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Molecular Chaperones in Ectothermic Marine Animals: Biochemical Function and Gene Expression[1]

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SYNOPSIS. The intertidal zone has historically functioned as an important natural laboratory for testing ideas about how physical factors such as temperature influence organismal physiology and in turn influence the distribution patterns of organisms. Key to our understanding of how the physical environment helps structure organismal distribution is the identification of physiological processes that have ecological relevance. We have focused on biochemical- and molecular-level physiology that would contribute to thermal tolerance and maintenance of a functional intracellular protein pool in the face of extreme and fluctuating environmental temperatures. Past research has addressed processes central to protein homeostasis (*e.g.*, protein ubiquitination) and the molecular ecology of molecular chaperones, a.k.a. heat shock proteins (Hsps), in ectothermic animals. In this presentation, we focus on two new developments regarding the biology of heat shock proteins as molecular chaperones in intertidal organisms. First, we present data on the functional characteristics of the transcriptional factor, HSF1 and discuss how these data relate to the plasticity of Hsp gene expression observed in intertidal organisms in nature. Second, we present data on the biochemical function of heat shock proteins purified from our non-model study organisms and discuss the temperature relationships of these molecules as they assist in protein folding *in situ.*

INTRODUCTION

As the rocky intertidal zone became a significant model system for experimental community ecology, it became clear that the ecology of the rocky intertidal was strongly influenced by its physical environment (see Connell, 1961; Newell, 1979; Menge and Sutherland, 1987). Due to the movements of tide and the alteration of aquatic and terrestrial-like conditions, the rocky intertidal is characterized by steep gradients in environmental factors such as temperature and desiccation stress. Thus, this marine ecosystem makes for an ideal natural laboratory in which to test the impact of physical factors and environmental variation on the physiology of the resident animals and algae. The rocky intertidal has historically been an important study system for physiological ecology and more recently the study of the ecological significance of heat shock proteins (Hsps) in rocky intertidal animals has become an area of particular interest. Other papers in this symposium series and elsewhere (Sanders, 1993; Hofmann, 1999) present different aspects of the study of Hsps in rocky intertidal animals. The goal of this paper is to present two new areas of investigation into the ecological significance of Hsps in the rocky intertidal environment. These two topics include the environmental regulation of Hsp gene expression and the biochemical function of Hsps as molecular chaperones in non-model ectotherms that synthesize and fold proteins at temperatures much different from those of the most often studied chaperones from endothermic vertebrates.

HEAT SHOCK PROTEINS AS MOLECULAR CHAPERONES

Many heat shock proteins are molecular chaperones, a class of proteins that assist in the folding of other proteins (Hartl, 1996; Gething, 1997; Fink, 1999). As molecular chaperones, Hsps perform numerous tasks in cells and assist with a variety of cellular processes such as protein translocation (Agarraberes and Dice, 2001), folding of newly translated proteins (Frydman, 2001) and regulation of apoptosis (Garrido *et al.*, 2001). From an organismal perspective, the role of Hsps has become clear—elevated levels of Hsps confer thermotolerance at the organismal level and does so presumably by stabilizing proteins that would otherwise be lost in response to sublethal denaturing stress (see Feder and Hofmann, 1999 for a review). The energetic consequences here are two-fold. Not only do denatured proteins need replacement, but also unstable proteins tend to aggregate into cytotoxic bodies that may cause further cellular damage much greater than that of the loss of a subset of proteins.

Hsps can be divided into two general categories—those that are expressed constitutively under normal physiological conditions (*i.e.*, Hscs) and those that are expressed only in response to protein-denaturing stress (*i.e.*, Hsps). The latter class of Hsp genes is synthesized during the stress response, a rapid up-regulation of a subset of genes that occurs in response to heat stress and other stressors (Lindquist, 1986). The Hsps are differentiated from the Hscs by a promoter and transactivation mechanism that is ''inducible'' and somehow sensitive to temperature and/or the increase in abnormal proteins in the cell. The temperature at

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which the Hsp gene products appear in cells is often called the threshold induction temperature or the induction set point. In general, the consensus signal for the activation of inducible Hsp genes is the presence in the cell of elevated levels of abnormal proteins. Experimentally, the stress response can be induced by injecting cells with abnormal proteins (Ananthan *et al.*, 1986) or by treating cells with a proteasome inhibitor such as MG 132 that causes the build up of ubiquitinated proteins that would otherwise be degraded by the proteasome (Lee and Goldberg, 1998), indicating the mechanistic basis for the response.

