Ecologically Relevant Variation in Induction and Function of Heat Shock Proteins in Marine Organisms

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SYNOPSIS. Ectothermic organisms often face dramatic traverses of environmental temperature on a daily or seasonal basis; exemplars among this group are invertebrates and fish of the rocky intertidal zone. Because of the extremes of temperature exposure, intertidal animals have served as an excellent study system to examine the expression of heat shock proteins (Hsps) in response to natural variation in environmental temperature. Ecologically relevant variation in Hsp expression has been observed with seasonal acclimatization, with small-scale temperature gradients that occur in microhabitats and between species with different intertidal distributions. The maturing understanding of Hsp expression patterns in marine organisms has established a solid foundation on which to build the next set of questions. In this paper, I present an overview of the variation of Hsp expression in intertidal animals in nature and then address two emerging areas of investigation in the ecological physiology of Hsps. One area addresses the plasticity of Hsp expression in marine invertebrates and focuses on the mechanism of regulation of Hsp gene expression by environmental temperature. A second emerging area of investigation concerns whether Hsps as molecular chaperones display functional diversity that correlates with species' adaptation temperature.

INTRODUCTION

Although the pursuit of the ecological meaning of heat shock proteins (Hsps) is well underway, there are numerous challenges that await the integrative and ecological physiologist (Feder and Hofmann, 1999). It is well established that organisms in nature have diverse patterns of Hsp expression. The observed variation encompasses a suite of traits that include expression of different isoforms within a family of Hsps, variations in endogenous levels of Hsps and changes in the temperatures at which Hsp genes are activated. However, the biological consequences of these variations and what significance the plasticity of Hsp gene expression has for the organism in nature have yet to be thoroughly revealed. Furthermore, the evolution of the plasticity of Hsp expression and how the transcriptional activation of Hsp genes is responsive to changes in environmental temperature are critical yet largely unexplored questions in the ecological physiology of the heat shock response.

The primary goal of this paper is to present an overview of the diversity in Hsp expression observed in marine organisms in nature and to place this variation in an ecologically relevant context. A second objective is to present emerging areas of investigation of the ecological physiology of Hsps using marine organisms. The two new areas presented here address how Hsps function as molecular chaperones in ectotherms and how Hsp gene expression may be regulated by environmental temperature.

The intertidal environment

By definition, the rocky intertidal zone is the area of the shore that is regularly covered and uncovered by the movement of the tides. Because of the tidal cycle, intertidal organisms are subjected to a regime of immersion and emersion that exposes them to a suite of abiotic stresses. The edge effect of the intertidal zone is characterized by a steep physical gradient and, within this gradient, each intertidal organism has a distribution pattern from high to low within the...
The pattern of intertidal zonation is thought to be maintained by a number of factors, including the effects of abiotic stresses (Menge and Farrell, 1989; Bertness and Leonard, 1997) such as temperature and biotic stresses such as predation and competition with neighboring organisms (Paine, 1974).

As a group, intertidal animals have attracted the attention of physiologists because of their unique tolerances to physical factors of the marine and terrestrial worlds (e.g., Newell, 1979). One goal of this article is to present intertidal organisms, particularly sessile invertebrates, as a study system to examine the patterns of Hsp expression in nature. It is not the intention of this article to propose that abiotic stresses such as temperature directly and solely structure the intertidal community. Rather, the intention is to illustrate the use of the intertidal environment and the animals that live there as a means to explore the biological consequences of living in such a steep gradient of physical stress.

