RESEARCH ARTICLE

Assisted protein folding at low temperature: evolutionary adaptation of the Antarctic fish chaperonin CCT and its client proteins

Jorge Cuellar1,*, Hugo Yebeñes1,*,‡, Sandra K. Parker2, Gerardo Carranza3, Marina Serna1, José María Valpuesta1, Juan Carlos Zabala3 and H. William Detrich, III2,§

ABSTRACT

Eukaryotic ectotherms of the Southern Ocean face energetic challenges to protein folding assisted by the cytosolic chaperonin CCT. We hypothesize that CCT and its client proteins (CPs) have co-evolved molecular adaptations that facilitate CCT–CP interaction and the ATP-driven folding cycle at low temperature. To test this hypothesis, we compared the functional and structural properties of CCT–CP systems from testis tissues of an Antarctic fish, Gobionotothen gibberifrons (Lönnberg) (habitat/body T = −1.9 to +2˚C), and of the cow (body T = 37˚C). We examined the temperature dependence of the binding of denatured CPs (β-actin, β-tubulin) by fish and bovine CCTs, both in homologous and heterologous combinations and at temperatures between −4˚C and 20˚C, in a buffer conducive to binding of the denatured CP to the open conformation of CCT. In homologous combination, the percentage of G. gibberifrons CCT bound to CP declined linearly with increasing temperature, whereas the converse was true for bovine CCT. Binding of CCT to heterologous CPs was low, irrespective of temperature. When reactions were supplemented with ATP, G. gibberifrons CCT catalyzed the folding and release of actin at 2˚C. The ATPase activity of apo-CCT from G. gibberifrons at 4˚C was ~2.5-fold greater than that of apo-bovine CCT, whereas equivalent activities were observed at 20˚C. Based on these results, we conclude that the catalytic folding cycle of CCT from Antarctic fishes is partially compensated at their habitat temperature, probably by means of enhanced CP-binding affinity and increased flexibility of the CCT subunits.

KEY WORDS: CCT, TriC, Chaperone, Chaperonin, Protein folding, Actin, Tubulin, Thermal adaptation, Evolution

INTRODUCTION

Protein quality control and maintenance of the proteome are essential for the health of cells and organisms (Hartl et al., 2011). Most proteins must acquire precise, but flexible and minimally stable, three-dimensional structures to function within cells. Because the “folding landscape” is complex, with many potential nonfunctional outcomes, cells produce molecular chaperones to guide efficient folding while preventing protein aggregation. Proteins that are irreversibly misfolded or aggregated are removed by the ubiquitin–proteasome system or by lysosomal autophagy (Ciechanover, 1998; Arias and Cuervo, 2011).

The chaperonin containing t-complex polypeptide-1 [CCT, aka TCP-1 ring complex (TriC)] plays a central role in cellular homeostasis by assisting the folding of ~10% of newly synthesized proteins, including tubulins and actins (“client proteins” or CPs) (Thulasiraman et al., 1999; Valpuesta et al., 2002; Dekker et al., 2008; Yam et al., 2008; Valpuesta et al., 2005). CCT is a cylindrical, 16-subunit toroid composed of eight distinct subunits (CCTα–CCTθ) that form two eight subunit, back-to-back rings, each containing a folding “cage” for CPs (Yebeñes et al., 2011; Leitner et al., 2012). Sequestration of CPs by CCT in a closed conformation and CP release require ATP binding, hydrolysis, and associated intra- and inter-ring allosteric signaling (Yebeñes et al., 2011; Leitner et al., 2012; Cong et al., 2012). In some cases, additional protein co-factors are required either to deliver CPs to CCT or to facilitate final maturation and oligomerization of CPs after their interactions with CCT (Valpuesta et al., 2002; Valpuesta et al., 2005; Yebeñes et al., 2011).

The Antarctic notothenioids are a unique, cold-adapted fish fauna whose evolution has been driven by the development of extreme low temperatures as the Southern Ocean cooled to the modern range, −1.9 to +2˚C, over the past 25–40 million years (DeWitt, 1971; Lawver et al., 1992; Lawver et al., 1991; Eastman, 1993; Eastman and Clarke, 1998; Scher and Martin, 2006; DeConto and Pollard, 2003). The acquisition of novel antifreeze proteins by the notothenioids (Chen et al., 1997; Cheng and Chen, 1999), their evolution of a cold-stable microtubule cytoskeleton (Detrich et al., 1989; Detrich et al., 2000; Redeker et al., 2004), their loss of an inducible heat shock protein (HSP) response (Hofmann et al., 2000; Buckley et al., 2004; Detrich et al., 2012), and the loss of hemoglobin expression by the icefish family (Cocca et al., 1995; Near et al., 2006; Zhao et al., 1998) are examples of novel traits that evolved over 5–10 million years of isolation in a perennially icy environment. Today, these stenothermal fishes are threatened by rapid warming of the Southern Ocean (~1–2˚C per century) over periods measured in centuries or less (Gille, 2002; Clarke et al., 2007; Ducklow et al., 2008).
Although High Antarctic notothenioids lack an inducible HSP response (Hofmann et al., 2000; Buckley et al., 2004; Buckley and Somero, 2009; Thorne et al., 2010), they do express constitutively many chaperones (Place et al., 2004; Place and Hofmann, 2005), including CCT (Pucciarelli et al., 2006), very likely to counteract the seemingly paradoxical cold-induced denaturation of proteins (Todgham et al., 2007; Lopez et al., 2008; Dias et al., 2010). Thus, we hypothesize that Antarctic notothenioids have evolved a chaperonin that is compensated, at least in part, to maintain folding activity at low temperature. To test this hypothesis, we have purified CCT from testis tissue of the Antarctic Humphead notothen, Gobionotothen gibberifrons (Lönnberg), and have compared its structural and functional properties to those of bovine testis CCT. The apparent affinity of G. gibberifrons CCT for homologous CPs is high at low temperature and declines as temperature increases, whereas the opposite behavior was observed for bovine CCT and CPs; affinity increased with increasing temperature. Furthermore, the ATPase activity of apo-CCT from the Antarctic fish is substantially greater at cold temperature than that of bovine CCT. We suggest that adaptation of the function of Antarctic fish CCT at low temperature is based on lowering the activation energy barrier(s) of the folding cycle through enhanced CP-binding affinity and increased subunit flexibility. Nevertheless, the thermal scope of the activity of G. gibberifrons CCT appears to be sufficient to tolerate temperatures as much as 5°C above their present habitat norm.

