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Temperature differentially affects adenosine triphosphatase activity in Hsc70 orthologs from Antarctic and New Zealand notothenioid fishes

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Abstract To test the temperature sensitivity of molecular chaperones in poikilothermic animals, we purified the molecular chaperone Hsc70 from 2 closely related notothenioid fishes—the Antarctic species *Trematomus bernacchii* and the temperate New Zealand species *Notothenia angustata*—and characterized the effect of temperature on Hsc70 adenosine triphosphatase (ATPase) activity. Hsc70 ATPase activity was measured using [α-^{32}P]-adenosine triphosphate (ATP)–based in vitro assays followed by separation of adenylates by thin-layer chromatography. For both species, a significant increase in Hsc70 ATPase activity was observed across a range of temperatures that was ecologically relevant for each respective species. Hsc70 from *T bernacchii* hydrolyzed 2-fold more ATP than did *N angustata* Hsc70 at 0°C, suggesting that the Antarctic molecular chaperone may be adapted to function more efficiently at extreme cold temperatures. In addition, Q_{10} measurements indicate differential temperature sensitivity of the ATPase activity of Hsc70 from these differentially adapted fish that correlates with the temperature niche inhabited by each species. Hsc70 from *T bernacchii* was relatively temperature insensitive, as indicated by Q_{10} values calculated near 1.0 across each temperature range measured. In the case of Hsc70 purified from *N angustata*, Q_{10} values indicated thermal sensitivity across the temperature range of 0°C to 10°C, with a Q_{10} of 2.714. However, Hsc70 from both *T bernacchii* and *N angustata* exhibited unusually high thermal stabilities with ATPase activity at temperatures that far exceeded temperatures encountered by these fish in nature. Overall, as evidenced by in vitro ATP hydrolysis, Hsc70 from *T bernacchii* and *N angustata* displayed biochemical characteristics that were supportive of molecular chaperone function at ecologically relevant temperatures.

INTRODUCTION

Recent physiological studies examining the effect of acute cold stress on the expression patterns of heat shock proteins (HSPs) have begun to highlight the importance of molecular chaperones in adaptation to cold temperatures (for a review see, Sonna et al 2002). For example, Kandror et al (2004) reported that yeast shows an adaptive response involving an accumulation of the chemical chaperone trehalose and the induction of hsps that sustain viability at freezing temperatures. These studies on yeast highlight the upregulation and potential low-temperature–specific role of molecular chaperones in a model research organism. However, numerous eukaryotes function at extremely low temperatures in nature, rendering the observations made in yeast highly relevant to the study of molecular chaperone biology in nonmodel organisms.

For example, Antarctic marine fishes of the family Nototheniidae, living in seawater hovering just above −2°C, provide an excellent study system to assess the function of molecular chaperones at near-freezing temperatures in natural populations. Early studies on protein synthesis rates in Antarctic notothenioids indicate that temperature selection may be acting on the protein synthesis machinery of these fishes. For example, Haschemeyer and Wil-

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liams (1982) showed that rates of protein synthesis in Antarctic notothenioid cell-free synthesis systems were differentially affected by temperature below 25°C when compared with studies on mammalian systems. Mammalian Q_{10} values (Q_{10} = 8–9 from 10°C to 25°C) indicate a significant temperature dependency at temperatures below 25°C (Brosnan et al 1976; Craig and Fahrman 1977), whereas rates in the cold-adapted notothenioid, *Trematomus bernacchii*, remained linear at these lower temperatures (Haschemeyer and Williams 1982). More recently, Place et al (2004) showed higher than expected levels of damaged proteins, as indicated by ubiquitin conjugate levels, in tissues of 2 wild-caught Antarctic notothenioids, *T bernacchii* and *Pagothenia borchgrevinki*. In addition, messenger ribonucleic acid for the inducible (*hsp*)*70*, commonly upregulated in the presence of cellular stressors perturbing protein homeostasis (reviewed in Iwama et al 1999), was constitutively expressed in field-acclimatized tissues of *T bernacchii* and *P borchgrevinki* (Place and Hofmann 2004; Place et al 2004). Taken in conjunction, these studies suggest that subzero temperatures may have a perturbing effect on protein synthesis in cold-adapted organisms, and, thus, a greater need for molecular chaperoning at these extreme cold temperatures may exist. Although the collective data have indicated adaptive changes in the thermal response of the protein biosynthesis machinery in Antarctic species, relatively little is known about the function of molecular chaperones, central players in the production of new proteins in these species. The focus of this study was to compare the functional activity of the generalist chaperone, heat shock cognate 70, from differentially adapted notothenioid fish to determine if evolution at a constant cold temperature had resulted in biochemical compensation.

