*Am J Physiol Regul Integr Comp Physiol* 288: R1195–R1202, 2005.
First published January 6, 2005; doi:10.1152/ajpregu.00660.2004.

# Comparison of Hsc70 orthologs from polar and temperate notothenioid fishes: differences in prevention of aggregation and refolding of denatured proteins

**Sean P. Place[1] and Gretchen E. Hofmann[1,2]**

[1]*Department of Ecology, Evolution, and Marine Biology,* [2]*Marine Science Institute, University of California, Santa Barbara, California*

Submitted 27 September 2004; accepted in final form 5 January 2005

**Place, Sean P., and Gretchen E. Hofmann.** Comparison of Hsc70 orthologs from polar and temperate notothenioid fishes: differences in prevention of aggregation and refolding of denatured proteins. *Am J Physiol Regul Integr Comp Physiol* 288: R1195–R1202, 2005. First published January 6, 2005; doi:10.1152/ajpregu.00660.2004.—Although a great deal is known about the cellular function of molecular chaperones in general, very little is known about the effect of temperature selection on the function of molecular chaperones in nonmodel organisms. One major unanswered question is whether orthologous variants of a molecular chaperone from differential thermally adapted species vary in their thermal responses. To address this issue, we utilized a comparative approach to examine the temperature interactions of a major cytosolic molecular chaperone, Hsc70, from differently thermally adapted notothenioids. Using in vitro assays, we measured the ability of Hsc70 to prevent thermal aggregation of lactate dehydrogenase (LDH). We further compared the capacity of Hsc70 to refold chemically denatured LDH over the temperature range of −2 to +45°C. Hsc70 purified from the temperate species exhibited greater ability to prevent the thermal denaturation of LDH at 55°C compared with Hsc70 from the cold-adapted species. Furthermore, Hsc70 from the Antarctic species lost the ability to competently refold chemically denatured LDH at a lower temperature compared with Hsc70 from the temperate species. These data indicate the function of Hsc70 in notothenioid fishes maps onto their thermal history and that temperature selection has acted on these molecular chaperones.

Antarctica; molecular chaperones; cold adaptation; notothenioids; protein refolding

TEMPERATURE HAS PERVASIVE EFFECTS on all levels of biological organization, and thus temperature-related adaptation can be expected to occur at the physiological, cellular, and molecular level. Although the links among temperature, biogeographical patterning, and protein structure have been a focus of research in comparative physiology (reviewed in Ref. 33), relatively little is known about the effect of temperature selection on the actual cellular processes central to protein biogenesis. One significant aspect of protein biogenesis that could be significantly perturbed by temperature is the function of molecular chaperones, cytosolic proteins that stabilize and maintain nascent polypeptides on productive pathways during translation (for reviews, see Refs. 22, 26, 29). Multiple classes of molecular chaperones are highly involved during the formation of nascent polypeptides (26), and for those molecular chaperones involved in protein synthesis, the general mechanism is to bind to exposed hydrophobic regions of nascent polypeptides as they emerge from the ribosome. This action prevents improper interactions until the full-length peptide is released from the ribosome and then subsequently promotes the formation of proper secondary and tertiary structures (22, 26, 29). Given the nature of their interaction with folding targets and the essential role they play in protein maturation, the function of these specialized molecules could be under stringent temperature selection. Thus the central goal of this study was to examine whether molecular chaperones from differently adapted organisms display biochemical function that correlates with the environmental temperature at which they function in situ. In the present study, we have compared the in vitro refolding characteristics of the molecular chaperone Hsc70 purified from three notothenioid fishes: *Notothenia angustata*, a benthic species found off the coast of the South Island of New Zealand (2) that experiences seawater temperatures that vary from 8 to 15°C annually (B. Dickson, personal communication), and two coastal Antarctic species, *Trematomus bernacchii* and *Pagothenia borchgrevinki*, that live at constant water temperatures of −1.86°C (16).

The notothenioid fishes provide an ideal comparative system to study the effects of cold temperature on physiological processes due to the isolation and unique thermal history of the Antarctic notothenioids and the existence of closely related temperate-adapted species (17). The establishment of the Antarctic circumpolar current ~25–22 million years ago produced the coldest, most stenothermic environment of the world's oceans with characteristic mean annual seawater temperatures of −1.86°C (9, 16). These waters only rarely deviate from this temperature, with infrequent warming periods reaching temperatures near −0.5°C for a few days during the austral summer (36). For decades, biologists have used this isolated environment as an ideal arena for the study of cold adaptation of physiological processes. Physiological modifications, such as the evolution of antifreeze proteins (8, 13, 14) and tubulin molecules that can polymerize at low temperatures (12), have conferred adaptive advantages on the fishes of the suborder Notothenioidei and have thus enabled Antarctic notothenioids to exist and thrive in the subzero, ice-laden waters that are characteristic of coastal Antarctica. In addition, characterization of myoglobins from several vertebrate species suggests the further occurrence of temperature selection in protein function within Antarctic fishes (6). Furthermore, studies of the function of cold-adapted mitochondria and proteins have shown that thermal history can affect structural and kinetic properties associated with function at the organism's habitat temperature, especially in extremely cold environments (11, 33, 35, 64).

Address for reprint requests and other correspondence: S. P. Place, S. C. Johnson Research Center, Mayo Clinic Scottsdale, Scottsdale, AZ 85259 (E-mail: place.sean@mayo.edu).

The costs of publication of this article were defrayed in part by the payment of page charges. The article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

  

Despite the advances in cold adaptation research on notothenioids, one area that has gone largely unstudied in these teleosts is that of protein biosynthesis. There are some data that suggest this process also may be under temperature selection. In a comparative study of Antarctic species, Haschemeyer and Williams (30) reported that temperature dependency of protein synthesis rates in Antarctic fish remained linear ($Q_{10}$ = 2.5–3) at temperatures below 25°C. These data contrasted with the steep rise in $Q_{10}$ values ($Q_{10}$ = 8–9) previously reported in rabbit reticulocyte lysate systems and rat hepatocytes at temperatures below 25°C (4, 10). The function of molecular chaperones in these fishes, however, has not been examined to date. To address this issue, we used a comparative approach to characterize the temperature sensitivity of the functional properties of the molecular chaperone Hsc70 in differently adapted members of the suborder Notothenioidei.