RESULTS

Purification of G. gibberifrons CCT

The eukaryotic class-II chaperonin CCT possesses biochemical and biophysical properties – subunit size and heterogeneity, oligomeric structure, etc. – that can be exploited to purify the complex from diverse sources. Here we employed ion-exchange chromatography, sucrose-gradient ultracentrifugation, and size-exclusion chromatography to isolate CCT from immature testis tissue of the Antarctic Humphead notothen, G. gibberifrons. Fig. 1 shows the purification of G. gibberifrons CCT at several stages: 1) a 30–50% ammonium sulfate cut of a testis high-speed centrifugal extract, which, after dialysis, was applied to a Heparin Sepharose column (Fig. 1A); 2) the elution of bound chaperonin from Heparin Sepharose (Fig. 1B) by application of a step gradient of NaCl (0.45–0.6 M); 3) the banding position of the ~25S CCT complex on a sucrose gradient (Fig. 1C); 4) its subunit complexity as revealed by SDS-PAGE (Fig. 1D); and 5) elution of CCT from a Superose 6 column at an apparent molecular weight of ~1000 kDa (Fig. 1E). The Superose-6-purified CCT is nearly homogeneous and is composed of multiple subunits of Mr ~55–60 kDa (Fig. 1D). The yield of CCT was 40 µg/g testis tissue, ~4-fold greater than obtained by our previous method (Pucciarelli et al., 2006).

CCT subunit identification and biochemical characterization

Using HPLC and mass spectrometry, we confirmed that G. gibberifrons CCT contained the eight canonical subunits (α, β, γ, δ, ε, ζ−1, η, θ) characteristic of the vertebrate chaperonin (Fig. 1F,G, Fig. 2); the mammalian ζ−2 variant was not detected. The pIs of five of the G. gibberifrons subunits were more basic than their bovine orthologs, and three had pIs that were more acidic (Table 1). The differences in pIs were generally concordant with compositional variation in charged amino acid residues (data not shown).

Muñoz et al. have shown that many subunits of bovine testis CCT are posttranslationally modified by small, charged moieties (thought to be acetate and phosphate groups) to give multiple modified variants (Muñoz et al., 2011), and the mouse CCT subunits β, γ, δ, ε, ζ, and η contain multiple acetylated and/or phosphorylated residues (UniProtKB and references therein). By contrast, CCT subunits from G. gibberifrons were generally homogeneous in isoelectric point, with the exception of the η chain (Table 1). [Although the γ and ε chains of G. gibberifrons each eluted as two peaks from the C4 Reversed-Phase HPLC column (Fig. 1F,G), they did not show evidence of isoelectric heterogeneity (Table 1).] Substantial isoelectric heterogeneity was observed for bovine CCT subunits β, γ, δ, ε, ζ−1, and η (data not shown), consistent with the results of Muñoz et al. (Muñoz et al., 2011) and in agreement with the mouse data. Therefore, CCTs expressed by the Antarctic notothenioids appear to be modified to a lesser extent than are mammalian CCTs, an observation that mirrors the reduced polyglutamylation of notothenioid tubulins (Redeker et al., 2004).

Structural characterization of G. gibberifrons CCT

Negative-stain EM and image averaging of the notothen apo-chaperonin at 4°C showed that it conforms to the classical end-on and side views of eukaryotic CCT – a toroid composed of two eight-subunit rings (Fig. 3A) in back-to-back orientation (Fig. 3B). Each ring contains a binding “cage” for CPs (Yébenes et al., 2011; Leitner et al., 2012). When G. gibberifrons apo-CCT was incubated either with denatured β-tubulin or with denatured β-actin, the binding cage was found to be occupied by a stain-excluding mass that crossed the cavity (Fig. 3C,E, respectively) in a CP-specific arrangement that was virtually identical to that observed with bovine CCT and its orthologous CP (Fig. 3D,F, respectively) at 25°C. [Note that the α and β chains of the bovine tubulin CP bind identically to CCT (Llorca et al., 2000).] Therefore, the structures of CCT–CP complexes from a psychrophilic fish are quite similar to those previously reported for CCT–CP complexes from mesophilic species, an observation consistent with the general structural conservation of chaperonins and their clients in organisms adapted to distinct thermal regimes.

Folding activity of G. gibberifrons CCT

The hallmark of chaperonin function is the capacity to assist the folding of its denatured clients. To assess the folding activity of G. gibberifrons CCT at an environmentally relevant temperature, we incubated the chaperonin with 35S-labeled α-actin at 2°C and then added ATP. Fig. 4 shows that G. gibberifrons CCT was able to bind denatured β-actin and release it in its native conformation. Release of the folded product first became apparent at 12 h (Fig. 4B), after which accumulation followed a sigmoidal path to a plateau attained at ~72 h (Fig. 4C,D). Thus, the folding cycle of Antarctic fish CCT at a temperature close to the physiological norm is considerably slower than that of mammalian CCT at 30°C (cf. Melki and Cowan, 1994), but this difference may simply be due to thermal scaling of the temperature coefficient, Q10. (Note that Q10 for the ATPase activity of G. gibberifrons apoCCT scales normally between 4 and 20°C; see ATPase activities of apoCCTs below.) The failure to observe folding of actin at 4 or 20°C in our previous
study of *N. coriiceps* CCT (Pucciarelli et al., 2006) probably resulted from the short incubation time (90 min) used in those assays.

**Temperature dependence of CP-binding by *G. gibberifrons* and bovine CCTs**

Given the dramatic effects of temperature change on the kinetics and energetics of biochemical reactions, we hypothesize that the CCT and CPs of Antarctic fishes co-evolved to give productive substrate folding in the cold and, therefore, may not perform efficiently at elevated temperature. Conversely, we predict that bovine CCT and CPs would interact more effectively at elevated temperatures near the body temperatures of mammals (+37°C). We tested these predictions by comparing the binding of homologous combinations of CCT and CPs (actins, tubulins) at temperatures between −4°C and +20°C by electron microscopy and image processing. Fig. 5A shows the binding of Antarctic fish β-actin to the open conformation of *G. gibberifrons* CCT (*hatched bars*) vs the binding the bovine cardiac actin to bovine CCT (*black bars*); Fig. 5B presents comparable analyses with homologous tubulins as the CPs. The trends in the data are clear – irrespective of CP, the percentage of *G. gibberifrons* CCT that bound client declined with increasing temperature, whereas the percentage of bovine CCT bound to CP increased. The data were well fit by linear regression (Pearson’s adjusted coefficient of determination, $R^2$, ≥0.95 for the four fits; data not shown), which indicates that the binding...
percentages were very likely determined by the assay temperature. Our results are consistent with the co-evolution of the interaction surfaces of CCT and CPs from Antarctic fishes to yield high binding affinities at low temperature.