Although heat shock proteins can be divided into classes by mechanisms of transcriptional regulation, many Hsps, as molecular chaperones, share a common mechanism in terms of how they interact with their substrate, nonnative proteins. Many cytoplasmic Hsps, such as the Hsp70 family, recognize and bind to hydrophobic patches in the primary protein structure. These regions are often exposed when the protein starts to unfold, and these regions are not normally exposed to the aqueous cellular milieu when the protein is in a folded conformation.

For ecological physiologists interested in the biological consequences of the physical environment, Hsps attract attention because of their cellular role in maintaining protein homeostasis—a fine balance between protein synthesis, protein degradation and protein refolding. Chaperones play a key role in regulating the recovery of thermally denatured proteins and returning them to the protein pool (Wickner *et al.*, 1999). Since proteins are arguably the most energetically expensive biomolecule that organisms synthesize and a large proportion of an organism's energy budget is dedicated to maintaining the protein pool (see Houlihan *et al.*, 1995), a process that ameliorates the cost of environmentally-induced irreversible protein damage would have adaptive value. For intertidal ectotherms that experience unpredictable and often times extreme variation in temperature (*e.g.*, Denny and Paine, 1998; Helmuth, 1998; Helmuth and Hofmann, 2001), Hsps are particularly significant as they represent a mechanism by which an organism can buffer the impact of environmental temperature on the protein pool without having to employ specialist protein isoforms to withstand high temperatures (Somero, 1995). Thus, investing in Hsps as the likelihood of high temperatures looms may be an effective strategy for intertidal animals. As discussed below, this is indeed the case; patterns of Hsp synthesis tracks thermal history very closely in the intertidal organisms thus far examined. However, lest we sing the virtues of Hsps and ask why not have huge stocks at all times, some research has demonstrated that organisms can have too much of a good thing, specifically that overexpression of chaperones may interfere with normal protein biogenesis (Feder *et al.*, 1992).

EXPRESSION PATTERNS OF HSPS IN NATURAL POPULATIONS

Certainly the most significant ecological benefit of Hsp synthesis is that Hsps confer thermotolerance to cells and organisms. As a result, intertidal ecophysiologists have focused on whether the stress response and the Hsps themselves have expression patterns that would support a protective role for these proteins. An accumulating body of research has demonstrated a great deal of variation in Hsp expression patterns in rocky intertidal animals. Most of these studies have results that support the role of Hsps in organismal thermotolerance. These variations fall into three broad categories of variation of response: as a function of thermal history, correlating to microhabitat, and between species.

First, variation in the stress response as a function of thermal history is one of the more compelling arguments for the ecological significance of Hsps. Total cellular levels of Hsps fluctuate with season (Hofmann and Somero, 1995; Chapple *et al.*, 1998; Buckley *et al.*, 2001) and with laboratory acclimation (Roberts *et al.*, 1997; Tomanek and Somero, 1999; Buckley *et al.*, 2001). In addition to changes in total amount of Hsps, induction set points are altered by thermal history with more warm-acclimated organisms displaying a higher threshold for induction than cold-acclimated organisms. A similar pattern is also observed in natural populations. For example, in intertidal mussels (*Mytilus trossulus*), threshold induction temperatures for Hsps varied by several degrees in a comparison of winter-*vs.* summer-acclimatized individuals (see Buckley *et al.*, 2001). Currently, there is no information regarding whether intertidal animals ''pre-synthesize'' Hsps as a means to prepare for potential heat stress during an impending low tide. Such a scenario has been suggested for desert ants (Gehring and Werner, 1995) but thus far rhythmicity of Hsp gene expression has not been recorded in animals of the rocky intertidal zone.

Second, although the stress response displays an element of plasticity, some components appear to be fixed and characteristic of a particular species. Different sets of congeners of rocky intertidal marine invertebrates have different stress responses despite being acclimated to the same temperature (*e.g.*, Hofmann and Somero, 1996; Tomanek and Somero, 1999). These results highlight the possibility that the stress response and the subsequent synthesis of Hsps may contribute to the physiological tolerance that sets species distribution limits.

