Extremophiles (2006) 10:537–549
DOI 10.1007/s00792-006-0528-x

ORIGINAL PAPER

Sandra Pucciarelli · Sandra K. Parker · H. William Detrich III · Ronald Melki

Characterization of the cytoplasmic chaperonin containing TCP-1 from the Antarctic fish *Notothenia coriiceps*

Received: 6 February 2006 / Accepted: 11 April 2006 / Published online: 13 June 2006

Abstract The cytoplasmic chaperonin containing TCP-1 (CCT) plays a critically important role in the folding and biogenesis of many cytoskeletal proteins, including tubulin and actin. For marine ectotherms, the chronically cold Southern Ocean (−2 to +2°C) poses energetic challenges to protein folding, both at the level of substrate proteins and with respect to the chaperonin/chaperone folding system. Here we report the partial functional and structural characterization of CCT from an Antarctic notothenioid fish, *Notothenia coriiceps*. We find that the mechanism of folding by the Antarctic fish CCT differed from that of mammalian CCT: (1) the former complex was able to bind denatured β-tubulin but (2) when reconstituted with rabbit Cofactor A, failed to release the protein to yield the tubulin/cofactor intermediate. Moreover, the amino acid sequences of the *N. coriiceps* CCT β and θ chains contained residue substitutions in the equatorial, apical, and intermediate domains that would be expected to increase the flexibility of the subunits, thus facilitating function of the chaperonin in an energy poor environment. Our work contributes to the growing realization that protein function in cold-adapted organisms reflects a delicate balance between the necessity of structural flexibility for catalytic activity and the concomitant hazard of cold-induced denaturation.

Communicated by K. Horikoshi

S. Pucciarelli (✉)
Dipartimento di Biologia, Molecolare,
Cellulare, ed Animale, Università di Camerino,
62032 Camerino, Italy
e-mail: sandra.pucciarelli@unicam.it
Tel.: +39-0737-403276
Fax: +39-0737-636216

S. K. Parker ·
H. W. Detrich III
Department of Biology, Northeastern University,
Boston, MA, 02115 USA

R. Melki
Laboratoire d'Enzymologie et Biochimie Structurales,
Centre National de la Recherche Scientifique,
91198 Gif-sur-Yvette, France

Keywords Protein folding · Chaperonin · CCT · Cofactor A · Tubulin · cpn60 · Antarctic fish

Abbreviation

CCT:	Cytoplasmic chaperonin containing tcp-1 ·
TCP1:	Tcomplex protein 1 ·
CofA:	Cofactor A ·
MES:	2-Morpholinoethanesulfonic acid, monohydrate
EGTA:	Ethylene glycol bis(2-aminoethyl ether)-*N*,*N*,*N*′*N*′-tetraacetic acid ·
DTT:	Dithiothreitol ·
SDS:	Sodium dodecyl sulfate ·
PAGE:	Polyacrylamide gel electrophoresis ·
EST:	Expressed sequence tags

Introduction

Since the advent of continental glaciation and the cooling of the Southern Ocean ~38 million years ago (Mya) (Eastman 1993; Peck et al. 2004), evolution of the Antarctic marine biota has been driven by the development of extreme cold temperatures. Although the cold climatic conditions have been interrupted by periods of modest warming, many temperate groups of marine organisms, including most taxa of fishes, became extinct. Together, the environmental and geographic isolation of Antarctic coastal waters and selection driven by cold temperatures have produced an endemic, highly cold-adapted fish fauna, which is dominated by species of a single suborder of perciform fishes, the Notothenioidei. This group, which has undergone a rapid radiation, now constitutes more than 50% of the species and 90% of the biomass of the Antarctic coastal fish fauna (DeWitt 1971).