Molecular chaperones have long been understood to play pivotal roles in protein maturation within the cellular milieu. Cellular strategies for promotion of native proteins through formation of local secondary structures require both cotranslational and posttranslational assistance to prevent the formation of nonproductive intermediates (Deuerling et al 1999; Fink 1999; Teter et al 1999; Bukau et al 2000; Frydman 2001; Ferbitz et al 2004). The mechanism of action in both prokaryotes and eukaryotes has been well studied and is highly conserved across taxa (for reviews see Feder and Hofmann 1999; Fink 1999; Bukau et al 2000; Frydman 2001). Through regulated conformational changes, molecular chaperones such as Hsc70 recognize and selectively bind exposed hydrophobic regions of nascent polypeptides (Fink 1999; Frydman 2001; Hartl and Hayer-Hartl 2002). The multiple cooperative interactions required for proper molecular chaperone function are often orchestrated through the actions of adenylates and cochaperones such as (Hsp)40. For instance, the function of Hsc70 during binding and release of target substrates is modulated by means of cycles of adenosine triphosphate (ATP) binding and hydrolysis (Palleros et al 1993; Schmid et al 1994; McCarty et al 1995; Hartl 1996). Furthermore, it has been suggested that Hsp40 facilitates Hsc70-substrate interactions and promotes ATP hydrolysis through the action of a highly conserved J-domain (Liberek et al 1991; Minami et al 1996; King et al 1997; Kelley 1998; Kosano et al 1998; Hernandez et al 2002).

The basic functions of molecular chaperones have been conserved across taxa, and therefore molecular chaperones are believed to function across the entire range of temperatures over which organisms are known to exist, approximately at −2°C in Antarctic organisms (Hunt et al 2003) to 121°C in hyperthermophiles (Kashefi and Lovley 2003). Nevertheless, the temperature sensitivity of this mechanism at the extremes of this temperature range has not been thoroughly examined. There is some evidence for biochemical adaptation within Hsc70 orthologs. For instance, single-turnover adenosine triphosphatase (ATPase) activity in a Hsc70 bacterial ortholog, DnaK, from *Thermus thermophilus* measured at 75°C was comparable with the activity of *Escherichia coli* DnaK measured at 25°C (Klostermeier et al 1998). In addition, Hsc70 purified from 2 Antarctic fish appear to exhibit temperature compensation in the classical refolding activities attributed to members of the HSC70 family (S.P. Place and G.E. Hofmann, unpublished data). Hsc70 isolated from *T bernacchii* and *P borchgrevinki* showed higher refolding activity at −2°C than the temperate-adapted notothenioid, *Notothenia angustata*, and the refolding activity of the Antarctic species rapidly decreased at temperatures above 20°C, whereas the refolding activity of *N angustata* remained elevated at these higher temperatures (Place and Hofmann, 2005). Although possible temperature adaptations have been observed in the peptide-binding region of proteins from these cold-adapted organisms, this is the first study to show biochemical adaptations in the ATPase region, similar to those seen in DnaK from *T thermophilus* (Klostermeier et al 1998), that have occurred in an organism adapted to extreme cold environments.

We characterized the biochemical and functional activities of Hsc70 from the Antarctic notothenioid, *T bernacchii*, a cold-adapted stenothermic teleost, and compared this activity with Hsc70 from a differentially thermally adapted confamilial species, *N angustata*. Both *T bernacchii* and *N angustata* are benthic species found in near-shore coastal waters; however; they differ greatly in their thermal histories. In the coastal Antarctic waters of McMurdo Sound where *T bernacchii* is commonly found, nearly constant water temperatures of −1.86°C are recorded yearround with only a few days of rare warming periods nearing −0.5°C (Hunt et al 2003). Antarctic notothenioids are thought to have evolved under these conditions for 14–25 million years, during which many physiological

adaptations have arisen in the nototheniid family (Eastman 1993; Clarke and Johnston 1996). In contrast, *N angustata*, commonly found off the coast of the South Island of New Zealand (Ayling and Cox 1982), experiences temperature variation on a daily as well as annual basis, with annual seawater temperatures that range from 8°C to 15°C annually (B. Dickson, personal communication). This temperate species is believed to have separated from polar notothenioid stocks approximately 15 million years ago and exhibits few of the biochemical adaptations found in the Antarctic notothenioids, although remnants of antifreeze glycoprotein genes have been identified in *N angustata* (Cheng et al 2003). Because *N angustata* probably diverged from other Antarctic notothenioids after the clade had adapted to the subzero environment, any temperature adaptations in this species are likely to have progressed from the cold-to-warm direction. The inclusion of *N angustata* in this study provided a comparison for the effects of temperature on chaperones from Antarctic fish and addressed the distinction between a true adaptive response vs one due solely to phylogenetic distance (ie, a trait that is characteristic of all the members of the notothenioid suborder regardless of evolutionary thermal history).

For this biochemical characterization study we purified Hsc70 from *T bernacchii* and *N angustata* white muscle and used a thin-layer chromatography(TLC)–based assay to examine the thermal stability and thermal sensitivity of the ATPase activity, a weak intrinsic activity exhibited by members of the 70-kDa Hsp gene family (McKay et al 1994). The results indicate that Hsc70 from these differentially thermally adapted notothenioid fishes displayed biochemical activity that differed in thermal sensitivity across the temperature range used in this study.