The analyses of the temperature dependence of the CCT–CP interaction in homologous combination suggest that the binding affinities of psychrophilic and mesophilic CCTs and CPs in heterologous combination would likely be low. Fig. 6 compares the binding of homologous and heterologous combinations of CCT and CPs at two temperatures, +4˚C and +20 ˚C. In homologous combinations (Fig. 6A,B), the results recapitulate those of Fig. 5A,B – the G. gibberifrons CCT–CP interaction was

Fig. 2. Identification of G. gibberifrons CCT subunits. Plugs containing the indicated protein bands were excised from the gel shown in Fig. 1G for in-gel tryptic proteolysis and mass spectrometric analysis. The identities of the presumptive G. gibberifrons CCT subunits were confirmed by querying the non-redundant NCBI protein database with the G. gibberifrons tryptic peptide sets. Each subunit possessed six, seven, or eight peptides that mapped perfectly to peptides of a bovine CCT subunit (red). Peptide sequence coverage ranged from 15–27% for the G. gibberifrons/bovine comparison, and higher values were found for comparison to CCT subunits from other fishes (data not shown). Percent coverage was highest for the b (67%) and h (55%) subunits of CCT from the Antarctic Bullhead notothen, N. coriceps, whose sequences had been established previously from cloned cDNAs (Pucciarelli et al., 2006). The calculated pIs correspond to the bovine CCT subunits.
stronger at low temperature for both actin (A) and tubulin (B), whereas the converse was true for the bovine system. In heterologous combination (Fig. 6C,D), by contrast, CCTs and CPs interacted at lower affinity regardless of temperature. [The apparent affinities observed in Fig. 6 were on average 15% greater than comparable values reported in Fig. 5. This disparity may be attributed to small variations in CCT preparations, which were made on multiple occasions over several years. Although the experiments cannot be directly compared numerically, the same patterns of temperature dependence of CCT binding affinity emerge from the two data sets.] There was an indication that the CCTs bind either actin substrate with greater affinity at the "physiological" temperature of the chaperonin (Fig. 6C), but this trend was not seen for tubulin substrates (Fig. 6D). Because binding of the heterologous pairings was analyzed in a single experiment, the results must be interpreted cautiously.

ATPase activities of apoCCTs

CCT possesses an intrinsic ATPase activity in the absence or presence of client proteins, and free energy released during the hydrolytic cycle drives the conformational cycle of the folding complex (Melki and Cowan, 1994). To determine whether CCT from G. gibberifrons exhibits thermal compensation of folding in the cold habitat experienced by the species, we compared its steady-state ATPase activities at psychrophilic and mesophilic temperatures to those of bovine CCT. Table 2 shows the ATPase activities of the two apoCCTs at 4 and 20°C, measured via a coupled-enzyme assay under conditions in which the concentration of CCT was the rate-limiting factor. At these temperatures, the ATPase activity of each CCT was linear for intervals >60 min, which indicates that neither the psychrophilic nor the mesophilic chaperonin denatured measurably during the assays. At 4°C, the ATPase activity of the notothen CCT was 2.6-fold greater than that of the bovine chaperonin, whereas the activities of the two CCTs were nearly identical at 20°C. The temperature coefficient, $Q_{10}$, for G. gibberifrons CCT was 2.6.

Table 1. Isoelectric points of CCT subunits from an Antarctic fish and a mammal

| CCT subunit | G. gibberifrons | B. taurus |
|-------------|----------------|----------|
| $\alpha$    | 5.4            | 5.6      |
| $\varepsilon$ | 5.9            | 5.8      |
| $\gamma$    | 6.15           | 6.5      |
| $\beta$     | 6.2            | 6.4      |
| $\alpha$    | 6.35           | 6.05     |
| $\zeta$–1   | 6.7            | 6.55     |
| $\eta$      | 6.75/6.9       | 6.6/6.9  |
| $\delta$    | >7             | 6.6/6.9  |
| $\zeta$–2   | —              | 7.0      |

Subunit pl was determined as the pH of the IPG well corresponding to highest subunit concentration. Some subunits gave two pls, presumably indicative of posttranslational variants.

Fig. 3. Structural characterization of G. gibberifrons CCT by EM: comparison to the bovine chaperonin. Two-dimensional average images of apo- and holo-CCTs were generated as described in Materials and Methods. (A) apo-CCT from G. gibberifrons, top view ($n=575$ particles). (B) apo-CCT from G. gibberifrons, side view ($n=486$ particles). (C) G. gibberifrons CCT in complex with N. coniceps $\beta$-tubulin, top view ($n=650$ particles). (D) CCT–$\beta$-tubulin complex from the cow, top view ($n=570$ particles). (E) G. gibberifrons CCT in complex with C. aceratus actin, top view ($n=710$ particles). (F) CCT–actin complex from the cow, top view ($n=657$ particles). Scale bar: 5 nm.

Fig. 4. G. gibberifrons CCT binds to, folds, and releases C. aceratus actin at physiological temperature. CCT was incubated with denatured actin at intervals from 0 to 96 h at 2°C in binding buffer containing 1 mM Mg$^{2+}$-ATP. Reaction products were analyzed at 2°C by non-denaturing electrophoresis on 4.5% polyacrylamide gels followed by autoradiography. (A) apo-CCT migrates as a single band as shown on this Coomassie Blue-stained gel. (B–D) Folded $\beta$-actin is detected at 12 h and increases in amount until a plateau is reached at 72–96 h. Large amounts of $\beta$-actin remained in complex with CCT. The positions of apo-CCT, of CCT–$\beta$-actin, and of folded actin monomer are indicated.

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close to the range of 2.0–2.5 that typifies biochemical reactions involving protein conformational changes at physiological body temperatures (Hochachka and Somero, 2002). This “normal” $Q_{10}$ is somewhat surprising considering that 20°C is distinctly outside the habitat temperature range of the Antarctic fish, but many psychrophilic enzymes and structural proteins have activity optima at temperatures near 20–30°C (cf. D’Amico et al., 2003; Detrich et al., 2000; Detrich et al., 1992). By contrast, $Q_{10}$ for bovine CCT was 5.0, which is consistent with a steep decline in catalytic performance with decreasing temperature. We conclude that $G.~gibberifrons$ CCT is at least partially compensated for the rate-depressing effects of low temperature and is sufficiently stable to retain catalytic activity at mesophilic temperature, whereas the activity of the bovine chaperonin is significantly compromised at psychrophilic temperature.

**DISCUSSION**

The successful radiation of Antarctic notothenioid fishes in the Southern Ocean involved constraints and trade-offs at many levels of biological organization: molecular, cellular, organismal, and ecological (Pörtner et al., 2007). At the molecular and cellular levels, numerous studies have documented compensatory thermal adaptation of individual proteins of these fishes relative to cool temperate notothenioids from South America and New Zealand and to temperate fishes in general (for reviews, see Coppes Petricorena and Somero, 2007; Somero, 2004). One may plausibly argue that widespread, cold-adaptive alteration of enzymes and structural proteins in the stenothermal Antarctic notothenioids would be disadvantageous, or perhaps lethal, should these fishes encounter the rapidly rising oceanic

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**Table 2. Temperature dependence of the ATPase activities of apocCTs from an Antarctic fish and a mammal**

|          | ATPase activity (nmol ATP/μmol CCT × min) |
|----------|------------------------------------------|
| CCT      | 4°C           | 20°C          |
| $G.~gibberifrons$ | 5.5          | 25.0          |
| $B.~taurus$       | 2.1          | 27.4          |

Assays were performed in triplicate; standard deviations were negligible.
temperatures projected along the Antarctic Peninsula during the
next century (Gille, 2002; Clarke et al., 2007; Dukelow et al.,
2007; Pörtner et al., 2007). Thus, Somero’s question – “how
many proteins ‘need’ to adapt (in ectotherms) when temperature
rises by a few degrees?” (Somero, 2011) – requires an expansive
functional analysis of proteins belonging to many structural
classes and different groups of organisms (Lockwood and
Somero, 2012). Must a few proteins evolve, many, or a lot?