Third, the stress response varies in natural populations across environmental gradients and with thermal microhabitat. Elements of the stress response in the intertidal mussel *Mytilus californianus* have been shown to vary with substrate angle (Helmuth and Hofmann, 2001). In studies that are focused on vertical zonation and the consequent gradient of stress due to the fluctuations of the tide, Dahlhoff *et al.*, (2001) have demonstrated changes in the physiological status of

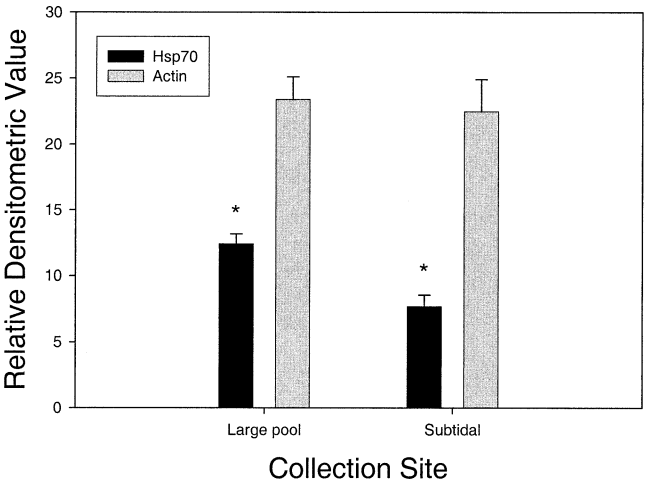

FIG. 1. Comparison of levels of the heat-shock protein Hsp70 in tube feet of purple sea urchins (*Strongylocentrotus purpuratus*) from tidepool and subtidal locations. Levels of Hsp70 were measured in the tube feet of urchins collected from Mayben's Beach, Vancouver Island, British Columbia, Canada. Western blotting followed by densitometric analysis was used to determine relative levels of Hsp70. For complete methods see Hofmann and Sewell, 2002. Measurement of actin was included to control for the direct effect of temperature on protein synthesis rates. For immunodetection of both actin and hsp70, equal amounts of protein (25 μg total protein) was loaded in each lane. Each bar represents the mean ± SEM for n = 5 different individuals from each location. * Significantly different using a *t*-test ($P < 0.05$).

whelks (*Nucella*). Investigations on the physiology of intertidal mussels on the Oregon coast found that there is a strong influence of intertidal location on characteristics of the stress response. In May, mussels from high and low intertidal locations induced Hsps at 23.3°C; by August this pattern had changed such that individuals from the low zone induced Hsps between 15–23.3°C and individuals in the high intertidal induced Hsps between 22 and 30°C (P. M. Halpin, unpublished results). The divergence here is due to the changes in the tide cycle as the summer progresses on the Oregon coast. At this latitude, low tide, and thus aerial exposure and the opportunity to experience fluctuations in body temperature, occurs increasingly earlier in the morning. Thus, mussels in the low portion of the mussel bed are less likely to experience thermal extremes that will activate expression of the heat-inducible Hsps genes and drive up the intracellular concentrations of Hsps. Finally, in a study on sea urchins focusing on thermal heterogeneity for continuously submersed intertidal invertebrates, Hsp levels and induction temperatures were different between intertidal populations of the purple sea urchin, *Strongylocentrotus purpuratus*, exposed to subtle warming cycles in a tidepool and an adjacent population of subtidal individuals in a much more cool, stable thermal environment (Fig. 1; see Hofmann and Sewell, 2002).

Overall, the centerpiece of the stress response data in natural populations is the observation that Hsp gene expression in rocky intertidal invertebrates is *plastic*—the activation temperature of the Hsp genes changes according to the temperature regime experienced by the animal. These studies raise the question as to how the environmental temperature signal is transduced to the genome of an organism to activate transcription of these protective genes.

ENVIRONMENTAL REGULATION OF HSP GENE EXPRESSION: HOW ARE HSP GENES INDUCED BY THERMAL STRESS?

How does temperature modulate Hsp gene expression in marine animals?

Building on these foundational data, we can address a set of mechanistic questions about how Hsp gene expression is influenced by the thermal history of the animal. Specifically, what is the physiologically significant aspect of the thermal signal? What cellular signaling pathway transduces environmental temperature to the nucleus? And, ultimately, how does environmental temperature modulate the transcriptional regulation of Hsp gene expression of ectothermic rocky intertidal animals?