Few studies have used a comparative approach to assess whether molecular chaperones in ectothermic organisms have functional activities that correlate to adaptation temperature. However, there is evidence that protein folding performed by molecular chaperones in vivo would be different in organisms adapted to different thermal regimes. For example, human chaperones have been shown to lose refolding activity above 41°C (24). In addition, studies by Zippay et al. (66) and Place and Hofmann (55) reported temperature effects on the function of Hsc70 purified from a eurythermal goby with an approximate upper environmental temperature exposure of 36°C. In these studies, Hsc70 lost refolding activity above 40°C (66), whereas the ATPase region maintained activity at significantly higher temperatures (55). Although these studies have begun to elucidate the effects of variable temperature environments on the function of molecular chaperones, the effects of extreme cold temperatures has not been considered. Therefore, the focus of this study was to determine whether evolution at a constant cold temperature has resulted in functional adaptation of molecular chaperones, in this case Hsc70, to effectively interact with target proteins at low temperature.

We began to address this question by characterizing the functional activities of Hsc70 from Antarctic teleosts. For functional characterization studies, we purified Hsc70 from white muscle and then employed two assays to test the thermal sensitivity of their primary functions in vitro (for reviews, see Refs. 18, 22, 26). We initially utilized an aggregation assay to determine the ability of Hsc70 to prevent irreversible aggregation, and we then applied a refolding assay to measure the ability of Hsc70s purified from our study organisms to restore the function of a chemically denatured substrate. Our data indicate that Hsc70 from these differentially thermally adapted notothenioid fishes effectively interacted with target substrates to prevent thermal denaturation, displaying protein refolding activity characteristic of the members of the 70-kDa heat shock protein (Hsp) gene family. Although refolding activity was measured at temperatures that greatly exceeded temperatures these fish encounter in nature, these data showed correlation of maximal refolding activity to the environmental temperatures of these fishes, suggesting that temperature selection may have contributed to the thermal sensitivity of Hsc70 in the Antarctic notothenioids.

## MATERIALS AND METHODS

*Collection of study organisms. T. bernacchii* (Boulenger, 1902) and *P. borchgrevinki* (Boulenger, 1902) specimens were collected using hook-and-line fishing through holes drilled through the sea ice in McMurdo Sound, Antarctica (S 77° 51.119′, E 166° 39.862′). The fish were transported back to McMurdo Station and held in flow-through seawater aquaria located in the Crary Laboratory facilities. Fish were killed via cervical transsection following MS-222 anesthesia and then dissected on ice in a 0°C environmental room. Dissected white skeletal muscle was immediately frozen in liquid nitrogen. *N. angustata* (Hutton, 1875) were collected with baited fish traps placed on the substrate at 2- to 10-meter depths near Portobello Marine Laboratories on the South Island of New Zealand (S 45.50°, E 170.38°). The fish were transported back to Portobello Marine Laboratories and held in flow-through seawater aquaria. Fish were killed on ice in a 10°C environmental room as described above, and white muscle was immediately frozen at −70°C. For all species, tissues were transported back to our home institution on dry ice and kept at −70°C until used for protein purification. This study was carried out in accordance with the American Physiological Society's "Guiding Principles for Research Involving Animals and Human Beings" (1). The animal use protocol was approved by the University of California, Santa Barbara, Institutional Animal Care and Use Committee (protocol # 10-02-634).

*Protein purification.* Hsc70 was purified from *T. bernacchii*, *P. borchgrevinki*, and *N. angustata* white skeletal muscle according to a modified two-step, low-pressure chromatography protocol previously reported by Place and Hofmann (55). For each purification procedure, 50–60 g of white muscle were homogenized in 5 volumes of homogenization buffer (10 mM Tris·HCl, pH 7.5, 10 mM NaCl, 0.1 mM EDTA, and 1.0 mM PMSF) in a Waring industrial blender. The homogenate was centrifuged at 10,700 *g* for 20 min at 4°C. The supernatant was then pooled and applied to a DEAE anion exchange column (2.5 × 20 cm, DEAE Sephacel; Pharmacia Biotech). The DEAE column was washed with a 50 mM NaCl solution and eluted with a buffer containing 200 mM NaCl. The eluted fractions were pooled and applied to an ATP-agarose affinity column (~10 ml; Sigma). The ATP affinity column was prewashed with affinity column buffer (A-CB) before the eluent was applied from the DEAE IE column. The column was washed with a salt gradient of 0.25–0.75 M NaCl (300 ml at a rate of 0.75 ml/min) followed by A-CB to remove the high-salt solution. The column was further washed with 1 mM GTP and then eluted with 3 mM ATP. The eluted fractions were pooled and condensed using Centricon-30 centrifugal concentrators (Millipore) at 5,000 *g* for 30 min. For a complete protocol, including detailed buffer constituents, see Place and Hofmann (55). Protein content was determined with a modified Bradford assay (Coomassie Plus; Pierce), and the purity of each preparation was determined using SDS-PAGE separation followed by silver staining (Bio-Rad Silver Stain Plus kit). Western blotting, as described by Hofmann and Somero (34), with the use of a monoclonal anti-Hsp70/Hsc70 primary antibody (MA3-001; Affinity Bioreagents) was performed to identify the purified protein.

*Thermal aggregation assays.* The thermal aggregation profile of lactate dehydrogenase (LDH) was examined using a modified protocol described by Langer et al. (44). Briefly, 150 nM LDH (rabbit type II; Sigma) was incubated in the presence or absence of 1 μM of purified Hsc70 in refolding buffer (25 mM HEPES, 50 mM KCl, 5 mM $MgCl_2$, 3 mM ATP, pH 7.2) in an ECHOtherm IC20 chilling/heating plate (Torrey Pines Scientific) at 55°C for 1 h. At the times indicated, 50-μl aliquots were monitored for light scattering at 320 nm in a quartz microcuvette at 25°C (37).

*Chemical denaturation and refolding assays.* Refolding of chemically denatured LDH was measured according to a modified protocol previously reported by Lee et al. (45). Native LDH was diluted to 13.5 μM in denaturation buffer (6 M guanidine hydrochloride, 30 mM

Tris·HCl, pH 7.4, 5 mM dithiothreitol, and 1 mM EDTA) and incubated for 30 min at 25°C. Refolding was initiated by diluting chemically denatured LDH into refolding buffer containing no additions, 1 μM Hsc70 alone, 0.2 μM of the mammalian Hsp40 cochaperone Hdj1 (SPP-400; Stressgen) alone, or 1 μM Hsc70 plus 0.2 μM Hdj1 with simultaneous vortexing to a final LDH concentration of 270 nM. Reactions were incubated across a temperature range of −2 to +45°C for 3 h. At the indicated times, 25-μl aliquots were removed and combined with 2 ml of assay cocktail (80 mM imidazole, pH 7.0, 150 μM NADH, and 2 mM pyruvate) and assayed for activity at 25°C.

*Statistical analysis.* All ANOVA measurements were performed using JMP 5 software (SAS Institute) for Windows. We verified that the ANOVA assumptions of normality were met by testing the fit distribution of each data set before performing a one-way ANOVA with a Tukey honestly significant difference analysis to test for significant differences, $P < 0.05$, between treatments. All data are presented as means ± SE.