To evaluate the thermal tolerance of the notothenioid proteome to
a warming marine environment, we have chosen to focus on the
gatekeeper of cellular protein homeostasis, the cytoplasmic
chaperonin CCT. This protein complex, which assists the folding
of a large number of proteins of multiple structural classes and
complex topologies (Thulasiraman et al., 1999; Dekker et al.,
2008; Yam et al., 2008), could constitute a metabolic bottleneck
in Antarctic notothenoid cells should its function be compromised by
elevated temperature. Alternatively, retention of the capacity of
notothenioid CCT to bind CPs and to assist their three-dimensional
maturation at 5°C above the current habitat temperature of this fish
group would support conjectures that a relatively small number of
proteins might require adaptive fine-tuning of function and stability
in the context of anticipated climate change (Somero, 2011; Powers
and Schulte, 1998; Ream et al., 2003; Lockwood and Somero, 2012).
Our results are consistent with the latter possibility, since the
psychrophilic CCT appears to be folding-competent, and at least
some of its CPs (e.g. tubulins) stable and active (Detrich et al.,
1989; Detrich et al., 1992), at temperatures 5–10°C above the physiological.

The apparent affinity of G. gibberifrons testis CCT for its
homologous actin and tubulin substrates at temperatures between
−4°C and +4°C approximates the affinity of bovine testis CCT for
its CPs at 20°C (Fig. 5). Since these temperatures are reasonable
proxies for their respective cellular environments, our results imply
that the two chaperonins have evolved corresponding states of the
conformational flexibility necessary for binding and release of
their CPs, and perhaps for the protein folding cycle as a whole.
How might this be achieved? Comparative analyses of orthologous
psychrophilic, mesophilic, and thermophilic enzymes have shown
that the rate-limiting step in enzymatic catalysis is the flexibility of
loops that must move to accommodate substrate binding and product
release during the catalytic cycle (reviewed by Somero, 2004). For psychrophilic enzymes, amino acid substitutions that
facilitate the flexibility of the hinge regions about which loops or
domains must move are the key adaptive changes that lower
activation energy barriers for catalytically critical conformational
changes. Therefore, we propose that adaptive evolution of CCT for
efficient function at psychrophilic temperatures may be based upon
flexibility-enhancing residue substitutions in the apical lid, which
appears to control the rate-limiting transition from the closed to
open state as ADP and P, are released (Reissmann et al., 2007). We
have not examined this step of the catalytic cycle, but our
measurements of the temperature dependence of the ATPase
activities of apoCCTs strongly supports this hypothesis – the
thermal coefficient of G. gibberifrons CCT is consistent with
maintenance of structural flexibility at 4°C, whereas that for bovine
CCT indicates a loss of flexibility at this temperature. We note,
however, that the interdomain and intersubunit cooperativity
intrinsic to the CCT folding cycle (Muñoz et al., 2011; Pereira et
al., 2012) suggests that sequence changes in the intermediate
and equatorial domains may also be involved in thermal adaptation of
the G. gibberifrons chaperonin.

CCT binds quasi-native actin or tubulin to specific ring
subunits via polar and electrostatic interactions (Rítcovonsovici
and Willson, 2000; Llorca et al., 2001; Gómez-Puertas et al.,
2004). Pucciarelli et al. have shown that the β and θ subunits of
CCT from the Antarctic Bullhead notothen N. coriiceps, contain
multiple flexibility-enhancing amino acid substitutions (bulky/
polar/charged residues in the CCT subunits of temperate fishes
and mouse replaced by Ala or Gly in the psychrophilic fish
subunits) in locations that should enable the conformational
changes necessary for binding and release of CPs to occur at
activation energies lower than those of mesophilic CCTs
(Pucciarelli et al., 2006). We anticipate that a comprehensive
survey of all G. gibberifrons CCT subunits will confirm this
observation and that comparative structural analysis of G.
gibberifrons and bovine CCTs (± bound CPs) will help to
refine our understanding of the molecular interactions and
catalytic mechanism of class II chaperonins. Conversely, we
note that psychrophilic CPs also show evidence of increased
structural mobility. Detrich et al. have shown that the α and β-
tubulins of Antarctic notothenioids have evolved more flexible M
and N loops (Detrich et al., 2000), which likely strengthen
interprotifilament interactions in microtubules at −1.9°C. Since
the M and N loops of tubulins contribute importantly to binding
to CCT (Gómez-Puertas et al., 2004), their increased flexibility in
Antarctic fish tubulins should also enhance the CCT–CP
interaction at low temperature. Together, these observations
support the hypothesis that CCT and some CPs have co-evolved
to maintain productive chaperonin-assisted folding reactions in a
psychrothermal environment.

Comparison of enzymes obtained from psychrophilic and
mesophilic organisms can be difficult due to differential thermal
stability. As one increases the experimental temperature from the
psychrophilic range (~0 to 15°C) to the mesophilic (~15–40°C),
the anticipated exponential increase in the activity of the
psychrophilic enzyme is likely to be compromised by an
increased rate of denaturation. In this work, however, we found
no evidence for denaturation-based decay of the ATPase activity of
G. gibberifrons CCT at the low mesophilic temperature of 20°C.
Similarly, we have shown that the brain and egg tubulins of
Antarctic fishes assemble and disassemble reversibly at
temperatures as high as 25°C with little evidence of denaturation
(Detrich et al., 1989; Detrich et al., 1992; Detrich et al., 2000),
whereas denaturation is clearly evident at 37°C. Thus, we suggest
that 20–25°C may a thermal “sweet spot” for comparing the
activities of psychrophilic and mesophilic enzymes.

Psychrophilic organisms, such as the Antarctic notothenioids,
that have evolved in stable thermal environments over millions of
years appear to be threatened by protein denaturation at both the
upper and lower limits of their narrow thermal regimes. The very
flexibility that maintains the functionality of their enzymes at
physiological temperatures renders these proteins both heat labile
and cold labile (Makhatadze and Privalov, 1995; Privalov, 1990;
D’Amico et al., 2003). Decreased stability and unfolding at low
temperature appear to be due to favorable changes in the contact
free energy between nonpolar groups and water, such that peripheral
penetration of water molecules weakens the hydrophobic effect and
causes mechanical instability in the protein core (Lopez et al.,
2008; Dias et al., 2010). The reality of cold-induced protein denaturation
is well illustrated by the observation of elevated protein turnover in
Antarctic fishes via ubiquitin-mediated proteasomal degradation
(Todgham et al., 2007). Thus, CCT and the suite of chaperones that
maintain protein homeostasis in Antarctic fishes provide novel
opportunities for mechanistic analysis of cold denaturation using
structural and biophysical strategies.
**Materials and Methods**

Materials

Unless otherwise stated, reagents were purchased from Sigma–Aldrich (St Louis, MO, USA). Water was purified by use of Milli-Q systems (Millipore, Bedford, MA, USA).