MATERIALS AND METHODS

Collection of study organisms

T bernacchii (Boulenger 1902) specimens were collected using hook and line fishing through holes drilled through the sea ice in McMurdo Sound, Antarctica (77°51′119″S, 166°39′862″E). The fish were transported back to McMurdo Station and held in flow through seawater aquaria located in the Crary Laboratory facilities. Fish were sacrificed by means of cervical transection after MS-222 anesthesia and dissected on ice in a 0°C environmental room. Dissected white skeletal muscle was immediately frozen in liquid nitrogen. Specimens of *N angustata* (Hutton 1875) were collected with baited fish traps placed on the substrate at 2- to 10-m depths near Portobello Marine Laboratories on the South Island of New Zealand (45.50°S, 170.38°E). The fish were transported back to Portobello Marine Laboratories and held in flow through seawater aquaria. Fish were sacrificed on ice in a 10°C environmental room as 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.

Protein purification

Hsc70 was purified from *T bernacchii* and *N angustata* white skeletal muscle according to a modified 2-step low-pressure chromatography protocol reported previously in Place and Hofmann (2001). Briefly, the supernatant of white muscle homogenate was applied to a diethylaminoethyl (DEAE) anion exchange column (2.5 × 20 cm, DEAE Sephacel, Amersham Pharmacia Biotech, Piscataway, NJ, USA) 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, St Louis, MO, USA) and subsequently eluted with 3 mM ATP. The eluted fractions were pooled and condensed using Centricon-30 centrifugal concentrators (Millipore, Billerica, MA, USA) at 5000 × *g* for 30 minutes. For a complete protocol including detailed buffer constituents, see Place and Hofmann (2001).

For characterization of each protein preparation, the protein content was determined with a modified Bradford assay (Coomassie Plus, Pierce, Rockford, IL, USA), and the purity of each preparation was determined by sodium dodecylsulfate–polyacrylamide gel electrophoresis (SDS-PAGE) separation followed by silver staining (BioRad Silver Stain Plus kit, BioRad, Hercules, CA, USA). Western blotting, as described in Hofmann and Somero (1995), using a monoclonal anti–Hsp70-Hsc70 primary antibody (MA3-001, Affinity Bioreagents, Golden, CO, USA) was performed to identify the purified protein.

Luciferase protection assays

Luciferase protection assays were performed using a modified protocol from Lu and Cyr (1998). Immediately before the assay, luciferase was diluted to 0.2 nM in 100 μL of refolding buffer (10 mM 3-(N-morpholino) propanesulfonic acid (MOPS), pH 7.2, 50 mM KCl, 5 mM $MgCl_2$, 1 mM ATP) and then 1 μM Hsc70, 0.2 μM Hdj1, a mammalian Hsp40 cochaperone (Stressgen, San Diego, CA, USA), and 50 μM ATP were added. Reactions were incubated at 38°C for 45 minutes. Activity was measured at 5-minute intervals by combining 10-μL aliquots of the reaction with 50 μL of luciferase assay reagent (Promega, Madison, WI, USA). Relative light units were then immediately measured using a luminometer.

ATPase assays

ATP hydrolysis was assayed by measuring the formation of [α-^{32}P]–adenosine diphosphate (ADP) from [α-^{32}P]-ATP, using TLC on Baker-flex polyethyleneimine (PEI)-cellulose plates (JT Baker, Phillipsburg, NJ, USA) in 0.5 M lithium chloride and 1 M formic acid, as described in Place and Hofmann (2001). In vitro activity assays were assembled on ice and consisted of 2 μg of Hsc70, 50 μl of assay buffer (AB) [20 mM Hepes-KOH, pH 7.0, 25 mM KCI, 1.0 mM $(NH_4)SO_4$, 2 mM magnesium acetate, 0.1 mM ethylenediaminetetraacetic acid, 1.0 mM dithiothreitol, 50 μM ATP], and 10 μCi of [α-^{32}P]-ATP. In some assays, reduced carboxymethylated α-lactalbumin (RCMLA, Sigma), an unfolded stable protein, was used as a target to measure the amount of stimulation of the intrinsic ATPase activity of Hsc70. Samples were incubated with 80 μM RCMLA for selected times and temperatures, and 1-μL aliquots were removed for nucleotide separation by TLC. Three replicate ATPase assays were performed for each treatment, and triplicate aliquots were measured for each data point taken. For all ATPase assays, the mean background ATP hydrolysis was obtained for assays containing only [^{32}P]-ATP. These background values were subtracted from the values of assays containing Hsc70 to yield a background-corrected value. Levels of product, [α-^{32}P]-ADP, were quantified with a Storm PhosPhor Imager system (Molecular Dynamics, Piscataway, NJ, USA) and densitometric analysis was performed using ImageQuant software (BioRad, Hercules, CA, USA).