Living in a stable and extremely cold habitat (−2 to +2°C), the notothenioid fishes have evolved numerous adaptations in their biochemical and physiological systems that compensate, at least in part, for the deleterious effects of low temperatures on reaction rates and equi-

libria. Many enzymes show temperature compensation of the catalytic rate constant, k_{cat}, such that metabolic flux through biochemical pathways is maintained at levels near or equal to those observed in temperate (mesophilic) organisms. Fields and Somero (1998) have shown that the k_{cat} of the skeletal muscle isoform of LDH from Antarctic notothenioid fishes is 4- to 5-fold greater than those of mammalian, avian, and thermophilic reptilian orthologues, when each is measured at 0°C. Since the active site chemistry of LDH (and of enzymes in general) is highly conserved evolutionarily, the most plausible explanation to account for cold adaptation is the evolution of greater flexibility in regions outside the active site that enable the true rate-limiting steps, the conformational changes necessary for binding of substrate and release of product, to occur at lower activation energies (NRC 2003). Similarly, temperature compensation of the polymerization of cytoskeletal proteins from Antarctic fishes, such as actins (Swezey and Somero 1982) and tubulins (Billger et al. 1994; Detrich et al. 2000), appears to be based, at least in part, on structural changes that increase the flexibility of these proteins.

Recent research on protein metabolism in cold-living organisms, from synthesis to folding, and ultimately to degradation, has revealed some startling departures from the eukaryotic norm. The translational machinery of Antarctic fishes, for example, shows clear evidence of cold adaptation (Haschemeyer 1983), with a rate of polypeptide chain elongation more than 10-fold greater than those measured in temperate fishes cooled to comparable temperatures. Protein synthesis and turnover by embryos of an Antarctic sea urchin, *Sterechinus neumayeri*, appear to be fully temperature compensated with respect to those of temperate urchins (Marsh et al. 2001). Surprisingly, the highly conserved eukaryotic heat shock response [i.e., the cellular induction of the family of heat-shock proteins (hsps) to prevent the aggregation of heat-damaged proteins and assist the refolding of misfolded proteins to their native states] is absent in at least one Antarctic notothenioid yet retained by two closely related, temperate species from New Zealand (Place et al. 2004). Place et al. (2004) propose that the low temperatures experienced by Antarctic notothenioids perturb native protein structure (cf. Privalov 1990), leading to high levels of denatured or misfolded proteins. In the absence of the selective pressure of rapid temperature change in Antarctic marine ecosystems, the "inducible' *hsp70* gene apparently has been co-opted to a constitutive role to provide for the increased requirement for molecular chaperones under in vivo environmental conditions. Motivated by these novel observations, we have chosen to investigate the functional and structural properties of the chaperonin CCT obtained from the cold-living Antarctic notothenioid *Notothenia coriiceps*.

The eukaryotic class-II chaperonin CCT (cytosolic chaperonin containing TCP-1) plays a critically important role in the folding and biogenesis of cytoskeletal proteins, including tubulin and actin (Hartl 1996), and many other proteins (Spiess et al. 2004). CCT (also known as TCP-1 complex or TriC) (Gao et al. 1992; Kubota et al. 1995; Frydman et al. 1992) is a cylindrical, 16-subunit double toroid composed of eight distinct polypeptides (designated α through θ) that are organized into two eight-subunit back-to-back rings (Kubota et al. 1995; Valpuesta et al. 2002). Three domains can be distinguished in each chaperonin subunit: (1) an equatorial domain that possesses ATPase activity; (2) an apical domain that is involved in the binding of target proteins and (3) an intermediate domain that functions as a flexible hinge connecting the equatorial and the apical domains (Kusmierczyk and Martin 2001). Misfolded actin and tubulin appear to bind to the apical domain of the CCT ring (Llorca et al. 1999; 2000) in a quasi-native conformation (Llorca et al. 2000; 2001a; b). Substrate binding is accompanied by a change in the conformation of CCT (Llorca et al. 1999; 2000; Melki et al. 1997). Subsequent nucleotide exchange and hydrolysis induce a further conformational change such that the affinity of CCT for misfolded target proteins (Melki 2001) is increased. Three-dimensional reconstructions of actin- and tubulin-CCT complexes show that CCT contributes actively to the folding of bound target proteins (Llorca et al. 2000) but its mechanism of action is not well understood. Upon release from CCT, the α- and β-tubulin chains interact with five additional cofactors (A–E) as they mature to yield the native tubulin heterodimer, the functional subunit of microtubules (Gao et al. 1994; Melki et al. 1996; Tian et al. 1999). Previously, we have described the folding of β-tubulin by the cold-adapted Antarctic ciliate *Euplotes focardii* (Pucciarelli et al. 2002). Our results demonstrated that this primitive eukaryote requires both CCT and Cofactor A (Cof A) to fold β-tubulin. However, further characterization of a psychrophilic CCT at the biochemical level necessitated that we examine a more tractable model. Here we describe the partial purification of CCT from the Antarctic yellowbelly rockcod *N. coriiceps*, investigate its functional properties, and report the amino acid sequences of two of its subunits. Our results indicate that either the energetics or mechanism of *N. coriiceps* CCT differ from that of mammalian CCT (Melki et al. 1997) and reveal important sequence changes in two CCT subunits that, by increasing their flexibility, are likely to adapt the complex to function at the chronically cold temperatures of the Southern Ocean.