**Collection of Antarctic fishes**

Specimens of the Humphead notothen, *Gobionotothen gibberifrons* (Lönnberg), were collected by bottom trawls or via baited fish traps deployed from the ARSV *Laurence M. Gould* south of Low Island or west of Brabant Island in the Palmer Archipelago (April–June, 2008 and 2010). The fish were transported alive to Palmer Station, Antarctica, where they were maintained in seawater aquaria at −1.5 to 0°C. All procedures, including euthanasia, utilizing live vertebrate animals at Palmer Station, Antarctica, were reviewed and approved by Northeastern University’s Institutional Animal Care and Use Committee.

**Purification of CCT from *G. gibberifrons* tests**

All steps were carried out at −1 to +1°C (unless otherwise noted).

Immature testes (stages 2–3) from *G. gibberifrons* were homogenized in a Teflon-glass tissue grinder at a ratio of 1 g tissue per ml buffer H [40 mM HEPES-KOH (pH 7.35), 20 mM KCl, 2 mM EDTA] containing 1 mM DTT and 1 mM PMSF; one protease inhibitor tablet (Complete EDTA-free Protease Inhibitor Cocktail Tablets, Roche Diagnostics, Indianapolis, IN, USA) was added per 50 ml buffer H. The homogenate was centrifuged at 9600 × g for 1 h at 4°C, and the supernatant was recovered and centrifuged again at 105,000 × g for 1 h at 4°C. The second supernatant (designated testis extract) was flash frozen in liquid nitrogen and stored at −70°C.

After thawing, testis extracts were precipitated by addition of 30% (w/v) ammonium sulfate, and the suspension was centrifuged at 14,500 g for 30 min at 4°C. The supernatant was recovered, ammonium sulfate was added to 50% (w/v), and the suspension was centrifuged again using the same parameters. The pellet was gently resuspended in a small volume of buffer A [50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 5 mM MgCl2, 10% (v/v) glycerol] containing 1 mM DTT and one protease inhibitor cocktail tablet per 50 ml, and the suspension was dialyzed overnight against the same buffer without protease inhibitors. The dialyzed extract was loaded by use of a peristaltic pump onto two sequentially coupled 5 ml HiTrap Heparin Sepharose columns (GE Healthcare Bio-Sciences, Pittsburgh, PA, USA) pre-equilibrated with buffer A plus 450 mM NaCl. Bound proteins were eluted from the column by application of buffer A plus 600 mM NaCl, fractions of 1.5 ml were collected, and the protein compositions of the fractions were analyzed by SDS-PAGE (Laemmli, 1970); gels were stained with Coomassie Brilliant Blue R-250. CCT-containing fractions were pooled; the solution was made 80% (w/v) in ammonium sulfate, and the precipitated proteins were collected by centrifugation at 12,000 × g at 4°C. Supernatants were discarded, the pellets were resuspended in small volumes of buffer A and pooled, and the CCT-enriched sample was dialyzed against 1× buffer A for at least 2 h with one buffer change. After dialysis, aliquots (1–1.5 ml) of the pool were loaded onto preformed sucrose gradients [10–50% (w/v)] in Beckman SW 28 open-top thick-wall polycarbonate centrifuge tubes, which were then centrifuged at 104,000 × g (28,000 rpm, rmax = 118.2 mm, Beckman SW-28 rotor) for 60 h at 4°C. Fractions (1 ml) were collected by lowering a glass needle, connected to a peristaltic pump, to the bottom of each tube. CCT-containing fractions, identified by SDS-PAGE, were pooled, flash frozen in liquid nitrogen, and stored at −70°C; some preparations were dialyzed against buffer A prior to flash freezing and storage. CCT was transported to our home institutions on dry ice.

The final step in the purification of *G. gibberifrons* CCT was size-exclusion chromatography of the sucrose-gradient-purified CCT on a Superose 6 10/300 GL column (GE Healthcare Bio-Sciences, Pittsburgh, PA, USA) equilibrated in buffer A and coupled to an ÄKTA Prime FPLC system (GE Healthcare Bio-Sciences, Pittsburgh, PA, USA) maintained at 6°C. The column was previously calibrated using the molecular size markers Dextran Blue (2000 kDa), thyroglobulin (670 kDa), ferritin (440 kDa), bovine serum albumin (67 kDa), and RNase (13.7 kDa). This purified preparation of *G. gibberifrons* CCT was then used for the subsequent experiments.

**Separation of *G. gibberifrons* CCT subunits by HPLC**

Purified *G. gibberifrons* CCT (280 µg) in buffer A was precipitated at 4°C by addition of trichloroacetic acid to 10%, and the suspension was centrifuged (15,000 × g, 4°C). The pellet was resuspended in 1 ml of 8 M urea, the suspension was diluted 8-fold with 0.1% trifluoroacetic acid, and the sample was centrifuged at 15,000 × g for 15 min (4°C). The supernatant was loaded (four injections of 2 ml each) on an XBridge BEH300 C4 Reverse-Phase HPLC column (2.1 × 50 mm; Waters Chromatografia S.A., Spain) coupled to an Etan LC chromatography system (GE Healthcare Bio-Sciences, Pittsburgh, PA, USA). Bound proteins were eluted by application of dual gradients of acetonitrile [50–82% (v/v)] and trifluoroacetic acid [0.1–0.075% (v/v)] in 15 column volumes; solvent A = 50% acetonitrile, 0.1% trifluoroacetic acid; solvent B = 82% acetonitrile, 0.075% trifluoroacetic acid. Fractions (250 µl) were collected; solvent was evaporated by use of a Savant Speed-Vac (Thermo Fisher Scientific, Pittsburgh, PA, USA), and protein compositions of the peaks were analyzed by SDS-PAGE on 8.5% gels.

**Mass spectrometric analysis of *G. gibberifrons* CCT subunits**

Protein bands containing CCT subunits were excised from Coomassie Blue-stained gels (see previous section), and automated in-gel protein digestion using modified porcine trypsin (sequencing grade; Promega, Madison, WI, USA) was performed on a Proteineer dp proteomics workstation (Bruker Daltonics, Bremen, Germany) according to established protocol (Shevchenko et al., 1996), with minor modifications. Peptide mass fingerprinting, MS/MS analysis, and peptide database searching were performed as described (Choi et al., 2013).