## RESULTS

*Purification of native Hsc70.* Native Hsc70 was purified from the white muscle of all three species to >90% homogeneity as determined by SDS-PAGE separation followed by silver staining (Fig. 1*A*). Identity of the molecular chaperone was verified using an antibody specific to 70-kDa Hsp gene family members. Western analysis yielded a single band corresponding to the 70-kDa size class of proteins (Fig. 1*B*). In general, 50- to 60-g white muscle routinely yielded ~0.01 mg of Hsc70, corresponding to <0.1% of the total protein content of the white muscle homogenate.

*Thermal aggregation.* To measure the basic action of our molecular chaperones, (i.e., recognizing and selectively binding nonnative proteins, thereby preventing aggregation), we measured the relative light scattering at 320 nm of a solution of LDH in the presence or absence of Hsc70 purified from *T. bernacchii*, *P. borchgrevinki*, or *N. angustata* incubated at 55°C for 1 h. LDH incubated at 55°C in the absence of Hsc70

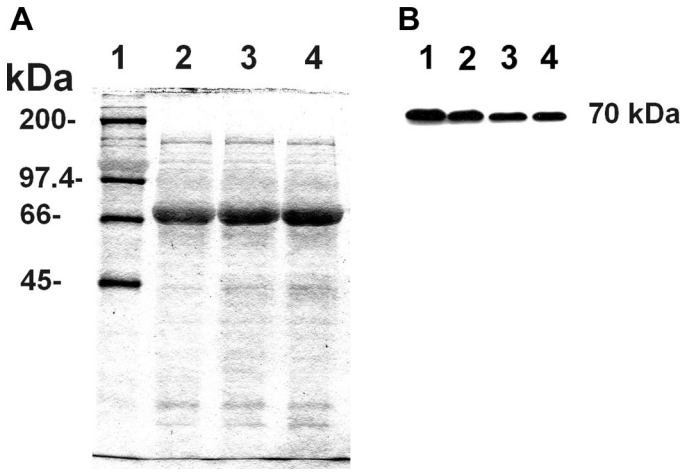


Fig. 1. *A*: silver-stained gel of purified Hsc70 from each species. For silver staining, 5 μg of total protein were applied to *lanes 1–4* and separated by electrophoresis on a 10% SDS-polyacrylamide gel. *Lane 1*, silver stain protein molecular mass standards; *lane 2*, Hsc70 from *Trematomus bernacchii* white muscle; *lane 3*, Hsc70 from *Pagothenia borchgrevinki* white muscle; and *lane 4*, Hsc70 from *Notothenia angustata* white muscle. *B*: Western blot detection of purified 70-kDa heat shock proteins (Hsps) 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 *T. bernacchii*; *lane 3*, 5 μg of Hsc70 purified from *P. borchgrevinki*; and *lane 4*, 5 μg of Hsc70 purified from *N. angustata*.

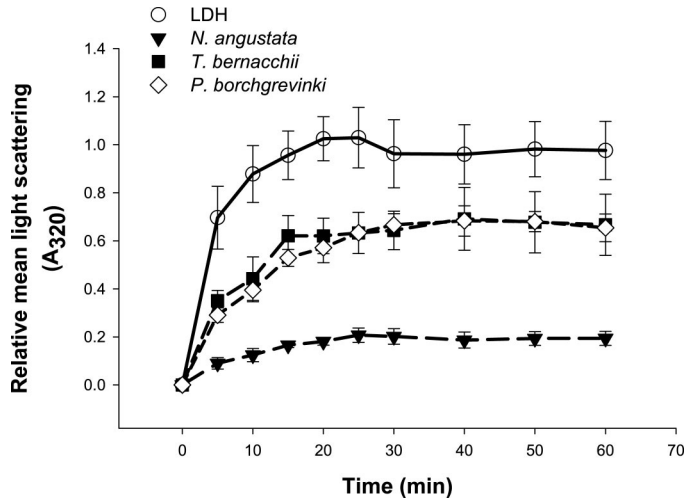


Fig. 2. Thermal aggregation of lactate dehydrogenase (LDH). In vitro assays containing 150 nM LDH were incubated at 55°C in the absence (○) or presence of 1 μM Hsc70 purified from *T. bernacchii* (■), *P. borchgrevinki* (◇), or *N. angustata* (▼). At the times indicated, 50-μl aliquots were removed and monitored for absorbance at 320 nm ($A_{320}$), which is indicative of light scattering due to LDH aggregation. Relative mean light scattering is expressed in arbitrary units.

rapidly denatured and aggregated, reaching a maximal absorption within 20 min (Fig. 2). When incubated at 55°C with purified Hsc70, LDH aggregation was significantly impeded. In the presence of Hsc70 purified from the two Antarctic species, *T. bernacchii* and *P. borchgrevinki*, the rate of aggregation of LDH was significantly slower; the maximum level of aggregation as measured by light scattering was twofold lower than that seen in reactions with LDH alone (Fig. 2). In the presence of Hsc70 purified from *N. angustata*, these effects were further amplified. The rate of aggregation in the presence of Hsc70 purified from *N. angustata* was depressed, and the maximum level of aggregation after 20 min was more than fivefold lower than the levels achieved when LDH was incubated alone (Fig. 2).

*Refolding of chemically denatured LDH.* To further characterize the effects of temperature on the function of orthologous variants of Hsc70, we measured the ability of Hsc70 purified from *T. bernacchii*, *P. borchgrevinki*, and *N. angustata* to competently refold chemically denatured LDH across the temperature range of −2 to +45°C. In these in vitro assays, chemically denatured LDH did not spontaneously refold when incubated alone or in the presence of 0.2 μM Hdj1 (Fig. 3, *A–C*). Low levels of activity (1–2% of the initial activity of a 270 nM solution of native LDH) were measured when denatured LDH was incubated with Hsc70 alone (Fig. 3, *A–C*). However, when supplemented with 0.2 μM of the cochaperone Hdj1, Hsc70 purified from each species was able to restore the activity of chemically denatured LDH across a wide range of temperatures (Fig. 3, *A–C*). When incubated across the temperature range of −2 to +45 °C, Hsc70 purified from the two Antarctic species maintained the ability to measurably refold denatured LDH at temperatures as high as 35°C, whereas Hsc70 purified from *N. angustata* restored activity of denatured LDH at temperatures as high as 40°C (Fig. 3, *A–C*). Maximum refolding activity was displayed at 20°C for Hsc70 purified from all three species; however, for both *T. bernacchii*

and *P. borchgrevinki* Hsc70, refolding activity readily decreased when refolding was carried out at temperatures higher than 20°C and was not detectable above control values (LDH alone) at temperatures above 40°C (Fig. 3, *A* and *B*). *N. angustata* Hsc70 maintained higher functional activities at temperatures above 20°C compared with the refolding activity of Hsc70 purified from the Antarctic species, and restored LDH activity did not fall below control values until refolding was carried out at 45°C (Fig. 3*C*).