Materials and methods

Collection of *Notothenia coriiceps*

Specimens of the yellowbelly rockcod, *N. coriiceps*, were caught by bottom trawling from the *R/V Polar Duke* or the *ARSV Laurence M. Gould* near Brabant and Low Islands in the Palmer Archipelago. The fish were transported alive to Palmer Station, Antarctica, where they were maintained in seawater aquaria at −1.5 to +1°C.

Preparation of tissue extracts

Muscle, testis (from gravid fishes), spleen, and pronephric (head) kidney from *N. coriiceps* were homogenized in buffer A containing 20 mM Tris (pH 7.5), 20 mM KCl, 1 mM EGTA, 1 mM $MgCl_2$, 1 mM DTT, and a protease inhibitor cocktail (2 mM PMSF, 10 μg/ml pepstatin A, 10 μg/ml leupeptin, 1 μg/ml aprotinin, 0.25 μg/ml TAME) at 4°C (100 g tissue per 120 ml of buffer A). Following centrifugation at 200,000*g* for 30 min (Beckman T45 rotor, 48,000 rpm, 4°C), the supernatant was clarified by filtration on Millipore membranes (47 mm, or 0.45 μm), or treated with cell debris remover (5 g/50 ml supernatant; Whatman). Reticulocyte extract was prepared as previously described (Melki et al., 1997).

CCT purification from testis

Testis extracts were loaded on a Q-Sepharose column (100 ml bed volume) equilibrated with buffer A. The column was washed with 300 ml of buffer A and eluted by application of a linear $MgCl_2$ gradient at 8°C (1–500 mM, 500 ml). Magnesium was chosen among the possible counter-ions in Q-Sepharose separation because it is the only one that reserves the folding activity of CCT. Fractions (4 ml) were analyzed for the presence of CCT by immunoblotting (see next section). All CCT-positive fractions were combined, diluted 2-fold with buffer B [20 mM Tris (pH 7.5), 20 mM KCl, 1 mM EGTA, 1 mM DTT], and applied to a 5-ml ATP-agarose column equilibrated in buffer C [20 mM Tris (pH 7.5) 20 mM KCl, 10 mM $MgCl_2$, 1 mM EGTA, 1 mM DTT] at 8°C. The resin was washed with 10 column volumes of buffer C, and CCT was eluted by application of buffer C supplemented with 15 mM ATP. Fractions containing CCT (determined by immunoblotting) were pooled, concentrated by ultrafiltration (Centricon 30, Amicon Inc., Beverly, MA, USA), and then applied to a Superose 6 column (HR 10/30, Pharmacia) equilibrated in buffer D [80 mM MES (pH 6.8) 1 mM EGTA, 1 mM $MgCl_2$, 1 mM DTT] at 4°C. Fractions with apparent molecular weights of 500–600 kDa, designated Nc CCT, were pooled and concentrated by ultrafiltration for subsequent study. The yield of CCT obtained was ~10 μg/ml extract, starting from 100 g of tissue (corresponding to about two testis) ground in 120 ml of buffer A. Other purification procedures were also tested to improve the yield of purified CCT without success.