**Isoelectric focusing**

Subunits of *G. gibberifrons* CCT, previously separated by HPLC and identified by mass spectrometry, were prepared for isoelectric focusing using an Agilent 3100 OFFGEL Fractionator and the OFFGEL pH 4–7 and 6–11 Kits (Agilent Technologies, Madrid, Spain) following the manufacturer’s instructions. After drying in a Savant Speed-Vac (Thermo Fisher Scientific, Pittsburgh, PA, USA), each subunit sample was resuspended in 1 ml OFFGEL buffer containing 8 M urea, 2 M thiourea, 70 mM DTT, and 1.2% (v/v) ampholytes: pH 4–7 (Agilent Technologies, Madrid, Spain) were used with subunits α, β, γ, δ, ε, ζ, and η, whereas pH 6–11 (GE Healthcare Bio-Sciences, Pittsburgh, PA, USA) was added to subunit η. Subunit samples were diluted to 3.6 ml by addition of OFFGEL fractionation buffer. Twenty-four aliquots of a subunit sample (150 µl each) were placed in the 24 wells of the OFFGEL tray, and focusing was performed on a 24-cm immobilized pH gradient (IPG) gel strip (linear pH gradient of 4–7 or 6.2–7.5, 50 μA) until 64 kVh was reached (48 h). After focusing, the 24 fractions were recovered and electrophoresed on an 8.5% SDS-PAGE gel. Each subunit pI was determined as the pH of the IPG well corresponding to highest subunit concentration. The pI of bovine CCT subunits were determined by focusing 300 µg of the purified complex on two IPG strips (pH 4.7 and 6.2–7.5) using the OFFGEL system described above. After SDS-PAGE of the 24 fractions from each strip, protein bands in the range 55–62 kDa were excised from each gel lane, and subunits were identified by mass spectrometry as described above. Subunit pIs were assigned by reference to the IPG pH gradient.

**Preparation of Antarctic fish client proteins (CPs)**

Notothenioid CPs were obtained by expression of brain actin and tubulin cDNAs from two species, *Chaenocephalus aceratus* (Lönnberg) and *Notoniathecoriaecips* (Richardson), that are closely related to *G. gibberifrons* (Eastman, 1993). *C. aceratus* β-actin (unpublished sequence, GenBank acc. no. KCS94078) and *N. coriiceps* β-tubulin (Detrich and Parker, 1993; acc. no. L08013), each cloned in PET 11a, were produced in *E. coli* as described (Gao et al., 1992) and modified (Pucciarelli et al., 2006). 35S-labeled CPs were expressed in medium containing 0.2 mCi of EasyTag®-L-[35S] methionine (NEG-709A, >1000 Ci/mmol, PerkinElmer, Waltham, MA, USA) and methionine-free amino acid mix. Unlabeled CPs were produced using complete amino acid mix. CPs were transferred to 7.5 M urea, 10 mM DTT, 20 mM Tris-HCl (pH 7.5) by gel filtration, and aliquots (5 mg/ml) were stored at −70°C.
Preparation of bovine CCT and CPs
CCT from bovine tests was purified by the method of Martín-Benito et al. (Martín-Benito et al., 2002). Bovine cardiac actin (>99%; cat. no. AD99) and bovine brain tubulin (>99%; cat. no. TL238) were purchased from Cytoskeleton, Inc. (Denver, CO, USA).

Structural characterization of CCT and CCT–CP complexes by EM
To compare the structures of apo- and holo-CCT particles from the psychrophilic fish to those of the mesophilic mammal, we generated two-dimensional average images by negative-stain EM. G. gibberifrons CCT was incubated in ATP-free binding buffer [50 mM Tris-HCl (pH 7.4), 500 mM NaCl, 5 mM MgCl2, 1 mM DTT, 10% (v/v) glycerol] (Cuellar et al., 2008) at 4°C in the absence or presence of homologous, denatured CPs (actin, tubulin), whereas bovine CCT was incubated with or without bovine CPs at 25°C. Denatured CPs from Antarctic fish or from the cow were diluted 100-fold into their respective CCTs to yield 10:1 molar ratios of NADH, 2 mM phosphoenolpyruvate, 15 mM MgCl2, 1 mM ADP, 1 mM DTT. Each sample was incubated for 5 min. Aliquots (5 μl) of each reaction were applied for 1 min to glow-discharged carbon-coated grids pre-cooled to the appropriate temperature. The samples were then stained for 1 min with 2% (w/v) uranyl acetate at the incubation temperature. Images were recorded at 0° tilt using a JEOL 1200EX-II electron microscope, operated at 100 kV, on Kodak SO-163 film at 20,000 x nominal magnification. Micrographs were digitized using a Zeiss SCAI scanner with a sampling window corresponding to 3.5 Å per pixel, and particles were automatically selected and classified using XMIPP software (Sorzano et al., 2009). Two-dimensional, reference-free averages of the images were generated for each CCT ± its CPs were averaged from ∼500–700 individual images (see legend to Fig. 3).

CCT–CP binding and folding assays
To assess chaperonin–client protein affinities, denatured CPs from Antarctic fish or from the cow were combined with CCTs as described in the previous section, and the samples were incubated for 5 min at four temperatures between −4°C and +20°C in ATP-free binding buffer. Homologous binding (fish CCT–fish CP, etc.) experiments were performed in triplicate, whereas heterologous binding experiments (fish CCT–cow CP, etc.) were performed once, albeit with a very large sampling population. Samples from each reaction were prepared for negative-stain EM as described in the preceding section. Unless otherwise noted, 2000 end-on view CCT particles from each binding reaction were scored automatically as apo- or holo-CCT (determined by the absence or presence of a stain-excluding mass in the chaperonin cavity) using maximum-likelihood procedures (Scheres et al., 2005). After particle classification, the apparent affinity of binding was measured as the percentage of CCT particles containing bound substrate [(holo-CCT/hoIo-CCT + apo-CCT) x 100%].

The folding activity of G. gibberifrons CCT was assessed at 2°C by diluting denatured °S-labeled C. aceratus β-actin 100-fold into chaperonin in binding buffer containing 1 mM ATP. At intervals, aliquots were withdrawn from the reaction, and the products (CCT–β-actin complex, folded actin) were analyzed on 4.5% non-denaturing polyacrylamide gels (Zabala and Cowan, 1992) run at the same temperature. Autoradiographs of the gels were scanned to quantify folded β-actin (Zabala and Cowan, 1992).