In order to begin to understand the mechanisms that explain the plasticity of Hsp induction, we have focused on the activity of heat shock factor-1 (HSF1), the transcription factor that regulates the transactivation of the stress-inducible Hsp genes (Wu, 1995). Not surprisingly, most of the available data have been gathered from model systems but the emerging mechanism of how stress-inducible Hsp synthesis is controlled at the transcriptional level has provided insight into mechanisms that might underlie the observed plasticity in rocky intertidal invertebrates. It will be useful to first review the transcriptional activation of the stress-inducible genes. There are numerous excellent reviews that address the regulatory aspects of Hsp gene expression (see Wu, 1995; Morimoto, 1998) and the nature of the family of HSFs that mediate Hsp gene expression (see Morano and Thiele, 1999; Pirkkala *et al.*, 2001). Basically, the transcriptional activation of stress-inducible genes via HSF1 involves three processes: (1) the oligomerization of monomeric HSF1 molecules into a trimer, (2) hyperphosphorylation of HSF1, and (3) the translocation of the HSF1 trimer into the nucleus (see Sarge *et al.*, 1993). As a result of the above processes, the HSF1 trimer obtains DNA binding activity, binds to the promoter of Hsp genes, and activates transcription. The timing of these events is complex and it should be noted that the transactivation of stress-inducible genes is not fully understood even in model systems. For example, DNA binding, the ability to bind the promoter of Hsp genes, is uncoupled from actual transcriptional activation in mammalian cells; full activation appears to be mediated by differential phosphorylation (*e.g.*, Cotto *et al.*, 1996).

Since HSF1 is subject to such complex regulation prior to transactivation, we reasoned that HSF1 might be a component in Hsp gene expression that could be leveraged in order to modulate gene expression in a temperature sensitive manner in the ectothermic ani-