SDS-polyacrylamide gel electrophoresis (PAGE) and immunoblotting

SDS-PAGE was performed by the method of Laemmli (1970). After electrophoresis, the gels (10% acrylamide) were blotted electrophoretically to nitrocellulose sheets (Scleicher and Schuell) as described previously (Pucciarelli et al. 1997). The blots were probed for the presence of the CCT α subunit using a rabbit polyclonal anti-hamster TCP-1α primary antibody (Melki et al. 1997) and peroxidase-conjugated goat anti-rabbit IgG secondary antibody (BioRad). Bound antibody was detected by Enzyme-Coupled Luminescence (ECL™, Amersham).

Characterization *of N. coriiceps* CCT by sedimentation velocity

Sedimentation velocity experiments on Nc CCT were carried out with a Beckman Optima XLA ultracentrifuge equipped with an AN 60Ti four-hole rotor and cells with two-channel 12 mm path length centerpieces. Measurements were made at 40,000 rpm and 8°C. Data were analyzed to provide the apparent distributions of sedimentation coefficients by means of the program DCDT (Stafford 1992).

Subcloning of the *N. coriiceps* β4-tubulin cDNA into the expression vector pET 11a

The *N. coriiceps* NcTbβ4-tubulin cDNA (Detrich et al. 2000) was transferred to the expression vector pET11a by PCR amplification of the coding sequence using primers that contained restriction sites for *Nde*I and *Bam*HI. The forward primer was 5′–TTGCAGCTCCATATGAGGGAA–3′, and the reverse primer was 5′–TAAAGGATCCTTTCACTCAAT–3′. The primers were designed to place an *Nde*I site immediately 5′ to the initiation codon ATG and a *Bam*HI site immediately 3′ to the stop codon TGA. The resulting construct, designated pET-β4, was sequenced [ABI Prism sequence analyzer Model 373A and Big Dye Terminator Methodology (PE Applied Biosystems)] to confirm that the NcTbβ4 coding sequence was placed in the proper reading frame for the heterologous expression in *Escherichia coli* (BL21 strain).

Preparation of labeled target proteins and in vitro folding assays

^{35}S-labeled, unfolded proteins, (NcTbβ4 tubulin, human β5 tubulin, and human β-actin) were expressed in *E. coli* and purified as inclusion bodies to perform folding reactions according to the protocol of Gao et al. (1992, 1993). Folding assays were performed in folding buffer (80 mM MES (pH 6.8), 1 mM EGTA, 1 mM $MgCl_2$, 1 mM ATP and 1 mM GTP), and the reaction products were analyzed on 4.5% non-denaturing polyacrylamide gels (Gao et al. 1993). Briefly, denatured target proteins (8 mg/ml) were diluted 100-fold in 20-μl folding buffer alone or in folding buffer supplemented with 2.5 μg CCT (*N. coriiceps* or rabbit). Rabbit Cof A was added to some reactions at final concentrations of 0.6–1 mg/ml.

The samples were incubated either at 30, at 20, or at 4°C for 90 min, the products were electrophoresed, and the gels were stained with Coomassie blue. After destaining, the gels were soaked in Amplify solution (Amersham), dried, and subjected to autoradiography. When present, the yield of 'native' products was estimated from radioactivity measurements to represent about 1% of the input substrates.

Preparation and use of the β-tubulin–chaperonin binary complex

Complexes of Nc CCT and ^{35}S-labeled NcTbβ4-tubulin were prepared by incubating a mixture of the former (2.5–6 μM, the molarity of Nc CCT is determined assuming a molecular weight of 400 kDa) and a 10-fold molar excess of the latter, in nucleotide-free folding buffer, for 30 min at 25°C. The NcTbβ4/Nc CCT complexes were then separated from unbound β4-tubulin chains by gel filtration on a Superose 6 column. The radioactive peak emerging at 670 kDa was concentrated to 2 mg/ml (2.4 μM) by centrifugal filtration (Centricon 30 filter units; Amicon Inc.). To evaluate the capacity of NcTbβ4 bound to Nc CCT to exchange into other empty chaperonins, NcTbβ4/Nc CCT complexes were incubated either with Cpn60 (5 mg/ml = 6 μM) or with rabbit CCT (5 mg/ml = 6 μM), in the absence or the presence of 1 mM ATP, at 30°C. Aliquots (16 μl) were withdrawn from each reaction at intervals (0.2, 1, 5, 20, and 45 min) and frozen on dry ice. The products formed during these incubations were analyzed by non-denaturing polyacrylamide gel electrophoresis.