Thermal stability measurements

Native Hsc70 was incubated in AB across the temperature range of 0°C to 80°C for 40 minutes. During the 40-minute incubation period, 2-μg aliquots were taken in triplicate every 10 minutes, combined with 10 μCi of [α-^{32}P]-ATP in 50 μL of AB, and placed in a 0°C water bath for *T bernacchii* Hsc70 or a 10°C water bath for *N angustata* Hsc70. The amount of ATP hydrolysis was measured after 15 minutes by TLC. Values are relative mean density (background corrected) ± standard error of the mean (SEM) ($n = 3$).

Data analysis

Computation of Q_{10} values

The effect of temperature on Hsc70 ATPase activity was expressed as a Q_{10} value (a Q_{10} value is the ratio of the rate of a reaction at a given temperature to its rate at a temperature 10°C lower, see Randall et al 1997). Q_{10} values were calculated using the van't Hoff equation as follows: $Q_{10} = (k_2/k_1)^{10/(t_2 - t_1)}$.

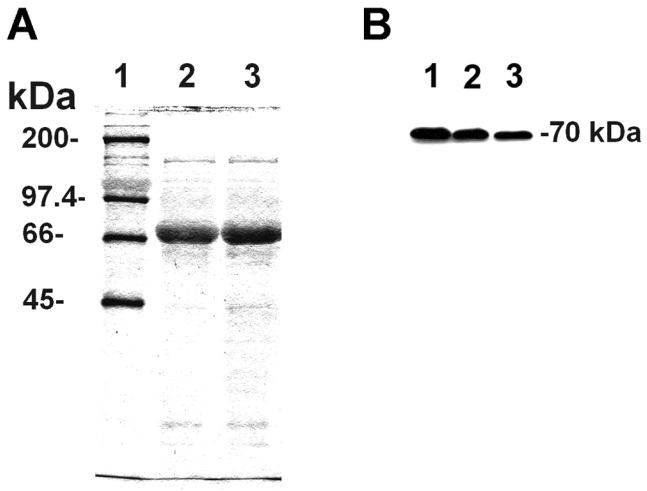

Fig 1. (A) Silver-stained gel of purified Hsc70 from each species. For silver staining, 5 μg total protein was applied to lanes 1–3 and separated by electrophoresis on a 10% sodium dodecylsulfate (SDS)–polyacrylamide gel. Lanes are as follows: (1) silver stain protein molecular weight standards; (2) Hsc70 from *Trematomus bernacchii* white muscle; (3) Hsc70 from *Notothenia angustata* white muscle. (B) Western blot detection of purified 70-kDa heat shock proteins using a rat monoclonal anti-Hsp70 antibody. Lanes are as follows: (1) 0.1 μg bovine brain Hsc70 standard; (2) 5 μg Hsc70 purified from *T bernacchii*; (3) 5 μg Hsc70 purified from *N angustata*.

Statistical analysis

All analysis of variance (ANOVA) measurements were performed using JMP 5 software (SAS Institute, Cary, NC, USA) for Windows. We verified that the ANOVA assumptions of normality were met by testing the fit distribution of each data set before performing a 1-way ANOVA with a Tukey's honestly significantly different test for significant differences, $P < 0.05$, between treatments. All data are presented as mean ± SEM ($n = 3$).

RESULTS

Protein purification

The purity of each preparation was determined by SDS-PAGE separation followed by silver staining (Fig 1A). The purity of the preparation routinely exceeded 90% homogeneity, and preparations with homogeneity lower than 90% were not used for the analyses. The identity of the molecular chaperone was confirmed by Western blotting using an antibody specific to 70-kDa HSP gene family members (Fig 1B). As in Place and Hofmann (2001), purified Hsc70 comprised less than 0.1% of the total protein content of fish white muscle.

Luciferase protection assay

To verify that the 70-kDa protein we had purified was a functional molecular chaperone, we measured the ability

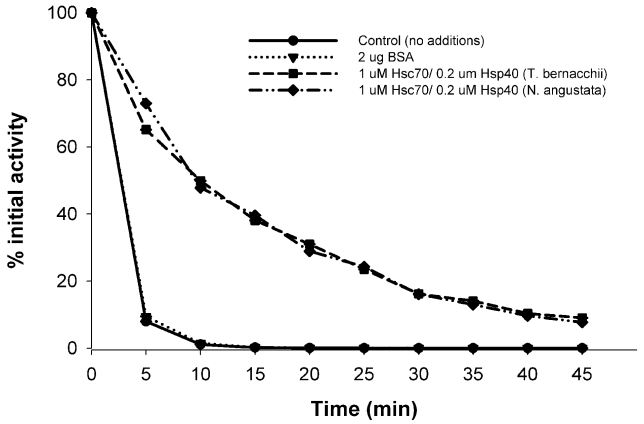

Fig 2. Prevention of thermal denaturation of luciferase at 38°C by *T bernacchii* Hsc70 and *N angustata* Hsc70. Samples of luciferase were incubated at 38°C for 45 minutes, 10 μL aliquots were removed, placed in 50 μL of luciferase assay reagent (LAR) (Promega), and relative light units were measured in a luminometer. The circles, triangles, squares, and diamonds represent, luciferase alone, 2 μM bovine serum albumin (BSA), 1 μM *T bernacchii* Hsc70 and 0.2 μM Hsp40, and 1 μM *N angustata* Hsc70 and 0.2 μM Hsp40, respectively.