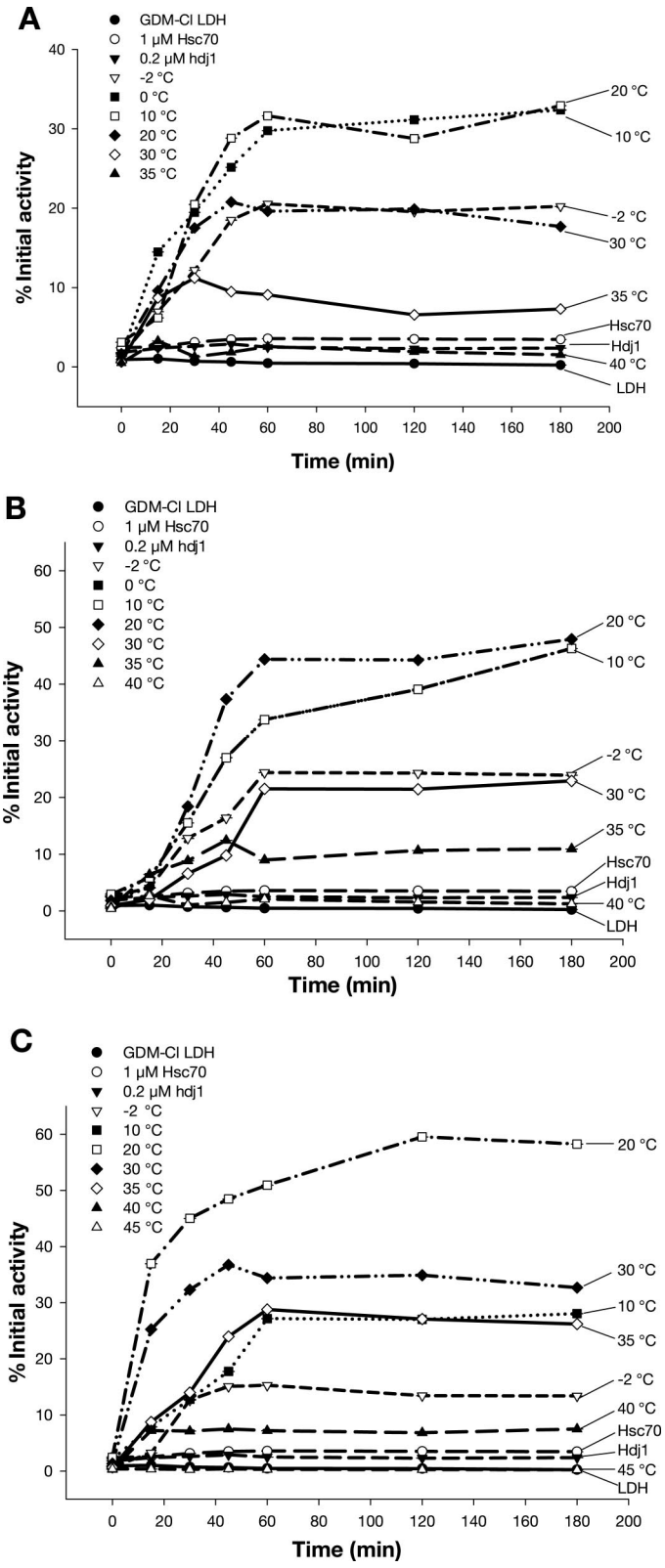


Fig. 3. LDH refolding assays. Native LDH was diluted to a final concentration of 13.5 μM and denatured in 6 M guanidine hydrochloride (GDM-Cl). Denatured LDH was then diluted 50-fold into refolding buffer supplemented with 0.2 μM Hdj1 and 1 μM Hsc70 purified from *T. bernacchii* (*A*), *P. borchgrevinki* (*B*), or *N. angustata* (*C*) to initiate refolding. Solutions were incubated across a temperature gradient of −2 to +45°C, and at the indicated times, 25-μl aliquots were assayed for LDH enzyme activity. As controls, solutions lacking Hsps (●), supplemented with 0.2 μM Hdj1 alone (▼) or with 1 μM Hsc70 alone (○), also were assayed for enzyme activity. Amounts of reactivation are expressed as mean percentages (±SE) relative to the activity of an equivalent amount of native LDH.

Summary data for the degree of LDH refolding by Hsc70 from all three species are shown in Fig. 4. At −2°C, the ecologically relevant temperature for *T. bernacchii* and *P. borchgrevinki*, Hsc70 purified from the Antarctic species displayed significantly higher refolding capacity compared with *N. angustata* Hsc70 (Fig. 4; $P < 0.0054$, ANOVA). Interestingly, there was no significant difference in refolding capacity among the species at 10°C (Fig. 4). However, for *N. angustata* Hsc70, percent refolding values increased significantly at temperatures above 10°C and remained significantly higher than Hsc70 from either *T. bernacchii* or *P. borchgrevinki* at temperatures above 10°C (Fig. 4; $P < 0.0003$, ANOVA). At 35°C, percent refolding values for *N. angustata* Hsc70 were over threefold higher than both *T. bernacchii* and *P. borchgrevinki* Hsc70, and at 40°C, refolding was nearly threefold higher with *N. angustata* Hsc70 compared with Hsc70 purified from either Antarctic species (Fig. 4).

## DISCUSSION

The primary focus of this study was to determine whether evolution at a constant cold temperature had resulted in functional adaptation of molecular chaperones, in this case Hsc70, to effectively interact with target proteins at low temperature. To this end, we purified the Hsc70 from three closely related and differently thermally adapted notothenioid fishes and compared the effect of temperature on the two characteristic activities attributed to this molecular chaperone: *1*) the ability to suppress improper protein interactions through recognition and interaction with exposed hydrophobic sites, and *2*) the promotion of competent folding intermediates (for reviews, see Refs. 22, 26, 29). All three species, *T. bernacchii*, *P. borchgrevinki*, and *N. angustata*, are phylogenetically closely related members of the suborder Notothenioidei, two of which have evolved in a dramatically different thermal environment for the last 15 million years (16). The New Zealand notothenioid, *N. angustata*, is distinguished by a very different thermal profile in nature than that encountered by the Antarctic notothenioids. *N. angustata* is a cold temperate-adapted species that encounters annual seawater temperature fluctuations between 8 and 15°C, whereas *T. bernacchii* and *P. borchgrevinki* are extreme cold stenotherms that experience mean annual temperatures of −1.86°C, with only rare temperature fluctuations during the austral summer (36).