ATPase activity measurements

Rates of ATP hydrolysis by *N. coriiceps* or rabbit CCTs (0.125 μM), either alone or after addition of denatured β-tubulin targets (100-fold dilutions of 25 μM target in 7.5 M urea), were measured in folding buffer at 30°C. Yields of acid-labile P_i were measured as described by Melki et al. (1990)

Cloning and sequencing of CCT β and θ cDNAs from *N. coriiceps*

CCT β and *θ* cDNAs from *N. coriiceps* were isolated by cross-hybridization to zebrafish CCT cDNAs/ESTs. The β-subunit cDNA was obtained from an *N. coriiceps* spleen cDNA library in λgt10 by probing 300,000 plaques with α ^{32}P-labeled zebrafish *CCT β* cDNA (generously provided by Dr. L.I. Zon, Childrens' Hospital, Boston) using methods described by Detrich et al. (2000). *N. coriiceps CCT θ* was purified from the same library by probing 120,000 plaques with a ^{32}P-labeled zebrafish *CCT-θ* clone (Open Biosystems I.M.A.G.E EST library). Phage inserts were sequenced in situ by the automated methods described above. The primary sequences of the encoded proteins were analyzed by use of the Clustal method provided by DNASTAR MegAlign. The sequences of the *CCT β and θ* cDNAs have been deposited in the GenBank™ database under the accession numbers AY823272 and AY823273, respectively.

Modeling of CCT subunit tertiary structures and other computational methods

The three-dimensional structures of *N. coriiceps* CCT β and θ subunits were modeled to the thermosome structure using the program SWISS-MODEL (Schwede et al. 2003; Guex and Peitsch 1997; Peitsch 1995). The isoelectric points of β tubulins were determined using WinPep (Hennig 1999).

Results

Distribution of CCT in *N. coriiceps* tissues

Figure 1 shows a Western blot of soluble proteins extracted from tissues of *N. coriiceps* after immunostaining with a polyclonal antibody to detect TCP-1α, a subunit proxy for the CCT complex. Muscle, testis, spleen, pronephric (head) kidney, and reticulocyte extracts each contained a single reactive protein of ~60 kDa, consistent with the presence of the CCT α subunit. Brain, by contrast, contained little TCP-1α. Because the testis of a sexually mature male can constitute 20% or more of the total body mass, we chose this tissue for the purification of *N. coriiceps* CCT.

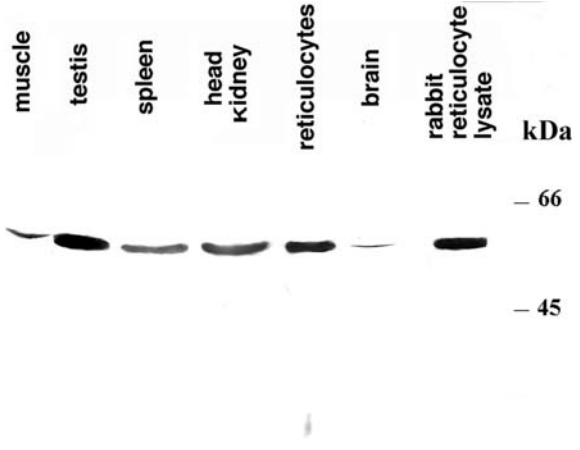

Fig. 1 Immunodetection of CCT α-subunit in *N. coriiceps* tissue extracts. *N. coriiceps* CCT was immunodetected by using a polyclonal rabbit anti-hamster TCP-1α primary antibody and a peroxidase-conjugated goat anti-rabbit IgG secondary. Equivalent amounts of each tissue (quantified by Bradford) were loaded. A sample of purified CCT from rabbit reticulocyte lysate was run in parallel as a control. The molecular weights of two protein standards (in kDa) are indicated on the *right*