of the purified product from both species to prevent the thermal denaturation of firefly luciferase at 38°C. As shown in Figure 2, Hsc70 purified from *T bernacchii* and *N angustata* greatly increased the half-life of luciferase. Luciferase incubated alone, or in the presence of 2 μM bovine serum albumin, lost 90% of its original activity within 5 minutes of incubation at 38°C (Fig 2). In contrast, in the presence of the mammalian Hsp40 cochaperone and Hsc70 from either species, luciferase lost only 38% of its original activity within the first 5 minutes of incubation. This represents a 2.5-fold stabilization of luciferase and, furthermore, luciferase incubated in the presence of Hsc70 did not experience a loss of 90% of its original activity until 40 minutes into the assay (Fig 2).

Thermal sensitivity of Hsc70 activity

The ATPase activity for stimulated and unstimulated Hsc70 from both species was measured across the temperature range of 0°C to 20°C to determine the effects of temperature on the ATPase activity. When the temperature effects were assessed within a species, *T bernacchii* Hsc70 showed no significant increase in ATPase activity when assayed from 0°C to 20°C (Fig 3A). In contrast, Hsc70 purified from *N angustata* showed a significant increase in activity when assayed at 5°C and 10°C as compared with the activity measured at 0°C (Fig 3A; ANOVA, $P < 0.05$). In addition, activity levels significantly increased when assayed at 10°C compared with the activity of *N angustata* Hsc70 at 5°C (Fig 3A; ANOVA, $P < 0.05$). Temperature comparisons among the 2 species used in this study indicated that Hsc70 purified from *T bernacchii*

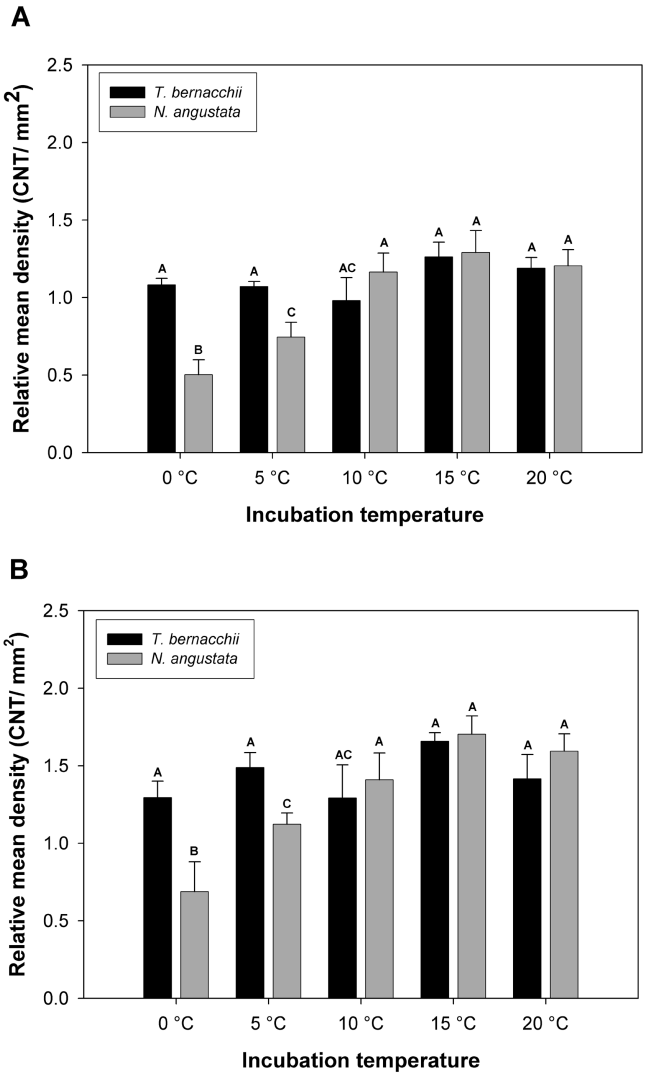

Fig 3. Effects of temperature on unstimulated (A) and reduced carboxymethylated α-lactalbumin (RCMLA)–stimulated (B) Hsc70 from *T bernacchii* and *N angustata*. The samples were incubated for 1 hour in the presence or absence of 80 μM RCMLA at the indicated temperatures and the amount of adenosine triphosphate (ATP) hydrolysis was measured by thin-layer chromatography. Values are relative mean density (background corrected) ± standard error of the mean (SEM) ($n = 3$ replicate assays). Levels not connected by the same letter are significantly different (analysis of variance [ANOVA]; $P < 0.01$).

displayed nearly 2-fold more ATP hydrolysis activity at 0°C and 5°C compared with the activity of *N angustata* Hsc70 (Fig 3A). When the assay was carried out at 10°C, *N angustata* Hsc70 ATPase activity significantly increased and was equivalent to the activity displayed by *T bernacchii* at 0°C (Fig 3A).