Overall, this study revealed two salient results: *1*) the chaperoning characteristics of Hsc70 from all three species was affected by temperature in a manner that correlated with each

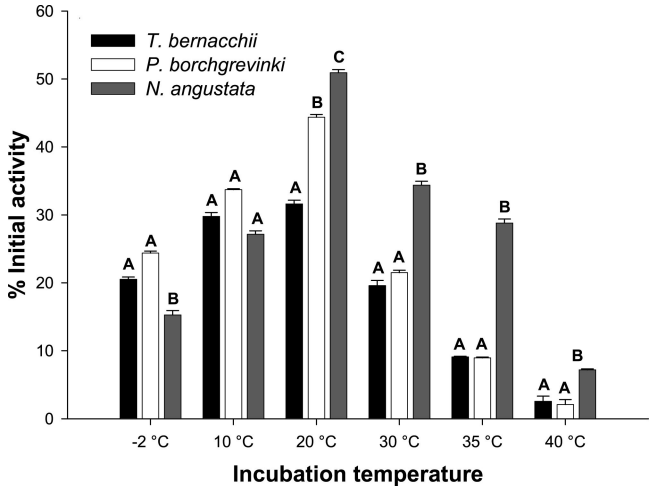


Fig. 4. Summary data of the refolding activity of Hsc70 purified from notothenioid fishes. Columns represent the mean percent refolding of chemically denatured LDH achieved within 60 min of incubation in refolding buffer supplemented with 0.2 μM Hdj1 and 1 μM Hsc70 purified from *T. bernacchii*, *P. borchgrevinki*, or *N. angustata* across the temperature range of −2 to +40°C. Levels within the indicated temperature with different letters are significantly different ($^{A,B,C}P < 0.005$, ANOVA). Amounts of reactivation are expressed as mean percentages (±SE) relative to the activity of an equivalent amount of native LDH.

species' respective ecological thermal niche, suggesting that adaptive compensation may have occurred in molecular chaperones of Antarctic notothenioids for efficient function at extreme cold temperatures; and *2*) the molecular chaperone Hsc70 from the cold stenothermic Antarctic fish maintained a surprising level of protein refolding activity at temperatures that far exceeded the normal environmental temperatures for these fish.

To verify that our purified fish Hsc70 possessed molecular chaperone activity, we followed the in vitro aggregation of LDH in the presence of purified chaperones to determine their ability to prevent improper interactions of nonnative LDH molecules. This approach has been used previously on chaperones from other organisms (44, 45, 65). As shown in Fig. 2, Hsc70 purified from all three species inhibited both the rate of LDH aggregation and the overall amount of aggregated LDH at 55°C. Although Hsc70 from all three species showed the ability to prevent aggregation of LDH, Hsc70 purified from the Antarctic species displayed a decreased capacity for interaction with nonnative substrate at this relatively high temperature compared with *N. angustata* Hsc70. These data indicate that the Hsc70s from the three fish species have different thermal sensitivities with respect to prevention of thermal aggregation.

To further assess changes in the interactions of Hsc70 with target substrates that were temperature sensitive, we subsequently examined the effects of temperature on the ability of the purified Hsc70s to refold chemically denatured LDH by measuring the recovery of LDH activity in vitro. Such assays are routinely used to assess the ability of molecular chaperones to refold various target proteins (24, 27, 42, 44–46, 66)

In our study, Hsc70 purified from the cold-adapted Antarctic species displayed greater refolding activity at the lower range of temperatures (−2 to +10°C), whereas Hsc70 from the temperate New Zealand species displayed elevated function at the higher temperatures (+30 to +40°C) compared with the Antarctic species, suggesting a perturbing effect on Hsc70 function in the Antarctic species at these high temperatures (Fig. 4). Purified Hsc70 from any of the species alone was not capable of significantly refolding chemically denatured LDH, even when the assay was performed at ecologically relevant temperatures for each fish (Fig. 3, *A–C*). Furthermore, the addition of Hdj1, a vertebrate ortholog of the cofactor Hsp40, was required for efficient refolding across the indicated temperature range (Fig. 3, *A–C*). As previously shown for several Hsc70 orthologs, the presence of an Hsp40 cofactor in solution in conjunction with Hsc70 is required for the efficient refolding of substrate, perhaps through the direction of protein interactions and modulation of substrate affinity (25, 33, 52). Although the use of Hdj1, a mammalian Hsp40 ortholog, with Hsc70 purified from our study organism could introduce variation within molecular chaperone interactions, there is extensive evidence for the conservation of function among Hsp40 orthologs from different species (32, 38, 39, 41). For example, previous studies have shown that the yeast Hsp40 ortholog Ydj1 functions comparably to human Hsp40 orthologs in Hsp40-dependent folding of steroid receptors and clathrin uncoating (32, 39). In light of these studies, we are confident that the variation in these data is not an artifact of molecular chaperone interactions.

Our protein folding data suggest that although molecular chaperones have retained function over a wide range of temperatures, temperature compensation may have occurred in the structure-function relationship of Hsc70 in Antarctic notothenioids that has resulted in a shift in the optimal functional temperature range for molecular chaperones from these fishes. Although the mechanism responsible for this shift is not yet understood, previous research on biochemical adaptations of proteins from cold-adapted species points to sequence variation as an evolutionary leverage point (20, 21, 33, 61, 62). Hochachka and Somero (33) suggested that adaptations in kinetic properties are generally not achieved through changes in amino acids directly responsible for substrate interaction; rather, adaptive changes in sequence must occur in regions outside an active site to maintain essential enzyme-ligand interactions. Therefore, enhanced activity in proteins of cold-adapted species potentially could be achieved through subtle changes in the primary sequence near a functional domain that affects the flexibility of variable hinge regions controlling conformational changes. Within Hsc70, two such functional regions exist: a larger ATPase domain near the $NH_2$ terminus, and a smaller peptide-binding domain near the COOH terminus. These domains are modulated through ATP-induced conformational changes and cooperative interactions with cofactors such as members of the 40-kDa family of Hsps. The cooperative actions of these two domains assist in the maintenance of protein homeostasis in the cellular milieu through regulated cycling of binding and dissociation events (5, 28, 31, 49, 63). Interestingly, a candidate region for adaptive sequence changes has been identified outside of these two functional domains. A regulatory motif at the extreme COOH terminus of Hsc70 affects substrate binding and cofactor interactions, as shown in mutational studies by Freeman et al. (25).