Purification of CCT from *N. coriiceps* testis and estimation of its mass

Following chromatography on ion-exchange and ATP-affinity columns, final purification of *N. coriiceps* CCT was accomplished by gel filtration of a concentrated, CCT-enriched sample on a Superose 6 column (4°C). Figure 2a shows the elution profile and Fig. 2b the anti-TCP1-α immunoblots of fractions from the two peaks: the first peak eluted at an apparent molecular weight of ~400 kDa and the second at ~60 kDa. The additional bands that are stained by the antibody in Fig. 2b may be either degraded TCP-1α subunit, or other polypeptides that cross-react with the polyclonal antibodies used here. Fractions from the first peak (23–30) were pooled to yield the purified complex, designated Nc CCT. Figure 2c illustrates the subunit composition of the Nc CCT preparation determined by SDS-PAGE. Three protein bands with the molecular weights anticipated for CCT subunits, ~55–60 kDa (arrow), were detected; one of which is immunostained by anti-TCP1-α (Fig. 2d). By contrast, rabbit reticulocyte CCT typically contains five bands when examined by denaturing electrophoresis. Two other proteins with molecular weights of 62 and 40 kDa were also present in the preparation. They may correspond to contaminating proteins [the ~60 kDA

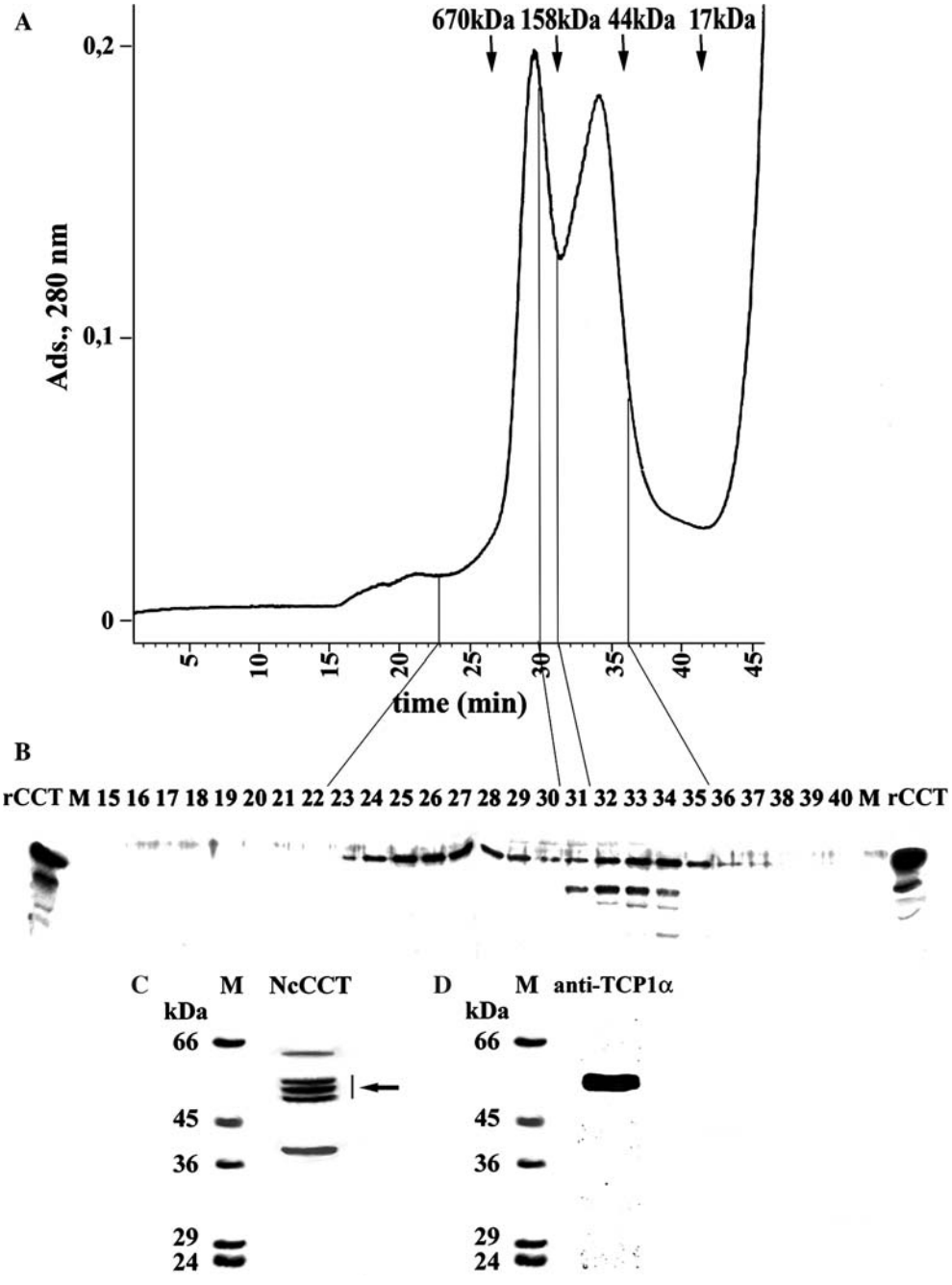

Fig. 2 Size exclusion chromatography of Nc CCT. Fractions containing CCT emerging from the ATP-agarose column were pooled, concentrated by ultrafiltration and then applied to a Superose 6 column at 4°C. **a** Chromatogram. *Arrowheads* show the positions of molecular size markers (thyroglobulin, 670 kDa; immunoglobulin G, 158 kDa; ovalbumin, 44 kDa; myoglobin, 17 kDa) run on the same column under identical conditions. **b** Immunoblot analysis of fractions from the Superose 6 column using the anti-TCP1-α antibodies. A sample of purified CCT from rabbit reticulocyte lysate (rCCT) was run in parallel as a control. **c** Protein composition of fractions 23–27 from the