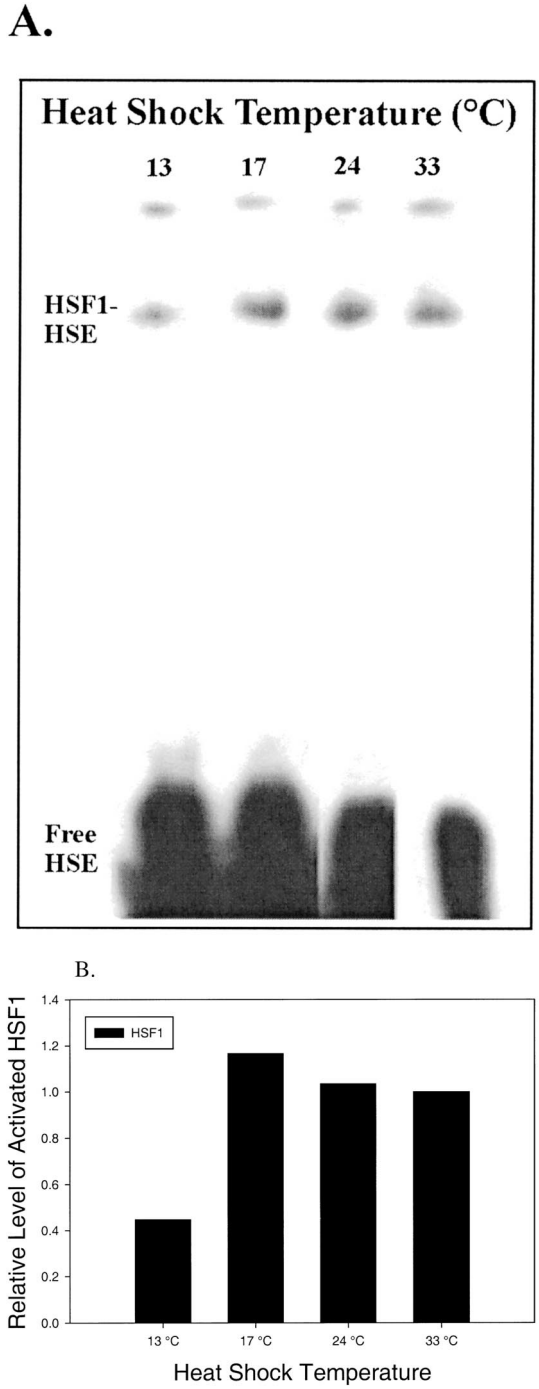

FIG. 2. An example of an electrophoretic mobility shift assay (EMSA) used to meaure HSF1 DNA binding activity. (A) This figure shows the result of an EMSA on liver tissue from the marine goby *Gillichthys mirabilis* heat shocked at the indicated temperatures. Equivalent amounts of cellular protein from a liver lysate were incubated with a ^{32}P-labeled oligonucleotide probe with a sequence that matched the sequence of the HSE in Hsp genes. After incubation, a sample of this assay was separated in a 4% polyacrylamide gel, dried and exposed to X-ray film. The autoradiograph of the EMSA displays a characteristic banding pattern with three important regions: a non-specific band (material that remains in the well of the gels, in this case), the HSF1-HSE complexes, and the free HSE probe at the bottom of the gel. See Buckley *et al.*, 2001 for EMSA methods. (B) An EMSA was used to measure the relative levels of activated HSF1 in liver tissue from specimens of the marine goby *Gillichthys mirabilis* that were acclimated to 13°C for 6 wk and then heat shocked at the given temperatures. Each bar represents a single densitometric value at the designated temperature.

mals of the rocky intertidal zone. Thus, we have used the ability of HSF1 to bind DNA as an indicator of Hsp gene activation in order to begin to look at the temperature sensitivity of Hsp gene expression. Given the apparent complexity of HSF1 regulation, focus on a single metric has potential shortcomings in terms of addressing the holistic pathway of the environmental regulation of Hsp gene expression. However, this 'first cut' analysis of HSF1 activity as a function of organismal thermal history provides a good starting point to determine whether any temperature-sensitive variation is observed. For our studies on rocky intertidal invertebrates, we developed an electromobility shift assay (EMSA) to measure the activity of the transcriptional factor HSF1 (Fig. 2A; see Buckley *et al.*, 2001 for methods). The EMSA is the most common method to assess the activity of HSF1 and relies on the HSF1 trimer binding *in vitro* to an oligonucleotide probe with the same sequence as the heat shock element (HSE), the region that the HSF1 trimer binds to *in vivo*. The complexes of HSF1-HSE can then be visualized on a gel using autoradiography or a chemiluminescent signal. This method has been used to assess activity of HSF1s from other nonmodel organisms (*e.g.*, Airaksinen *et al.*, 1998; Zatespina *et al.*, 2000; Lerman and Feder, 2001) and is considered to be a reliable measure of the percentage of the population of HSF1 molecules in an active trimerized state.

Experiments on intertidal mussels indicated that HSF1 DNA binding activity does vary with the temperature where HSF1-HSE complexes increased as temperature exposure of gill tissue increased (Buckley *et al.*, 2001). Interestingly, although HSF1 activity varied as a function of organismal temperature acclimation, the amount of HSF1 did not change (Buckley *et al.*, 2001). However, different amounts of cellular HSF1 have been linked to different Hsp induction patterns in intertidal snails of the genus *Tegula* (Tomanek and Somero, 2002). In addition to the mussel study, similar data on the activity of HSF1 have been collected on HSF1 in estuarine gobies. Using an electromobility shift assay to measure DNA binding activity, the relative amount of activated HSF1 in liver tissue increased with *in vitro* heat shock temperature (Fig. 2B). With regard to the observed plasticity of Hsp induction, thermal-history dependent changes in HSF1 DNA binding have been observed in ectothermic animals; estuarine gobies acclimated to different temperatures displayed different amounts of HSF1-HSE complexes (Buckley and Hofmann, 2002). The next phase of this investigation is to address the temperature sensitivity of HSF1 itself, whether it is a molecule regulated by other proteins such as Hsps, and whether the sequence and DNA binding activity of HSF1 plays a

role in modulating the response to temperature stress. The HSF1 project is very exciting as we now have a direct look at part of the ''cellular thermostat,'' a complex cellular process that transduces temperature to the nucleus (Craig and Gross, 1991) and alters gene expression in ectothermic animals in a temperature-responsive manner.