On a typical day in the intertidal, several factors determine the intensity of the thermal stress to the animals. The data in Figure 1 show body temperature data for a group of intertidal animals from various study sites and exemplify the environmentally-related changes in body temperature that intertidal animals experience. First, the timing of low tide, climatic conditions, cloud cover and microhabitats are all key determinants in the level of solar irradiance and wind that reaches the animals. During a midday low tide, ectothermic marine invertebrates may experience up to a 25°C change in body temperature and this increase can be rapid, up to 3°C per hour (Fig. 1A; Owen and Hofmann, 1998; Hofmann and Somero, 1995) or at an even greater rate (see Hel- muth, 1999). There is a distinct element of chance; extreme high temperatures on a day of a low mid-day tide may prove to be lethal (e.g., Garrity, 1984). Second, the zonation pattern of a species determines the amount of time an organism is emersed and thus, sets limits on the cumulative intensity of the thermal and dessication stress. With respect to temperature, there is an interaction between time of exposure and intensity. Mussels found high on the shore will be emersed for longer periods of time and will experience a greater bout of thermal stress (Fig. 1B). The zonation effect is also observed on an interspecific basis. Figure 1C shows body temperature data for three limpet species at a study site in the San Juan Islands. Limpets (Lottia digitalis) that are found in the upper intertidal reach higher body temperatures than other species found in the mid (L. pelta) or the lower intertidal (Tectura scutum). Third, there is an effect of season; body temperatures of mussels are on average much lower in winter than in summer (Fig. 1A).

Variation in Hsp expression in intertidal invertebrates

In intertidal invertebrates, the variation in Hsp expression has been observed in three general cases: 1) on a seasonal basis, 2) intraspecific differences as a function of distribution gradients in the intertidal and 3) in comparisons of species with different distribution patterns. Evidence from all these studies support a role for Hsps in stress tolerance where the direction of the variation in expression correlates with the direction of change of environmental temperature.

Seasonal differences have been observed for two features of Hsp expression: endogenous levels of Hsp protein and threshold induction temperatures, the temperature at which the hsp genes are activated. In the intertidal mussel, Mytilus trossulus, endogenous concentrations of hsp70 in gill tissue were significantly elevated in summer versus winter-acclimatized mussels (Hofmann and Somero, 1995). Similarly, levels of hsp70 isoforms were higher in gill from a congenic species of mussel, M. californianus, collected in summer as compared to those collected in the winter (Roberts et al., 1997). In intertidal invertebrates, the threshold induction temperatures also show seasonal differences, rising to higher temperatures in the summer. Figure 2 shows the results of an induction experiment where metabolic labeling was used to determine the temperature at which hsps were synthesized in gill tissue from a mussel. The threshold induction temperature is deter-
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Fig. 1. In situ body temperatures of marine intertidal invertebrates. All data were collected using a hand-held digital thermometer. (A) Mytilus trossulus from San Juan Islands. Body temperatures (solid symbols) were collected every 20 min on the same 5 individuals. Points represent mean ± SEM; for air temperature (open symbols), points are single determinations. From Hofmann and Somero, 1995. (B) Mytilus californianus at high and low-intertidal sites from an outer coast location (Strawberry Hill, Oregon). Each point represents the mean for 5 mussels; error bars are ±1 SEM. Data from Roberts et al., 1997. (C) Limpet species sampled at Iceberg Point, Lopez Island WA. Each point represents the mean of 5 limpets. Since limpets lose considerable water when removed from their substrate and this greatly affects body temperature over the course of the low tide, different individuals were measured at each time point. Limpets were selected haphazardly from a population of limpets at the median point of the zonation for each species.

mined to be the temperature at which hsp synthesis is first observable. In the summer mussels, the induction temperature is 28°C; however, the first appearance of hsps shifts to 23°C in winter-collected mussels. Overall, there was a 6°C change in induction temperatures that occurs on a seasonal basis. The interpretation here is that the animals are turning on hsp genes at a "later" point following heat stress. The shift in the
Temperature (°C)

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FIG. 2. Heat shock protein induction profile in gill from Mytilus trossulus. The fluorogram is from an induction experiment on isolated gill tissue from one individual mussel. At each temperature, a single piece of gill tissue from the same individual was incubated with 50 μCi of [35S] methionine/cysteine amino acid labeling mixture and then processed for SDS-PAGE. Proteins synthesized de novo during metabolic labeling were separated on a 10% SDS-polyacrylamide gel and fluorography was used to visualize the protein bands. Samples of gill extracts containing 600,000 CPM were loaded in duplicate lanes. 14C protein molecular weight markers are shown in kdaltons in the left lane.