Superose 6 column (10% SDS-polyacrylamide gel), pooled and concentrated by ultrafiltration. The bands in the gel were revealed by silver staining. Molecular mass markers (in kDa) are shown on the *left*. **d** Immunoblot analysis of purified *N. coriiceps* CCT (shown in **c**), using a polyclonal rabbit anti-hamster TCP-1α and a peroxidase-conjugated goat anti-rabbit IgG

probably corresponds to fish equivalent of hsc or hsp 70, and/or Hop/p60 (Gebauer et al. 1998), which are known to co-purify with CCT], to the unusual behavior of one of CCT subunits, or to substrates that remained bound to Nc CCT during purification. Assuming that the 55–60 kDa proteins constitute the subunits of Nc CCT, we estimate the purity of the preparation to be ≥75%.

Sedimentation velocity analysis

To determine the oligomeric state of Nc CCT, we performed sedimentation velocity experiments to evaluate the apparent distribution of sedimentation coefficients, $g^*(s)$, of the preparation in the absence of free ATP (i.e., the ADP-bound CCT complex). Figure 3 shows that Nc CCT resolved into two boundaries, designated fast and slow, at 8°C. The experimental data were modeled to a non-interacting, two-component system (solid lines), yielding best-fit sedimentation coefficients of 14.4 and 26.6s in the proportions of 52 and 41%, respectively. The sedimentation coefficient of the faster species is similar to that of ADP-bound rabbit CCT (25.2 S extrapolated to zero concentration), which corresponds to the two-ring complex (Melki et al. 1997), whereas the sedimentation coefficient of the slower species is consistent with that expected for single ring particles (Viitanen et al. 1992). Although the boundaries were modeled as non-interacting, the absence of a plateau between them suggests that the double- and single-ring particles of Nc CCT may be in rapid and reversible equilibrium under these conditions. Confirmation of this hypothesis would require examination the concentration dependence of the $g^*(s)$ boundaries.