In this light, our data, in conjunction with that of previous studies of Hsc70 from warm-adapted organisms, suggest that potential temperature compensations are a result of changes affecting the substrate binding region rather than the ATPase

region of this molecular chaperone. Our study found that both substrate interactions, as measured by LDH aggregation, as well as refolding activity, as measured by recovery of LDH activity, vary among the three species and correlate with thermal adaptation. These data suggest that substrate interactions within the peptide-binding domain are affected in a temperature-sensitive manner. Evidence from the literature supports this perspective that protein binding by molecular chaperones is temperature sensitive. Although there are few examples, and none to our knowledge that have used a comparative approach, other studies have shown that in vitro refolding correlates with body or environmental temperature. For example, human Hsp70 has been shown to lose the ability to refold proteins above 41°C, only 4°C above human body temperature (24). In addition, an Hsc70 ortholog in the eurythermal goby *Gillichthys mirabilis* has been shown to lose refolding activity at 40°C, a temperature near the upper thermal limits encountered by this species (66). These values correspond closely to the melting temperature ($T_m$) of 40.5°C recorded for the thermal denaturation profile attributed to the loss of secondary structure within the peptide-binding region of DnaK from *Escherichia coli* (53). Finally, the lack of specificity associated with the multiple ligand interactions within the peptide-binding domain may be indicative of greater sequence variation within this region of the molecule (59).

In contrast, there is ample evidence indicating that of the two functional domains of Hsc70, conservation of function and sequence is greater in the ATPase region (51). Functionally, similar characteristics have been observed in ATPase activity of Hsc70 orthologs across taxa (50, 54, 55, 60). In two species included in the current study, *T. bernacchii* and *N. angustata*, Hsc70 ATP hydrolysis activity has been measured at temperatures above 65°C (56). Similarly, hydrolysis of ATP by the ATPase region of the Hsc70 ortholog purified from *G. mirabilis* has been measured at temperatures as high as 70°C (55). Finally, in a prokaryotic chaperone, Palleros et al. (53) described a second step in the thermal denaturation profile of DnaK corresponding to the loss of secondary structure in the ATPase domain that proceeded at a much higher temperature with a $T_m$ of 72°C. Interestingly, there is some evidence for biochemical adaptation within the ATPase region of DnaK isolated from the hyperthermophilic bacterium *Thermus thermophilus*. Single-turnover ATPase activity of *T. thermophilus* DnaK measured at 75°C was comparable to the activity of *E. coli* DnaK measured at 25°C (40).

Although there is some support for the mechanism of these adaptations in temperature sensitivity, there is little known about the effects of extreme cold temperatures on protein structure and function that may be driving the necessity for these adaptations. However, we can make predictions based on what we know about the perturbing effects of high temperatures. The effect of high temperature has long been understood to perturb proteins via several mechanisms. Increased temperatures weaken hydrogen bonding and increase "conformational breathing", resulting in decreased protein stability. At the same time, elevated temperatures strengthen hydrophobic interactions, exacerbating the formation of protein aggregates (22, 33, 61). These effects of high temperatures lead to the prediction that cold temperatures would thus enhance protein stabilities and substrate interactions. Continuing along this line of reasoning, a decrease in temperature would decrease hydrophobic effects and at the same time strengthen hydrogen bonds and electrostatic interactions (15), perhaps increasing protein stability and thereby reducing the need for molecular chaperone assistance. Recombinant expression studies have, in fact, demonstrated that a decrease in temperature correlates with an increased yield of stable recombinant proteins (47). However, research on the cold denaturation of proteins is quickly changing these assumptions. It is now understood that extreme cold temperatures can have equally perturbing effects on the tertiary structure of proteins and their interactions with solutes and target substrates (for reviews, see Refs. 43, 48, 58). Protein synthesis efficiencies reported in Antarctic limpets (23) and expression patterns of the molecular chaperones Hsc71/Hsp70 in conjunction with measurements of ubiquitin conjugate levels in tissues of Antarctic fish indicate that these extreme cold-adapted species may possess a greater need for molecular chaperone assistance (57).

In summary, molecular chaperone function requires the cooperation of multiple binding sites that could be disturbed by low temperatures and thus may have resulted in adaptive changes within this variable region, promoting proper interaction with partially unfolded proteins at extreme cold temperatures. Previous studies have shown that as little as a single amino acid substitution in a nonconserved region of LDH orthologs from differentially adapted fish species can result in changes in enzyme kinetics (19–21). Similarly, although all three species in this study displayed a general ability to competently refold chemically denatured LDH over a wide range of temperatures in vitro, overall, the optimal refolding activity of each species mapped onto their thermal history, indicating a change in function of Hsc70 across these differentially adapted species. Further kinetics studies, studies of structure-function relationships, and sequence data will be an important part of understanding how molecular chaperones have been adapted to function in situ in the extreme cold environments inhabited by Antarctic fishes.

## ACKNOWLEDGMENTS

We are indebted to many individuals who assisted during the course of this project. In particular, we thank the field team members of Bravo 134 (Dr. Brad Buckley, Mackenzie Zippay, Dr. Ali Whitmer, and Beverly Dickson) for logistical support in obtaining Antarctic specimens. We thank the United States Antarctic Program and Raytheon Polar Services Corporation for logistical and field support at McMurdo Station, Antarctica. We thank the University of Otago (Dunedin, New Zealand) for access to research facilities at Portobello Marine Laboratory (PML). In addition, we gratefully acknowledge Chris Osovitz, Susan Lund, and the staff of PML, especially Beverly Dickson (Lab Manager), Bill Dickson, and S. A. Monteith, for assistance with the collection of *Notothenia* specimens. Finally, we thank Drs. Bruce Sidell and Arthur DeVries for helpful discussions on evolution of notothenioid fishes and Drs. David F. Smith and George Somero for informative discussions on the biology of molecular chaperones.

## GRANTS

This research was supported by National Science Foundation (NSF) Grants IBN 0096100 and OPP 0301927 (to G. E. Hofmann) and by Susan and Bruce Worster in support of G. E. Hofmann as the Worster Scholar at the University of California, Santa Barbara. Supplementary funding for research in New Zealand was provided by NSF International Programs. In addition, a University of California, Santa Barbara Fee Fellowship from the Department of Ecology, Evolution, and Marine Biology supported S. P. Place during parts of this project.