Finally, although HSF1 DNA binding behavior is a promising entry into the workings of how cells sense thermal stress, there are many perspectives to consider. From a phylogenetic angle, there may be different HSF1 isoforms with different temperature sensitivities (Råbergh *et al.*, 2000). However, some research suggests that the isoform-specific induction temperature may be more closely linked to the presence of regulatory factors in the cellular environment than to the activity of a species-specific HSF1 (*e.g.*, Clos *et al.*, 1993). Cell signaling may be the real key here. However, the consensus mechanism by which a cell senses temperature stress is largely unknown. Even in model systems, the specific phosphorylation sites of HSF1 and the phosphatases and kinases involved have yet to clearly defined (*e.g.*, Dai *et al.*, 2000; see also Pirkkala *et al.*, 2001). However, Holmberg *et al.* (2001) have recently demonstrated that phosphorylation of serine 230 is essential for the transcriptional activation of human HSF1. Alternatively, there is substantial evidence that HSF1 itself is temperature sensitive and may undergo conformational changes that trigger transactivation capacity in the absence of other factors such as phosphorylation (Newton *et al.*, 1996; Zhong *et al.*, 1998). Finally, promoter architecture may drive interspecific differences in patterns of Hsp gene expression (L. Tomanek, personal communication). And, while it is unlikely that wholesale sequence changes in promoter regions explains the pattern of Hsp expression within the timeframe of this physiological plasticity, promoter strength could act to influence the plasticity of the onset temperature for Hsp synthesis that is observed in intertidal animals exposed to different temperature regimes.

TEMPERATURE RELATIONSHIPS OF HSPS AS MOLECULAR CHAPERONES

Are Hsps as molecular chaperones ''fine-tuned'' to operate at temperatures at which they evolved?

Despite the fact that the stress response and Hsps are found in almost all taxa thus far examined, there is virtually no information as to whether the Hsps and their chaperoning capacity exhibit any functional diversity that correlates with species' adaptation temperature. Especially among ectotherms, one might hypothesize that Hsps display patterns of molecular evolution and functional diversity that correlates with species' average habitat temperature. Alternatively, since molecular chaperones are so conserved across divergent taxa, as a group, Hsps may have relatively temperature-insensitive activity and function over a broad range of temperatures regardless of the species in which they evolved. In order to test the divergence of function of Hsps as molecular chaperones, we have examined biochemical properties and protein folding capacities of homologous Hsp genes, in this case, Hsc70, a constitutively expressed member of the 70 kDa Hsp multigene family, from closely related marine fishes that have evolved at different temperatures. Due to the ease with which Hsps are purified from fish as a result of the large amount of white skeletal muscle, these studies have been initiated on fish. Future experiments will include rocky intertidal marine invertebrates that are of particular interest because many species function across broad ranges of environmental temperature.

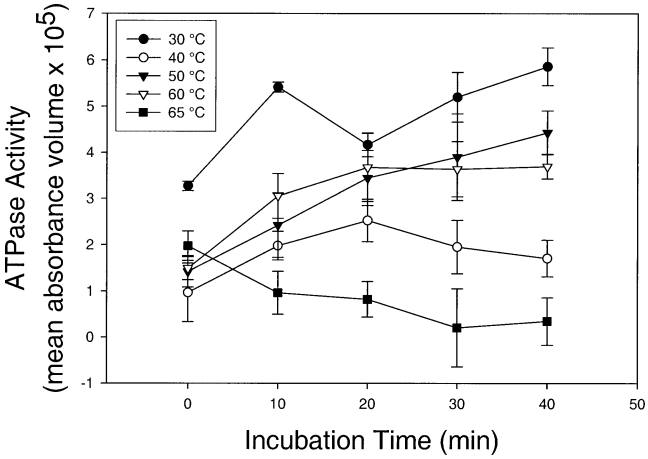

FIG. 3. Thermal stability of the ATPase activity of Hsc70 purified from white skeletal muscle of the goby *Gillichthys mirabilis*. For these assays, native Hsc70 was incubated in assay buffer for 40 min at the temperatures shown in the figure legend. During the assay, 2 μg samples were removed in triplicate every 10 min, combined with 10 μCi of [(α-^{32}P]ATP in 50 μl of assay buffer and incubated at 23°C. The amount of ATP hydrolysis was determined after 15 min using thin-layer chromatography. Values are mean pixel absorbance volumes ± SD for n = 3 samples at each time point. See Place and Hofmann (2001) for complete methods for Hsc70 purification and ATPase assay conditions.