induction temperature reflects seasonal acclimatization and a sort of heat hardening that results from repeated bouts of exposure to thermal stress during summer low tides. The explanation for the plasticity in the induction temperature for Hsp genes on a seasonal basis is unknown. However, the mechanism that regulates Hsp gene activation is of considerable interest and is discussed in a following section.

Expression patterns of Hsps in intertidal invertebrates are also affected by distribution characteristics such as microhabitat conditions and vertical height of occurrence within the intertidal zone. In each of these two cases, we have observed that the subtle and sublethal variations in the temperature regime have a surprisingly strong effect on Hsp expression. For example, in a comparison of adult sea urchins (Strongylocentrotus purpuratus), animals from tidepools displayed Hsp expression that suggested these animals experienced more thermal stress than subtidal conspecifics. Sea urchins in the tidepools were exposed to cyclically warming water temperatures where the tidepool would warm when the tide was out; the pool fluctuated from 2–6°C above ambient seawater temperature of 12°C once each day (Hofmann, unpublished data). When features of Hsp expression were examined, Hsp induction temperature and Hsp70 levels were both significantly elevated in tube feet from the tidepool specimens. In addition, levels of ubiquitin conjugates were higher, indicating that there was a higher degree of irreversible protein damage in the tissues from the tidepool urchins. All three of the biochemical indices exhibited changes demonstrating that mi-
Crohabitat differences had an impact on the state of the intracellular protein pool. Similarly, Hsp expression has been shown to differ for mussels that have different heights of occurrence in the intertidal zone. Within the predominant band of mussel bed that typifies the outer rocky coast intertidal zone of the Northeast Pacific, specimens of *Mytilus californianus* that occur in locations higher on the shore displayed elevated levels of Hsp70 isoforms compared to mussels from lower portions of the mussel bed (Roberts *et al.*, 1997). These results are consistent with the observation that individuals in the high-intertidal sites experience greater amounts of thermal stress.

Finally, intertidal invertebrates display interspecific differences in Hsp expression. The most informative studies have used congeneric species to test whether there are interspecific differences in Hsp gene expression (e.g., Bosch *et al.*, 1988; Sanders *et al.*, 1991; Dietz and Somero, 1992; Hofmann and Somero, 1996; Tomanek and Somero, 1999). Investigation of Hsp expression patterns in different species has been approached with two different strategies. In some cases, investigators have acclimated the study species to the same temperature and then assessed parameters of Hsp expression. A second strategy has been to collect individuals of congeneric species and test the Hsp expression profiles immediately following collection from the field. The advantage of the acclimation approach is that the thermal exposure is the same for each species and Hsp expression patterns can be tested using *a priori* assumptions based upon the temperature range for each species. For example, following acclimation to 13°C of two *Mytilus* congeners (*M. trossulus* and *M. galloprovincialis*), the levels of Hsp70 isoforms and induction threshold temperatures were tested in gill tissue from specimens of each congener (Hofmann and Somero, 1996). Since *M. trossulus* has a more northerly distribution (Alaska to central California) than *M. galloprovincialis* (central California to Baja California, Mexico; McDonald and Koehn, 1988), it was hypothesized that *M. galloprovincialis* may be better adapted to cope with heat stress than its northern congener.

The results of the study and the nature of Hsp expression supported this hypothesis. The northern species exhibited elevated levels of one Hsp70 isoform (Fig. 3), different Hsp induction profiles and elevated levels of thermally damaged proteins (Hofmann and Somero, 1996).

