrated the in vitro chaperone activity (i.e. protein refolding action) of molecular chaperones, few have studied the action of the chaperone in question with respect to the ecological or evolutionary relevance of temperature. For example, Schumacher et al. (1996) showed that the human chaperones Hsp70 and yeast Ydj-1 renature luciferase at 25 °C; in addition, the yeast Hsp70 homologue, Ssa-1, has refolding activity at 25 °C while rat Hsc70 has been shown to refold luciferase at 30 °C (Lu and Cyr, 1998; Lüders et al., 1998). However, none of these studies considered the role of temperature in an ecological context. As an exception to this trend in model study systems, Freeman and Morimoto (1996) noted that in vitro protein refolding by human-derived chaperones failed at 42 °C, a temperature 5 °C above the adaptation temperature of the source organism. These data from a mammalian chaperone system are in stark contrast to the observation of thermal insensitivity observed for *G. mirabilis* Hsc70 in the current study.

After confirming that Hsc70 purified from *G. mirabilis* possessed in vitro chaperoning activity, thermal stability assays were conducted in order to test the folding capacity of Hsc70 at non-physiological temperatures. Although these types of measures have little ecological relevance, thermal stability assays are often used in comparative biochemistry to address emergent properties of the protein constituency of ectothermic organisms. Often the in vitro thermal stability of a particular protein is routinely measured as well above the organism's actual habitat temperature, and many studies have suggested that global protein stability correlates with evolutionary adaptation temperature (reviewed in Hochachka and Somero, 2002). With respect to the thermal stability of Hsc70 with preservation of refolding activity as an indicator of biochemical function, the ability to refold luciferase decreased significantly when *G. mirabilis* Hsc70 was pre-incubated at 45 °C (Fig. 4); this temperature is 10 °C in excess of the presumed upper thermal maximum (approx. 35–38 °C) for this species in the field (Place and Hofmann, 2001). Place and Hofmann (2001) found that the ATPase activity of purified *G. mirabilis* Hsc70 was thermally stable up to 50 °C and began to display reduced activity at 62.5 °C. Taken together, these two studies indicate that the two functional regions of the Hsc70 molecular chaperone—the ATPase moiety and the protein binding region—are differentially sensitive to temperature, with the protein binding region displaying a much greater perturbation by non-physiological temperatures in vitro.

In summary, the results of this study suggest that molecular chaperones from ectothermic animals may function across a broad range of temperatures and are able to participate in protein synthesis at ecologically relevant temperatures.

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