As a starting point, we characterized Hsc70 from a eurythermal estuarine goby *Gillichthys mirabilis* and found that the ATPase activity of Hsc70 was very thermostable, functioning up to 62.5°C, a temperature that exceeds the average environmental exposure for this subtropical species by over 30°C (Fig. 3; see also Place and Hofmann, 2001). However, tests of the other functional component of the Hsc70 molecule, the protein-binding region, showed that this aspect of Hsc70 activity was temperature sensitive. Using denatured luciferase as a model unfolded protein, *in vitro* chaperoning assays showed that *G. mirabilis* Hsc70 displayed thermal sensitivity starting at 35°C (Fig. 4). The ability of Hsc70 to rescue and refold luciferase declined further at higher temperatures with precipitous loss of refolding activity at 40°C (M. L. Zippay, unpublished results). Interestingly, the maximal thermal exposure for *G. mirabilis* in nature is thought to be around 37°C; thus, 40°C is an extreme temperature for this fish and

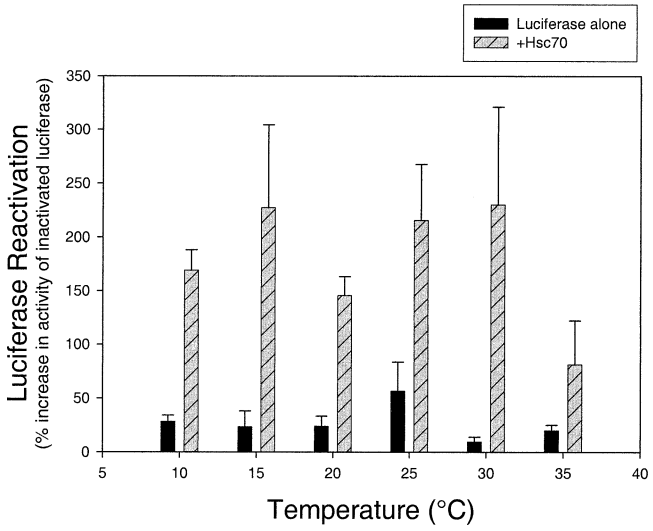

FIG. 4. Luciferase refolding activity of *Gillichthys mirabilis* Hsc70. Luciferase was diluted to 2.19×10^{-10} M in refolding buffer (25 mM Hepes, 50 mM KCl, 5 mM $MgCl_2$, pH 7.4) and thermally inactivated at 38°C for 2 hr. For the *in vitro* refolding assays, 2 μM of *G. mirabilis* Hsc70 was incubated with 14.7 μg/μl thermally-denatured luciferase for 60 min at the given temperatures. Luciferase reactivation was measured in a luciferase assay where 4 μl aliquots were removed in triplicate and combined with 50 μl of luciferase assay reagent containing the substrate luciferin (Promega). Relative light units were measured in a luminometer and luciferase reactivation was expressed as the percent increase in activity over the activity of denatured luciferase at time zero. Although some spontaneous refolding does occur *in vitro* in the absence of Hsc70 (solid bars), luciferase reactivation occurs much more readily in the presence of the chaperone, Hsc70 (hatched bars). Bars represent the mean of triplicate assays at each temperature; error bars are ± SD of the mean.

may perturb the weak interactions that mediate the interaction of the chaperone with its protein target.

These data are among the first on chaperones from non-model species and they currently present a two-part answer about the temperature relationships of molecular chaperones from ectothermic species—the biochemical characteristics of Hsc70 appear to be fairly conserved while the functional characteristics of protein chaperoning appear to be much more temperature sensitive. Since the Hsc70 chaperone are comprised of two functional regions—an ATPase activity and a protein-binding region—these different regions of the gene may have evolved in response to temperature selection in different ways. This outcome has opened up other avenues of investigation, including sequencing of *hsc70* and its gene product, exploring the specificity of protein binding by the chaperone and additional species comparisons.

SUMMARY

The study of the stress response and Hsps in an ecological and physiological context has established a rich area for investigation of how animals respond to variation in environmental temperature. In many ways, the stress response is an ideal system to study temperature adaptation, as Hsps are *a priori* known to be activated by temperature and capable of offsetting the negative consequences of irreversible protein damage. Future directions in the field will certainly reveal more about the temperature physiology of rocky intertidal invertebrates. However, given the interesting natural history of these organisms and their adaptation to the physical extremes of the rocky, wave-swept intertidal zone, studies of these organisms may also contribute to questions on a much more global scale. For example, the study of thermal stress in the rocky intertidal and the way in which invertebrates cope with this stress may elucidate the cell signaling pathways of cells that are routinely exposed to thermal stress. Furthermore, the study of the stress response in nature also affords the opportunity to address central questions in biology with the integration of ecology and physiology. Most notably, directed studies of the stress response contribute to our understanding of the factors that limit species' biogeographical distributions. In other words, are species really more stressed at the extremes of their range? Thus, the study of heat shock proteins provides an ideal study system in which to test ecological hypotheses.

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