**Plasticity of Hsp expression in marine invertebrates and environmental regulation of gene expression**

A developing area of investigation in Hsp expression in intertidal invertebrates is addressing the nature of the cellular thermometer. How do ectotherms sense changes in temperature and then adjust Hsp gene expression accordingly to deal with elevated levels of thermally damaged proteins? In the physiological literature, there are numerous examples of how functional processes are altered in response to temperature changes (e.g., enzyme concentrations, physical characteristics of membranes) and the evolution of phenotypic plasticity of thermal acclimation is beginning to be addressed (Kingsolver and Huey, 1998). However, the mechanisms that are responsible for *sensing* changes in abiotic factors in the environment and transducing these changes to the cell are only beginning to be explored (Morimoto *et al.*, 1994; Kültz and Burg, 1998; Kwast *et al.*, 1998; Owen and Hofmann, 1998).

A first approach to dissecting the mechanism of how changes in environmental temperature are transduced to the genome was defining how the heat shock response changed with temperature acclimation and monitoring putative molecular factors that might regulate Hsp gene expression. The observation that Hsp induction temperatures were subject to acclimatization had been made previously (Dietz and Somero, 1992; Roberts *et al.*, 1997) but other factors that might have a regulatory role either had not been measured or were assayed in separate studies. Using intertidal mussels (*Mytilus trossulus*) as a study organism, we conducted an experiment designed as a preliminary test of a favored model in cell biology that describes the transcriptional activation of Hsp genes (Craig and Gross, 1991; Morimoto, 1993; Morimoto *et al.*, 1994; Kültz and Burg, 1998; Kwast *et al.*, 1998; Owen and Hofmann, 1998).
Specifically, we measured the following three features of the heat shock response in a single acclimation study: (1) the endogenous concentrations of hsp70, (2) the levels of a transcriptional factor, HSF1 (responsible for activating heat-inducible Hsp genes) and (3) the threshold induction temperatures for Hsps. Our goal was to begin to understand how these features interact and account for the observed plasticity in the temperature at which hsp genes are activated in ectotherms in the context of the proposed model (shown in Fig. 4).

With regard to the transcriptional activation of Hsp genes, it is well described that the inducible genes are controlled by a single transcriptional factor, HSF1, which activates the hsp genes in response to heat...
stress. In the model, the most proximal known step in hsp gene activation is that HSFl undergoes a conformational change in response to heat stress, trimerizes and binds to the heat shock element (HSE) in the promoter of hsp genes to activate transcription (Fig. 4). Under normal physiological conditions, it is proposed that a heat shock protein, Hsp70 in this case, binds to the monomeric form of HSFl in the cytoplasm and prevents trimerization. According to the model, exposure to stress in the form of heat or chemicals will result in elevated levels of denaturing proteins that compete for cytoplasmic Hsp70 and effectively remove it from the complex with HSFl. Monomeric HSFl is then free to move into the nucleus, trimerize and activate the expression of the Hsp gene. There is now considerable speculation about exactly what mechanism/pathway transduces cellular stress to the transcriptional apparatus and modulates the activity of HSFl. Hypotheses generally fall into two categories. One is that cellular stressors such as temperature directly induce conformational changes and trimerization of HSFl (Larson et al., 1995; Zhong et al., 1998) and the alternative hypothesis is the interaction with hsp70 in a regulatory fashion as illustrated by the role of Hsp70 in the model above (Morimoto, 1993).

We tested this model by acclimating mussels in the laboratory to a single temperature and compared them to mussels that remained in the intertidal and were subjected to varying and high temperatures. As anticipated, acclimation to a constant and lower temperature resulted in alteration of Hsp gene expression and the interaction of the measured characteristics were consistent with the model. First, the threshold induction temperature for hsp70 (i.e., the temperature at which the heat-inducible hsp70 gene was expressed) in mussel gill tissue was reduced by 6°C following the acclimation treatment. Mussels acclimated in seawater tables to a constant temperature of 12°C for 6 weeks displayed a threshold induction temperature of 20°C as compared to 26°C for individuals that were acclimatized in the intertidal for the same interval of time (Fig. 5; arrows indicate hsp70 bands). Second, the shift in the setpoint for the induction of hsp70 genes from 26°C to 20°C was correlated with a 7-fold concomitant decrease in cellular levels of hsp70 isoforms. Finally, using standard immunocytochemical techniques and an antibody specific for HSFl, no change in the levels of HSFl in gill tissue were detected (Fig. 6).