In addition, we examined the effect of an unfolded substrate on the ATPase activity of Hsc70 from both species. Figure 3B shows the effect of 80 μM RCMLA on the ATPase activity of Hsc70 purified from *T bernacchii* and *N angustata*. The presence of an unfolded substrate sig-

Table 1 Calculated Q_{10} values[a] for unstimulated and RCMLA-stimulated rates of ATP hydrolysis by Hsc70 purified from the white muscle of the Antarctic species, *Trematomus bernacchii*, and the New Zealand species, *Notothenia angustata*[b]

	***T bernacchii* Hsc70**		***N angustata* Hsc70**	
Temperature range (°C)	**Unstimulated**	**Stimulated**	**Unstimulated**	**Stimulated**
0–10	0.677	0.550	2.714	2.678
5–15	1.178	1.114	1.730	1.518
10–20	1.6	1.115	0.663	0.665

[a] Q_{10} values were calculated for the temperature intervals shown using the van't Hoff equation as follows: $Q_{10} = (k_2/k_1)^{10/(t_2-t_1)}$.
[b] RCMLA, reduced carboxymethylated α-lactalbumin; ATP, adenosine triphosphate.

nificantly stimulated the hydrolysis of ATP by *T bernacchii* and *N angustata* Hsc70 when compared with unstimulated activities (ANOVA, $P < 0.001$). However, the presence of RCMLA did not alter the influence of temperature on the ATPase activity. The overall activity profile of unstimulated and RCMLA-stimulated Hsc70 from *T bernacchii* and *N angustata* was similar in each species (Fig 3 A,B).

To further assess the thermal sensitivity of Hsc70, Q_{10} values were calculated using the van't Hoff equation for stimulated and unstimulated Hsc70 activity as measured by ATP hydrolysis (Table 1). Hsc70 from *T bernacchii* was relatively temperature insensitive, as indicated by Q_{10} values calculated near 1.0 across each temperature range measured (Table 1). Hsc70 purified from *N angustata* showed thermal sensitivity across the temperature range of 0°C to 10°C, with a Q_{10} of 2.714 and 2.678 for unstimulated and RCMLA-stimulated ATPase activity, respectively (Table 1). Across the ecological temperature range of *N angustata*, 5°C to 15°C, the ATPase activity of Hsc70 from these fish displayed thermal sensitivity closer to the average physiological value of 2.0 (Table 1). Q_{10} values calculated for *N angustata* Hsc70 at temperatures above 15°C fell below 1.0 for both unstimulated and stimulated ATP hydrolysis rates (Table 1).

Thermal stability of the ATPase activity of Hsc70

We used the heat stability of the ATPase activity as an indicator of tertiary stability within this region. After 40-minute incubations at each treatment temperature, Hsc70 isolated from *T bernacchii* and *N angustata* displayed activity at temperatures that far exceeded the temperature that each species encounters in nature. For *T bernacchii* Hsc70, the ATPase activity remained relatively unchanged between 0°C and 65°C (Fig 4A). However, at temperatures of 70°C and 80°C, ATPase activity decreased rapidly within the first 10 minutes of incubation and levels remained below 40% of the initial (0 minute) activity. However, ATP hydrolysis remained detectible above background after incubation at temperatures as high as