ATPase assays
Rates of ATP hydrolysis by apoCCT from G. gibberifrons or from the cow were measured spectrophotometrically in a buffer containing a coupled-enzyme, ATP-regenerating system (Sot et al., 2002). Reaction mixtures (50 mM Tris-HCl, 10 mM MgCl2, 100 mM KCl, 0.2 mM NADH, 2 mM phosphoenolpyruvate, 15 μg/ml pyruvate kinase, and 30 μg/ml lactate dehydrogenase, pH 7.5) were pre-equilibrated at the desired temperature (either 4 or 20°C) for 10 min in the thermostated cuvettes of a Shimadzu CPS-240A spectrophotometer. ATP (2 mM final concentration) was added to each cuvette, and the assay mixtures were incubated isothermally for 2 min. Finally, CCT (0.38 μM final concentration) was added, and the decrease in absorbance at 340 nm was followed for intervals up to 120 min. Triplicate assays were performed at each temperature. We verified that the activities of the coupling enzymes were sufficiently high at both temperatures such that the ATPase activity of CCT was the limiting factor controlling the oxidation of NADH.

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Competing interests
The authors have no competing interests to declare.

Author contributions
J.C., H.Y., J.M.V., J.C.Z. and H.W.D. conceived and designed the experiments; J.C., H.Y., S.P., G.C., M.S. and J.M.V. performed the experiments and collected the data; J.C., H.Y., J.M.V., J.C.Z. and H.W.D. interpreted the findings; J.M.V., J.C.Z. and H.W.D. drafted and revised the manuscript.

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References
Arias, E. and Cuervo, A. M. (2011). Chaperone-mediated autophagy in protein quality control. Curr. Opin. Cell Biol. 23, 184-189.
Buckley, B. A. and Somero, G. N. (2009). cDNA microarray analysis reveals the capacity of the cold-adapted Antarctic fish Trematomus bernacchii to alter gene expression in response to heat stress. Polar Biol. 32, 403-415.
Buckley, B. A., Place, S. P. and Hofmann, G. E. (2004). Regulation of heat shock proteins in isolated hepatocytes from an Antarctic fish, Trematomus bernacchii. J. Exp. Biol. 207, 3649-3656.
Chen, L., DeVries, A. L. and Cheng, C.-H. C. (1997). Evolution of antifreeze glycoprotein gene from a trypsinogen gene in Antarctic nototheniid fish. Proc. Natl. Acad. Sci. USA 94, 3811-3815.
Cheng, C.-H. C. and Chen, L. (1999). Evolution of an antifreeze glycoprotein. Nature 401, 443-444.
Choi, H., Juarez, S., Ciordia, S., Fernandez, M., Bagiela, R., Albar, J. P., Mazumdar, V., Antón, B. P., Kasif, S., Ferrer, M. et al. (2013). Biochemical characterization of hypothetical proteins from Helicobacter pylori. PLOS ONE 8, e66056.
Chechpanov, A. (1998). The ubiquilin-proteasome pathway: on protein death and cell life. EMBO J. 17, 7151-7160.
Clarke, A., Murphy, E. J., Meredith, M. P., King, J. C., Peck, L. S., Barnes, D. K. and Smith, R. C. (2007). Climate change and the marine ecosystem of the western Antarctic Peninsula. Philos. Trans. R. Soc. B 362, 149-166.
Coca, E., Ratnayake-Lecamwasam, M., Parker, S. K., Camardella, L., Ciaramella, M., di Prisco, G. and Detrich, H. W., III (1995). Genomic remnants of alpha-globin genes in the hemoglobinless Antarctic icefishes. Proc. Natl. Acad. Sci. USA 92, 1817-1821.
Cong, Y., Schröder, G. F., Meyer, A. S., Jakana, J., Ma, B., Dougherty, M. T., Schmid, M. F., Reissmann, S., Levitt, M., Ludtke, S. L. et al. (2012). Symmetry-free cryo-EM structures of the chaperonin TRiC along its ATPase-driven conformational cycle. EMBO J. 31, 720-730.
Coppes Petricorena, Z. L. and Somero, G. N. (2007). Biochemical adaptations of nototheniid fishes: comparisons between cold temperate South American and New Zealand species and Antarctic species. Comp. Biochem. Physiol. 146, 799-807.
Cuellar, J., Martin-Benito, J., Scheres, S. H., Sousa, R., Moro, F., López-Viñas, E., Gómez-Puertas, P., Muga, A., Carrascosa, J. L. and Valpuesta, J. M. (2008). The structure of CCT–Hsc70 NBD suggests a mechanism for Hsp70 delivery of substrates to the chaperonin. Nat. Struct. Mol. Biol. 15, 808-814.
D’Amico, S., Marx, J. C., Gerday, C. and Feller, G. (2003). Activity-stability relationships in extremophilic enzymes. J. Biol. Chem. 278, 7891-7896.
DeConto, R. M. and Pollard, D. (2003). Rapid Cenozoic glaciation of Antarctica induced by declining atmospheric CO2. Nature 421, 245-249.
Decker, C., Sterling, P. C., McCormack, E. A., Filippone, H., Paul, A., Brost, R. L., Costanzo, M., Boone, C., Leroux, M. R. and Willisson, K. R. (2008). The interaction network of the chaperonin CCT. EMBO J. 27, 1827-1839.
Detrich, H. W., III and Parker, S. K. (1993). Divergent neural beta tubulin from the Antarctic fish Notothenia coriiceps neglecta: potential sequence contributions to interaction network of the chaperonin CCT. EMBO J. 12, 1827-1839.
Detrich, H. W., III, Johnson, K. A. and Marchese-Ragona, S. P. (1989). Polymerization of Antarctic fish tubulins at low temperatures: energetic aspects. Biochemistry 28, 10085-10093.

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Detrich, H. W., III, Fitzgerald, T. J., Dinsmore, J. H. and Marchese-Ragona, S. P. (1992). Brain and egg tubulins from Antarctic fishes are functionally and structurally distinct. J. Biol. Chem. 267, 18766-18775.

Detrich, H. W., III, Parker, S. K., Williams, R. C., Jr, Nogales, E. and Downing, K. C. (2000). Cold adaptation of microtubule assembly and dynamics. Structural interpretation of primary sequence changes present in the alpha- and beta-tubulins of Antarctic fishes. J. Biol. Chem. 275, 37038-37047.

Detrich, H. W., III, Buckley, B. A., Doolittle, D. F., Jones, C. D. and Lockhart, S. J. (2012). Sub-Antarctic and High Antarctic notothenioid fishes: ecology and adaptational biology revealed by the ICEFISH 2004 cruise of RVIB Nathaniel B. Palmer. Oceanography 25, 184-187.

DeWitt, H. H. (1971). Coastal and Deep-Water Benthic Fishes of the Antarctic (Antarctic Expedition, Fall 15) ed. V. C. Bushnell, pp. 1-10. New York, NY: American Geographical Society.

Dias, C. L., Ala-Nissila, T., Wong-ekkatub, J., Vattulainen, I., Grant, M. and Karimtunen, M. (2010). The hydrophobic effect and its role in cold denaturation. Cytobios 60, 91-98.

Ducklow, H. W., Baker, K., Martinson, D. G., Quetin, L. B., Ross, R. M., Smith, R. C., Stamm, J. E. S., Vernet, M. and Fraser, W. (2007). Marine pelagic ecosystems: the west Antarctic Peninsula. Phils. Trans. R. Soc. B 362, 67-94.