## REFERENCES


1. **American Physiological Society.** Guiding principles for research involving animals and human beings. *Am J Physiol Regul Integr Comp Physiol* 283: R281–R283, 2002.
2. **Ayling T and Cox GJ.** *Collins Guide to the Fishes of New Zealand.* Auckland, NZ: Collins, 1982.
3. **Blond-Elguindi S, Fourie AM, Sambrook JF, and Gething MJH.** Peptide-dependent stimulation of the ATPase activity of the molecular chaperone BiP is the result of conversion of oligomers to active monomers. *J Biol Chem* 268: 12730–12735, 1993.
4. **Brosnan ME, Myron DR, Feltham LAW, and Sells BH.** Comparison of the temperature sensitivity of protein synthesis by cell-free systems from liver of rat and skate (*Raja ocellata*). *Biochim Biophys Acta* 447: 360–374, 1976.
5. **Buchberger A, Schröder H, Hesterkamp T, Schönfeld HJ, and Bukau B.** Substrate shuttling between the DnaK and GroEL systems indicates a chaperone network promoting protein folding. *J Mol Biol* 261: 328–333, 1996.
6. **Cashon RE, Vayda ME, and Sidell BD.** Kinetic characterization of myoglobins from vertebrates with vastly different body temperatures. *Comp Biochem Physiol* 117B: 613–620, 1997.
7. **Chang TC, Hsiao CD, Wu SJ, and Wang C.** The effect of mutating arginine-469 on the substrate binding and refolding activities of 70-kDa heat shock cognate protein. *Arch Biochem Biophys* 386: 30–36, 2001.
8. **Chen LB, DeVries AL, and Cheng CHC.** Convergent evolution of antifreeze glycoproteins in Antarctic notothenioid fish and Arctic cod. *Proc Natl Acad Sci USA* 94: 3817–3822, 1997.
9. **Clarke A and Johnston IA.** Evolution and adaptive radiation of Antarctic fishes. *Trends Ecol Evol* 11: 212–218, 1996.
10. **Craig N and Fahrman C.** Regulation of protein synthesis by temperature in mammalian cells: noninvolvement of the plasma membrane. *Biochim Biophys Acta* 447: 478–490, 1977.
11. **Dahlhoff E and Somero GN.** Effects of temperature on mitochondria from abalone (genus *Haliotis*)—adaptive plasticity and its limits. *J Exp Biol* 185: 151–168, 1993.
12. **Detrich HW and Parker SK.** Divergent neural β tubulin from the Antarctic fish *Notothenia corriceps neglecta*: Potential sequence contributions to cold adaptation of microtubule assembly. *Cell Motil Cytoskeleton* 103: A544, 1993.
13. **DeVries AL.** The role of antifreeze glycopeptides and peptides in the freezing avoidance of Antarctic fishes. *Comp Biochem Physiol* 90B: 611–621, 1988.
14. **DeVries AL and Wohlschlag DE.** Freezing resistance in some Antarctic fishes. *Science* 163: 1073–1075, 1969.
15. **Dill KA.** Dominant forces in protein folding. *Biochemistry* 29: 7133–7155, 1990.
16. **Eastman JT.** *Antarctic Fish Biology: Evolution in a Unique Environment.* San Diego, CA: Academic, 1993.
17. **Eastman JT.** Antarctic notothenioid fishes as subjects for research in evolutionary biology. *Antarct Sci* 12: 276–287, 2000.
18. **Feder ME and Hofmann GE.** Heat-shock proteins, molecular chaperones, and the stress response: evolutionary and ecological physiology. *Annu Rev Physiol* 61: 243–282, 1999.
19. **Fields PA and Somero GN.** Amino acid sequence differences cannot fully explain interspecific variation in thermal sensitivities of gobiid fish $A_4$-lactate dehydrogenases ($A_4$-LDHs). *J Exp Biol* 200: 1839–1850, 1997.
20. **Fields PA and Somero GN.** Hot spots in cold adaptation: Localized increases in conformational flexibility in lactate dehydrogenase $A_4$ orthologs of Antarctic notothenioid fishes. *Proc Natl Acad Sci USA* 95: 11476–11481, 1998.
21. **Fields PA, Wahlstrand BD, and Somero GN.** Intrinsic versus extrinsic stabilization of enzymes. The interaction of solutes and temperature on $A_4$-lactate dehydrogenase orthologs from warm-adapted and cold-adapted marine fishes. *Eur J Biochem* 268: 4497–4505, 2001.
22. **Fink AL.** Chaperone-mediated protein folding. *Physiol Rev* 79: 425–449, 1999.
23. **Fraser KPP, Clarke A, and Peck LS.** Low-temperature protein metabolism: seasonal changes in protein synthesis and RNA dynamics in the Antarctic limpet *Nacella concinna* Strebel 1908. *J Exp Biol* 205: 3077–3086, 2002.
24. **Freeman BC and Morimoto RI.** The human cytosolic molecular chaperones hsp90, Hsp70 (hsc70), and hdj-1 have distinct roles in recognition of a non-native protein and protein refolding. *EMBO J* 15: 2969–2979, 1996.
25. **Freeman BC, Myers MP, Schumacher R, and Morimoto RI.** Identification of a regulatory motif in Hsp70 that affects ATPase activity, substrate-binding and interaction with HDJ-1. *EMBO J* 14: 2281–2292, 1995.
26. **Frydman J.** Folding of newly translated proteins in vivo: The role of molecular chaperones. *Annu Rev Biochem* 70: 603–647, 2001.
27. **Gebauer M, Zeiner M, and Gehring U.** Proteins interacting with the molecular chaperone hsp70/hsc70: physical associations and effects on refolding activity. *FEBS Lett* 417: 109–113, 1997.
28. **Hartl FU.** Molecular chaperones in cellular protein folding. *Nature* 381: 571–580, 1996.
29. **Hartl FU and Hayer-Hartl M.** Protein folding. Molecular chaperones in the cytosol: from nascent chain to folded protein. *Science* 295: 1852–1858, 2002.
30. **Haschemeyer AEV and Williams RC.** Temperature dependency of cell-free protein synthetic systems from Antarctic fish. *Mar Biol Lett* 3: 81–88, 1982.
31. **Hermawan A and Chirico WJ.** N-ethylmaleimide-modified Hsp70 inhibits protein folding. *Arch Biochem Biophys* 369: 157–162, 1999.
32. **Hernandez MP, Chadli A, and Toft DO.** HSP40 binding is the first step in the HSP90 chaperoning pathway for the progesterone receptor. *J Biol Chem* 277: 11873–11881, 2002.
33. **Hochachka PW and Somero GN.** *Biochemical Adaptation: Mechanism and Processes in Physiological Evolution*. New York: Oxford University, 2002, p. 290–449.
34. **Hofmann GE and Somero GN.** Evidence for protein damage at environmental temperatures—seasonal changes in levels of ubiquitin conjugates and hsp70 in the intertidal mussel *Mytilus trossulus*. *J Exp Biol* 7: 1509–1518.
35. **Holland LZ, McFall-Ngai M, and Somero GN.** Evolution of lactate dehydrogenase-A homologs of barracuda fishes (genus *Sphyraena*) from different thermal environments: differences in kinetic properties and thermal stability are due to amino acid substitutions outside the active site. *Biochemistry* 36: 3207–3215, 1997.
36. **Hunt BM, Hoefling K, and Cheng CHC.** Annual warming episodes in seawater temperatures in McMurdo Sound in relationship to endogenous ice in notothenioid fish. *Antarct Sci* 15: 333–338, 2003.
37. **Jaenicke R and Rudolph R.** Folding proteins. In: *Protein Structure: A Practical Approach*, edited by Creighton TE. Oxford, UK: IRL, 1989.
38. **Kelley WL.** The J-domain family and the recruitment of chaperone power. *Trends Biochem Sci* 23: 222–227, 1998.
39. **King C, Eisenberg E, and Greene L.** Effect of yeast and human DnaJ homologs on clathrin uncoating by 70 kilodalton heat shock protein. *Biochemistry* 36: 4067–4073, 1997.
40. **Klostermeier D, Seidel R, and Reinstein J.** Functional properties of the molecular chaperone DnaK from *Thermus thermophilus*. *J Mol Biol* 279: 841–853, 1998.
41. **Kosano H, Stensgard B, Charlesworth MC, McMahon N, and Toft D.** The assembly of progesterone receptor-hsp90 complexes using purified proteins. *J Biol Chem* 273: 32973–32979, 1998.
42. **Kubo Y, Tsunehiro T, Nishikawa S, Nakai M, Ikeda E, Toh-e A, Morishima N, Shibata T, and Endo T.** Two distinct mechanisms operate in the reactivation of heat-denatured proteins by the mitochondrial Hsp70/Mdj1p/Yge1p chaperone system. *J Mol Biol* 286: 447–464, 1999.
43. **Kunugi S and Tanaka N.** Cold denaturation of proteins under high pressure. *Biochim Biophys Acta* 1595: 329–344, 2002.
44. **Langer T, Lu C, Echols H, Flanagan J, Hayer MK, and Hartl FU.** Successive action of DnaK, DnaJ and GroEL Along the pathway of chaperone-mediated protein folding. *Nature* 356: 683–689, 1992.
45. **Lee GJ, Pokala N, and Vierling E.** Structure and in vitro molecular chaperone activity of cytosolic small heat-shock proteins from pea. *J Biol Chem* 270: 10432–10438, 1995.
46. **Lee GJ and Vierling E.** A small heat shock protein cooperates with heat shock protein 70 systems to reactivate a heat-denatured protein. *Plant Physiol* 122: 189–197, 2000.
47. **Li ZJ, Xiong F, Lin QS, d'Anjou M, Daugulis AJ, Yang DSC, and Hew CL.** Low-temperature increases the yield of biologically active herring antifreeze protein in *Pichia pastoris*. *Protein Expr Purif* 21: 438–445, 2001.
48. **Marques MI, Borreguero JM, Stanley HE, and Dokholyan NV.** Possible mechanism for cold denaturation of proteins at high pressure. *Phys Rev Lett* 91: 138103, 2003.