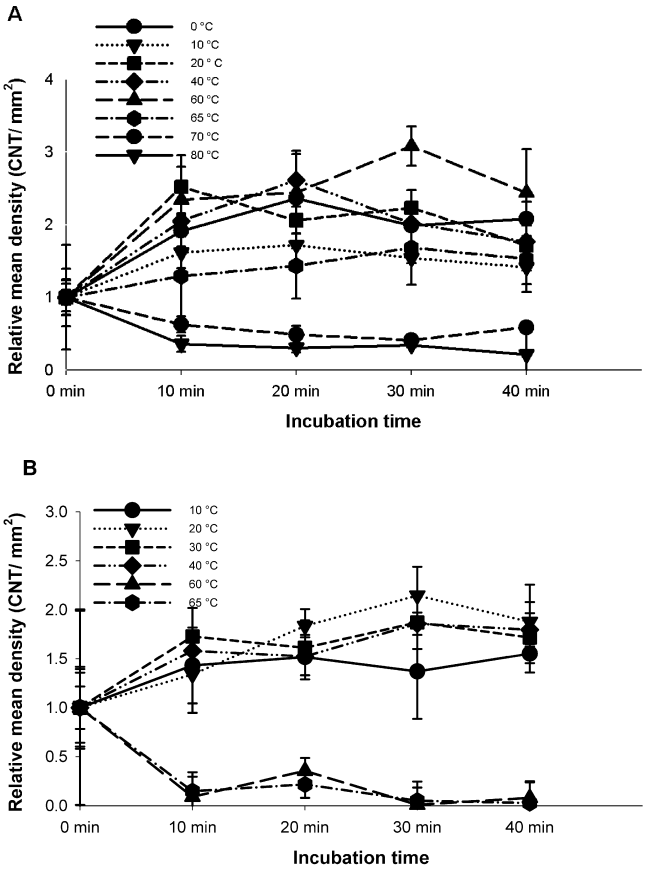

Fig 4. Thermal stability of the adenosine triphosphatase (ATPase) activity of *T bernacchii* Hsc70 (A) and *N angustata* Hsc70 (B). Native Hsc70 was incubated in the assay buffer at the indicated temperatures for 40 minutes. At indicated times during the incubation period, 2 μg aliquots were removed and combined with 10 μCi of [α-^{32}P]–adenosine triphosphate (ATP) in 50 μL of assay buffer and placed in a 0°C ice bath for *T bernacchii* Hsc70 and 10°C water bath for *N angustata*. The amount of ATP hydrolysis was measured after 15 minutes by thin-layer chromatography (TLC). Values are relative mean density (background corrected) ± standard error of the mean (SEM) ($n = 3$ replicate assays).

80°C for 40 minutes (Fig 4A). Similar results were observed with *N angustata* Hsc70, although the rapid loss of function occurred at a lower temperature than that seen for *T bernacchii* Hsc70; ATPase activity of *N angustata* Hsc70 decreased significantly with the hydrolysis of ATP becoming undetectable within 40 minutes when incubated at 60°C (Fig 4B).

DISCUSSION

To assess the effects of temperature on the biochemical function of molecular chaperones that have been selectively adapted to extreme cold temperatures, we purified the molecular chaperone Hsc70 from 2 notothenioid fishes and compared the thermal sensitivity of their ATPase activities. The 2 species chosen for this study are phylogenetically related members of the suborder Notothen-

ioidei that are distinguished by very different thermal profiles in nature and have been previously used in comparative studies to examine physiological adaptations to subzero temperatures (Eastman and DeVries 1986; Carpenter and Hofmann 2000; Egginton et al 2001; Guynn et al 2002). Carpenter and Hofmann (2000) showed that there was no apparent difference in the number of Hsp70 isoforms expressed in gill tissue between *T bernacchii* and *N angustata*. The amount of the molecular chaperone Hsp70 was found to be lower in the Antarctic species as compared with *N angustata* (Carpenter and Hofmann 2000). Studies by Guynn et al (2002) showed that warm acclimation of *T bernacchii* and *N angustata* had differential effects on the Na^+/K^+-ATPase pump activity. This study revealed a 2-fold increase in the Na^+/K^+-ATPase pump activity in warm-acclimated *T bernacchii*, but this acclimation had no effect on the activity in *N angustata* (Guynn et al 2002). Overall, these studies mentioned previously demonstrate that some measures of biochemical function of Hsc70 from the cold-adapted species, *T bernacchii*, displayed temperature compensation at near-freezing temperatures. However, in addition to possible temperature compensation for ATP hydrolysis at low temperatures, Hsc70 from both species displayed a stable ATPase activity at temperatures uncharacteristic of those encountered by these marine fish in nature. Interestingly, *T bernacchii* Hsc70 displayed greater stability within the ATPase region, maintaining hydrolytic activity at temperatures 20°C higher than could be detected with *N angustata* Hsc70. These results are a further indication of functional differences between these 2 molecular chaperones, although, with questionable physiological relevance for these organisms.

Once we had confirmed chaperone function in Hsc70 purified from each species, we proceeded to profile the thermal sensitivity of the ATPase activity of this molecular chaperone. The ATPase activity has often been used as an indicator of temperature dependence of Hsc70 because it is known to be an important regulator of the binding and release of substrate (Palleros et al 1993; Schmid et al 1994; McCarty et al 1995; Hartl 1996). Hsc70 purified from both *T bernacchii* and *N angustata* were relatively stable proteins as has been described for other members of the HSC70 family (eg, McCarty and Walker 1991; Place and Hofmann 2001). Both chaperones maintained activity far above the average habitat temperature range for each respective species; however, their relative stability did not reflect their thermal histories. Hsc70 from the cold-stenotherm *T bernacchii* maintained detectible activity at 80°C, a considerably higher temperature than *N angustata*, where ATPase activity was not detectible above 60°C (Fig 4A). The gradual rise in activity observed as the incubation period at each temperature increased may be an indication that these proteins are perhaps chaperoning themselves, leading to a substrate stimulation effect (Fig 4 A,B). The failure of thermal stabilities to correlate with habitat temperature has also been shown for 2 congeneric gobies, *Gillichthys mirabilis* and *G seta*, in which heat denaturation profiles for lactate dehydrogenase (LDH) purified from both species indicated greater thermal stability in the LDH ortholog from the colder-adapted *G mirabilis* (1997). Several studies have proposed that changes in amino acid sequence outside the active region of an enzyme can have significant effects on the kinetics and stabilities of a protein, as could be the case for our protein of interest (Holland et al 1997; Fields and Somero 1998). However, further studies show that interspecific variation in thermal sensitivities can also be found between orthologs with identical sequences and, therefore, may be attributed to other factors such as variable folding conformations and solute interactions (Fields and Somero 1997; Fields et al 2002).