Eastman, J. T. (1993). Antarctic Fish Biology: Evolution in a Unique Environment. San Diego, CA: Academic Press.

Eastman, J. T. and Clarke, A. (1998). A comparison of adaptive radiations of Eastman, J. T. and Clarke, A. (2002). Warming of the Southern Ocean since the 1950s.

Go´ mez-Puertas, P., Martı´n-Benito, J., Carrascosa, J. L., Willison, K. R. and Mun˜ oz, I. G., Ye´benes, H., Zhou, M., Mesa, P., Serna, M., Park, A. Y., Bragado-Nilsson, E., Beloso, A., de Cárden, G., Malumbres, M. et al. (2011). Crystal structure of the carboxyl terminus of the mammalian chaperonin CCT in complex with tubulin. Nat. Struct. Mol. Biol. 18, 14-19.

Near, T. J., Parker, S. K. and Detrich, H. W., III (2006). A genomic fossil reveals key steps in hemoglobin loss by the Antarctic icefishes. Biol. Mol. Evol. 23, 2008-2016.

Pereira, J. H., Ralston, C. Y., Douglas, N. R., Kumar, R., Lopez, T., McMordie, R. P., Knee, K. M., King, J. A., Frydman, J. and Adams, P. D. (2012). Mechanism of nucleotide sensing in group II chaperonins. EMBO J. 31, 731-740.

Place, S. P. and Hofmann, G. E. (2005). Constitutive expression of a stress-inducible heat shock protein gene, hsp70, in phylogenetically distant Antarctic fish. Polar Biol. 28, 261-267.

Place, S. P., Zippay, M. L. and Hofmann, G. E. (2004). Constitutive roles for inducible genes: evidence for the alteration in expression of the inducible hsp70 gene in a sub-Antarctic nototheniid fishes. Am. J. Physiol. 287, R432-R436.

Pörtner, H. O. and Farrell, A. P. (2008). Ecology. Physiology and climate change. Science 322, 690-692.

Pörtner, H. O., Peck, L. and Somero, G. (2007). Thermal limits and adaptation in Antarctic ectotherms: an integrative view. Philos. Trans. R. Soc. B 362, 2233-2258.

Power, D. A. and Schulte, P. M. (1998). Evolutionary adaptations of gene structure and expression in natural populations in relation to a changing environment: a multidisciplinary approach to address the millenary-saga of a small fish. J. Exp. Zool. 282, 71-94.

Privatoy, P. L. (1990). Cold denaturation of proteins. Crit. Rev. Biochem. Mol. Biol. 25, 281-306.

Pucirelli, S., Parker, S. K., Detrich, H. W., III and Melki, R. (2006). Characterization of the cytoplasmic chaperonin containing TCP-1 from the Antarctic fish Notolitiiota fisheri. Extremophiles 10, 537-549.

Ream, R. A., Johns, G. C. and Somero, G. N. (2003). Base compositions of genes encoding alpha-actin and lactate dehydrogenase-A from differently adapted vertebrates show no temperature-adaptive variation in G + C content. Mol. Biol. Evol. 20, 105-110.

Reddy, C. R., Frankfurter, A. K., Parker, S. K., Ross, R., Jesch, J. and Detrich, H. W., III (2004). Posttranslational modification of brain tubulins from the Antarctic fish Notolitiiota coriiceps: reduced C-terminal glutamylation correlates with efficient microtubule assembly at low temperature. Biochemistry 43, 12265-12274.

Reddy, C. R., R. Chiu, W. and Detrich, H. W. (2007). Essential function of the built-in lid in the allosteric regulation of eukaryotic and archaean chaperonins. Nat. Struct. Mol. Biol. 14, 432-440.

Ritzo-Vonsovici, M. and Willison, K. R. (2000). Defining the eukaryotic cytosolic chaperonin-binding sites in human tubulins. J. Mol. Biol. 304, 81-98.

Scher, H. D. and Martin, E. E. (2006). Timing and climatic consequences of the opening of Drake Passage. Science 312, 428-430.

Scheres, S. H., Valle, M., Ruiz, R., Sorzano, C. O., Marabini, R., Herman, G. T. and Carazo, J. M. (2004). Reference refinement for electron microscopy images. J. Mol. Biol. 348, 139-149.

Shevchenko, A., Wilm, M., Vorm, O. and Mann, M. (2006). Mass spectrometric sequencing of proteins from silver-stained polyacrylamide gels. Anal. Chem. 68, 850-858.

Somero, G. N. (2004). Adaptation of enzymes to temperature: searching for basic “strategies”. Comp. Biochem. Physiol. 139B, 321-333.

Somero, G. N. (2005). Linking biogeography to physiology: Evolutionary and acclimatory adjustments of thermal limits. Front. Zool. 2, 1.

Somero, G. N. (2011). Comparative physiology: a “crystal ball” for predicting consequences of global change. Am. J. Physiol. 301, R1-R14.

Somero, G. N. (2010). Evolution of functions: the west Antarctic Peninsula. J. Struct. Biol. 171, 824-829.

Valpuesta, J. M., Carrascosa, J. L. and Willison, K. R. (2007). Salt acclimation in the Antarctic fish Trematomus bernacchii (family Nototheniidae).

Wellauer, L. A., Royer, J.-Y., Sandwell, D. T. and Scutese, C. R. (1991). Evolution of the Antarctic continental margins. In Geologic Evolution of Antarctica (ed. M. A. Thomson, J. A. Crame and J. A. Thomson), pp. 533-539. Cambridge: Cambridge University Press.

West, A. G., milf, M. and Shaw, J. (1998). The major adult alpha-globin gene of the Antarctic fish Trematomus bernacchii (Nototheniidae). Cytoskeleton 34, 289-295.

Woolf, D. K., Robin, B. E., Perron, E. R. and Frampton, M. (2003). In vivo newly translated eukaryotic chaperonin function of the cytosolic chaperonin CCT. In Protein Folding Handbook ed. D. T. Todgham, A. E., Hoaglund, E. A. and Hofmann, E. A. (2003). Linking biogeography to physiology: Evolutionary and acclimatory adjustments of thermal limits. Front. Zool. 2, 1.

Yam, A. Y., Xia, Y., Lin, H. T., Burlingame, A., Gerstein, M. and Frydman, J. (2008). Defining the TRiC/CCT interactome links chaperonin function to stabilization of newly made proteins with complex topologies. Nat. Struct. Mol. Biol. 15, 1255-1262.

Yamada, K. H., M. R. A. Thomson, J. A. Crame and J. A. Thomson), pp. 533-539. Cambridge: Cambridge University Press.

Yamada, K. H., M. R. A. Thomson, J. A. Crame and J. A. Thomson), pp. 533-539. Cambridge: Cambridge University Press.