49. **Martin J and Hartl FU.** Chaperone-assisted protein folding. *Curr Opin Struct Biol* 7: 41–52, 1997.
50. **McCarty JS and Walker GC.** DnaK as a thermometer—threonine-199 is site of autophosphorylation and is critical for ATPase activity. *Proc Natl Acad Sci USA* 88: 9513–9517, 1991.
51. **McKay DB, Wilbanks SM, Flaherty KM, Ha JH, O'Brien MC, and Shirvanee LL.** Stress-70 proteins and their interaction with nucleotides. In: *The Biology of Heat Shock Proteins and Molecular Chaperones*, edited by Morimoto RI, Tissières A, and Georgopoulos C. New York: Cold Spring Harbor Laboratory, 1994.
52. **Minami Y and Minami M.** Hsc70/Hsp40 chaperone system mediates the Hsp90-dependent refolding of firefly luciferase. *Genes Cells* 4: 721–729, 1999.
53. **Palleros DR, Shi L, Reid KL, and Fink AL.** Hsp70-protein complexes—complex stability and conformation of bound substrate protein. *J Biol Chem* 269: 13107–13114, 1994.
54. **Palleros DR, Welch WJ, and Fink AL.** Interaction of Hsp70 with unfolded proteins—effects of temperature and nucleotides on the kinetics of binding. *Proc Natl Acad Sci USA* 88: 5719–5723, 1991.
55. **Place SP and Hofmann GE.** Temperature interactions of the molecular chaperone Hsc70 from the eurythermal marine goby *Gillichthys mirabilis*. *J Exp Biol* 204: 2675–2682, 2001.
56. **Place SP and Hofmann GE.** Temperature differentially affects ATPase activity in Hsc70 orthologues from Antarctic and New Zealand notothenioid fishes. *Cell Stress Chaperones*. In press.
57. **Place SP, Zippay ML, and Hofmann GE.** Constitutive roles for inducible genes: evidence for the alteration in expression of the inducible hsp70 gene in Antarctic notothenioid fishes. *Am J Physiol Regul Integr Comp Physiol* 287: R429–R436, 2004.
58. **Privalov PL.** Cold denaturation of proteins. *Crit Rev Biochem Mol Biol* 25: 281–305, 1990.
59. **Rüdiger S, Germeroth L, Schneider-Mergener J, and Bukau B.** Substrate specificity of the DnaK chaperone determined by screening cellulose-bound peptide libraries. *EMBO J* 16: 1501–1507, 1997.
60. **Sadis S and Hightower LE.** Unfolded proteins stimulate molecular chaperone Hsc70 ATPase by accelerating ADP/ATP exchange. *Biochemistry* 31: 9406–9412, 1992.
61. **Somero GN.** Proteins and temperature. *Annu Rev Physiol* 57: 43–68, 1995.
62. **Somero GN.** Temperature and proteins: little things can mean a lot. *News Physiol Sci* 11: 72–77, 1996.
63. **Takeda S and McKay DB.** Kinetics of peptide binding to the bovine 70 kDa heat shock cognate protein, a molecular chaperone. *Biochemistry* 35: 4636–4644, 1996.
64. **Weinstein RB and Somero GN.** Effects of temperature on mitochondrial function in the Antarctic fish *Trematomus bernacchii*. *J Comp Physiol* 168B: 190–196, 1998.
65. **Zietara MS and Skorkowski EF.** Thermostability of lactate-dehydrogenase LDH-$A_4$ isoenzyme—effect of heat-shock protein Dnak on the enzyme-activity. *Int J Biochem Cell Biol* 27: 1169–1174, 1995.
66. **Zippay ML, Place SP, and Hofmann GE.** The molecular chaperone Hsc70 from a eurythermal marine goby exhibits temperature insensitivity during luciferase refolding assays. *Comp Biochem Physiol* 138A: 1–7, 2004.