Collectively, these results support the proposed model for the adjustment of the cellular thermostat. Our data for mussels
Temperature (°C)

14 17 20 23 26 29 32

70 kDa

Acclimated Field-collected

FIG. 5. Heat shock induction profile experiment on acclimated and field collected mussels (Mytilus trossulus). The fluorogram shows threshold induction temperatures for Hsp synthesis in gill tissue (arrow indicates the appearance of a 70 kDa band). Metabolic labeling and fluorography techniques are similar to those described in the legend for Figure 2. Data from Owen and Hofmann (1998).

show that the setpoint at which the hsp genes are activated and the cellular hsp concentration track each other; gill from warm-acclimated mussels displays higher threshold induction temperatures and higher endogenous concentrations of hsps. According to the proposed cellular thermometer model, higher levels of hsp70 would effectively deal with denatured proteins up to a point until a threshold was reached and the hsp70 chaperones complexed with HSF1 would be recruited, HSF1 released and Hsp genes activated. This scenario effectively explains how the induction temperature can change in cells of an ectothermic organism on a daily or seasonal basis. It should be noted that Hsp70 is a protein that has an expected half-life of several days and thus the consequences of synthesizing additional Hsp70 can be lasting and cumulative. The combination of repeated bouts of exposure to heat stress in the intertidal and sequential activation of inducible Hsp genes can begin to explain some of the induced seasonal thermostolerance that is observed in intertidal invertebrates.

Another salient finding of this study was that the cellular concentrations of HSF1 did not change dramatically with acclimation. One prediction that can be made using the model is that the change in threshold induction temperature could be due to a quan-
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...change in HSF1 levels where HSF1 levels decline during acclimation to warmer conditions and thus the genes are activated more slowly. We are currently performing experiments to address alternative hypotheses and are performing experiments to study variation in the DNA-binding activity of HSF1 using gel shift assays. Although intraspecific differences and the physiological plasticity of Hsp gene expression may be due to regulatory mechanisms that are independent of HSF1 characteristics, interspecific differences may be related to differential activity of HSF1. Evidence does exist that HSF1 may have some species-specific activity or activation setpoints (Wu, 1995). Regardless of how HSF1 is regulated, what is clear is that the activity of HSF1 determines when the mussel tissues activate expression of Hsp genes. The important question here is: What is the ecologically relevant variation in the HSF1 function that transduces environmental temperature changes in the genome?

Hsps as molecular chaperones in fish

Despite the fact that the stress response and hsps are observed in all taxa thus far examined, there is virtually no information as to whether hsps and the protein chaperoning functions they perform exhibit any functional diversity that correlates with species' thermal distributions. Especially among ectothermic organisms, one might hypothesize that hsps display a pattern of molecular evolution and functional changes with species' adaptation temperatures. An alternative hypothesis is that molecular chaperones are conserved across the phyla that, as a group, hsps display 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 are comparing biochemical properties and protein folding capacities of homologous hsp gene products, in this case members of the 70kDa multigene family called hsp70s, from closely related marine fishes that have evolved at different temperatures. For the purposes of this paper, the data will focus on a comparison of the congeneric gobies, Gillichthys mirabilis and G. seta. Both of the species are eurythermic and are exposed to a broad range of temperatures on an annual basis with the upper end of the range being quite extreme for teleosts at 35–40°C (Fields and Somero, 1997). Of the two gobies, G. seta is an intertidal species with a distribution that is limited to the Northern Gulf of California. During low tides, G. seta remain in small tidepools or in crevices among rocks and are exposed to extremely warm seawater temperatures that can exceed 40°C in summer (Hofmann, unpublished data). G. mirabilis occurs in coastal sloughs and estuaries and has a broader biogeographic distribution than G. seta; it is found from Baja California to Central California and also occurs in coastal regions of the Northern Gulf of California. Temperatures in the estuarine habitat of G. mirabilis tend to be lower than the tidepool temperatures that G. seta experience. However, G. mirabilis have been caught in upper reaches of small estuaries where seawater temperature was 35–37°C and remained at this temperature for several hours (Hofmann, unpublished data). Thus, although G. seta may be exposed to a higher maximal temperature, G. mirabilis may experience a cumulatively similar amount of thermal stress due to exposure to high temperatures for extended periods of time in shallow estuarine waters.