A second significant finding of this study with regard to temperature effects on Hsc70 was that when ATPase activity of Hsc70 was measured at ecologically relevant temperatures for each species, 0°C for *T bernacchii* and 5°C to 15°C for *N angustata*, we found that Hsc70 from each species displayed a different thermal response that indicated possible temperature compensation in the ATPase region of Hsc70 from the cold-adapted notothenioid. Specifically, for *T bernacchii*, we calculated Q_{10} values near 1.0 across the temperature range of 0°C to 20°C, indicating a decreased sensitivity to temperature change within this molecular chaperone (Table 1). Alternatively, a Q_{10} of 2.714 between 0°C and 10°C, indicative of temperature sensitivity of the rate of hydrolysis of ATP by Hsc70 from this temperate species, was calculated for *N angustata* (Table 1). Across the ecologically relevant temperature range for *N angustata* (5°C to 15°C), calculated Q_{10} values fell closer to 2, indicating a characteristic physiological response of Hsc70 ATP hydrolysis rates in these fish (Table 1). In addition, relative levels of ATP hydrolysis by *T bernacchii* Hsc70 were 2-fold higher than those observed for *N angustata* Hsc70 at 0°C and nearly 2-fold higher when the ATPase assays were performed at 5°C (Fig 3A). Interestingly, at 0°C, relative levels of *T bernacchii* Hsc70 ATPase activity were equivalent to the relative levels of *N angustata* Hsc70 activity at 10°C, a temperature commonly experienced by *N angustata* in nature (Fig 3A). These data indicate that Hsc70 from the cold-adapted Antarctic teleost is differentially affected by temperature and may have undergone adaptive compensation to allow for proper function at subzero temperatures. In addition to this apparent biochemical adaptation within the ATPase region, possible temperature selection acting on these fish has affected functions associated with the peptide-binding region of Hsc70 purified from *T bernacchii* as well (Place and Hofmann 2005). Although the coop-

erative function of these 2 regions is required for proper substrate interactions, we feel that the changes in refolding activity within this molecule are not driven by the temperature sensitivity of the ATPase region described here. In fact, Hsc70 purified from this cold-adapted species maintained high ATPase activity above 35°C (Fig 4A), the temperature at which the ability of Hsc70 from *T bernacchii* to refold denatured LDH failed (Place and Hofmann 2005). These data suggest that Hsc70 has either undergone multiple adaptations to efficiently function at extreme cold temperatures, or more likely, a single adaptive change outside these functional regions has similarly affected the function of both domains.

In addition to the thermal characterization of Hsc70, we examined the stimulation of the ATPase activity by the addition of a stable unfolded protein, RCMLA (Fig 3B). As observed for other members of the 70-kDa molecular chaperone family (Palleros et al 1991; Freeman et al 1995; Place and Hofmann 2001), the ATPase activity was positively stimulated by the addition of the unfolded substrate RCMLA. However, the degree of stimulation by this unfolded protein was lower than that reported previously in other Hsc70 orthologs (Palleros et al 1991; Freeman et al 1995). This may in part be caused by a stimulation of the intrinsic ATPase activity by contaminant proteins already present in the native preparations. Furthermore, the presence of an unfolded substrate did not affect the overall temperature profile of these molecular chaperones (Fig 3B). The stimulation of the ATPase activity is a common kinetic characteristic of the chaperoning cycle of molecular chaperones of the 70-kDa family. Although the precise kinetics of this reaction are not fully understood, the binding and release of unfolded proteins is suspected to be closely coupled to nucleotide binding and hydrolysis of ATP to ADP (Palleros et al 1991; Sadis and Hightower 1992; McCarty et al 1995).

In summary, our data suggest that ATP hydrolysis rates of Hsc70 from these notothenioid fishes were differentially responsive to temperature changes within an ecologically relevant range for each species. Interestingly, although *T bernacchii* Hsc70 displayed higher ATPase activities at low temperatures, the temperate and polar forms of Hsc70 also differed when ATPase assays were performed at temperatures above 60°C, with thermal stability assays indicating that *T bernacchii* Hsc70 was relatively more stable at elevated temperatures. Hsc70 from both species were able to bind target proteins and hydrolyze ATP far above any temperature at which they would function in nature. There is evidence indicating that there is a high degree of sequence conservation within the ATPase domain of members of the HSC70 family (McKay et al 1994), lending support to the similar characteristics observed in orthologs of Hsc70 ATPase activity (McCarty and Walker 1991; Palleros et al 1991; Sadis and Hightower 1992; Place and Hofmann 2001).

To further understand the effects of extreme cold temperatures on Hsc70 and the effects of temperature on molecular chaperones in poikilotherms in general, it will be important to begin to use the powerful applications of structure-function studies as well as sequence analysis to determine how these adaptive responses have come about. These experiments, as well as comparison of data from the current gene sequence project underway in our lab, will be an important part of understanding how molecular chaperone function has been conserved across taxa while still allowing organisms to adapt to a wide range of temperatures in nature.

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