Our approach in these experiments has been to purify an hsc70 homologue from fish white skeletal muscle and then to test various biochemical characteristics of the native chaperone (Hofmann and Place, 1998). Although purification of a native protein is a labor-intensive task, it is necessary in order to accurately assess the function of the hsps as they would be in situ. Recombinant protein techniques, while advantageous because of large yields of pure protein, have been reported to result in chaperones that have two-fold different ATPase activities as compared to the native form of the chaperone (Blond-Elguindi et al., 1993). The dissimilarity between the native and recombinant protein has been attributed to misfolding of the recombinant protein (Gao et al., 1996). Thus, interspe-
specific comparisons of chaperone function will be best performed using natural chaperones that have been folded in situ and represent the closest approximation to the native functional state of the protein.

Once the protein is purified, an ATPase assay is used as a functional assay for chaperone performance. Members of the 70 kDa hsp family hydrolyze ATP during the chaperoning cycle (McKay et al., 1994) and the weak ATPase activity is readily measured in vitro (Sadis and Hightower, 1992). The data in Figure 7 show the effect of temperature on the ATPase activity of hsc70 purified from, *G. mirabilis* and *G. seta*. For these assays, the protein was incubated at the specified temperature and activity was followed for 1 hr. The data points in the curve reflect the amount of $^{32}$P that was released from $\gamma$-labeled $^{32}$P-ATP during the incubation period. Results for both species show that the activity of native, purified hsc70 was within the range of ecological temperatures. For each species, activity is optimal at 37°C and begins to dissipate at 42°C (Hofmann and Place, 1998).

In conclusion, current results on the chaperoning activity of hsps from ectothermic organisms support a hypothesis that the hsps are "fine-tuned" to chaperone proteins at a temperature that is within physiological limits for the species. Other studies that address whether chaperoning activity of Hsps occurs at physiologically relevant temperatures are remarkably few. Bovine hsc70 has been shown to have optimal chaperoning function at physiological temperatures for mammalian cells (Leung et al., 1996) and DnaK, the hsp70 homologue from *E. coli*, hydrolyzes ATP from 20 to 53°C (McCarty and Walker, 1991). Additional experiments are in progress and a more detailed picture will emerge once hsc70s from other species are examined. At present, these data provide completely novel insight into the evo-
lutionary physiology of the hsp as molecular chaperones in ectotherms.

**Summary**

Research thus far on Hsps in marine organisms has laid a substantial foundation on which to build the next set of questions about Hsp expression in nature. We now have a study system to examine mechanisms underlying the physiological plasticity of Hsp gene expression and ask how environmentally-induced changes in body temperature are transduced to the organism. The pathways of hsp gene regulation have rarely been placed in an environmental context and, for eurythermal ectotherms, these mechanisms are vital to understanding organismal-level adjustment in thermotolerance and, species-level differences in responses to environmental temperature. In addition, new information about the Hsps as molecular chaperones is emerging. A next step in the study of the ecophysiology of the stress response is to build a connection between the temperature range at which an organism must perform physiologically and the biochemical function of hsp as molecular chaperones at ecologically relevant temperatures.

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**References**