Folding capacity of expressed NcTbβ4 tubulin

The ability of NcTbβ4 tubulin to fold, either autonomously or by a CCT-assisted pathway, was determined by use of the components of the well-characterized rabbit reticulocyte CCT system. Labeled, denatured protein was diluted into folding buffer alone or in the presence of (1) purified rabbit CCT, (2) rabbit CCT in combination with rabbit Cof A, and (3) rabbit reticulocyte lysate (Fig. 4) at 30°C. NcTbβ4 alone did not fold spontaneously, but rather generated a series of high molecular weight oligomers (first lane, upper arrow), as does β tubulin (Fig. 4, lane 4). The addition of CCT led to the formation of an NcTbβ4/CCT complex (second

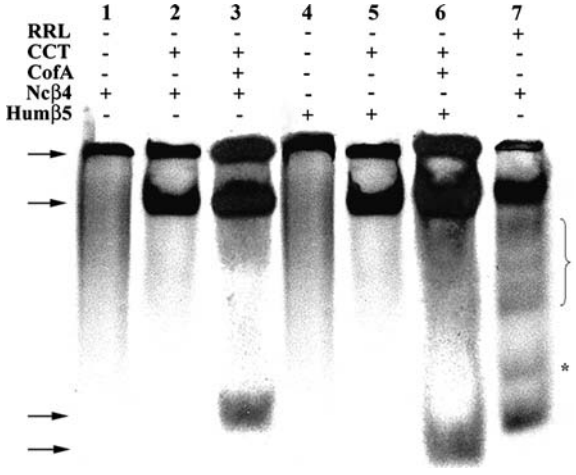

Fig. 4 NcTbβ4 tubulin: capacity for folding by a heterologous CCT. Folding of NcTbβ4 by the rabbit chaperonin, at 30°C. In vitro folding reactions of labeled denatured NcTbβ4 tubulin in the presence (+) or the absence (−) of purified rabbit CCT and Cof A, and in rabbit reticulocyte lysate (*lanes 1*, *2*, *3*, and *7*). For comparison, a folding reaction containing human β5 (Hum β5) tubulin and purified rabbit CCT and Cof A was run in parallel (*lanes 4*, *5*, and *6*). The *upper arrowhead* indicates the location of the series of high molecular weight β tubulins oligomers. The *second arrow* from the top indicates the NcTbβ4/CCT and Hum β5/CCT complexes. The *third arrowhead* the position of the β4/Cof A complex, and the *lower arrowhead* the location of the Hum β5/Cof A complex. The *asterisk* indicates the position of hybrid tubulin dimers composed of rabbit α chains and NcTbβ4 tubulin. The *bracket* on the *right side* of the gel indicates additional intermediates that probably correspond to NcTbβ4 in association with other cofactors (Tian et al. 1999)

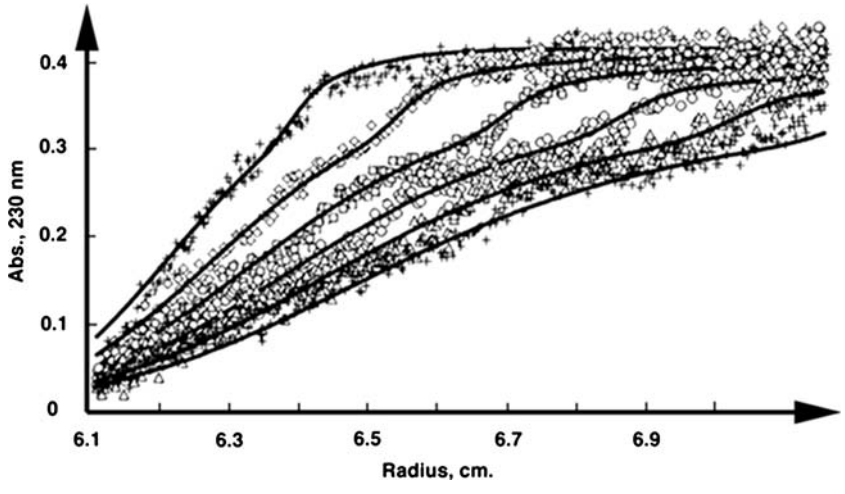

Fig. 3 Sedimentation behavior of CCT from *N. coriiceps*. Typical sedimentation velocity data for Nc CCT (2.5 μM), at 20°C. The positions of the moving boundaries were recorded at 5 min intervals by spectrophotometric scanning at 230 nm. The *solid lines* are best fits of the experimental data to a two-component model (see Results)

lane, second arrow from the top) in a manner similar to what is observed in the case of $\beta 5$ tubulin (Fig. 4, lane 5, and Melki and Cowan 1994). When CCT was combined with Cof A, NcTb$\beta 4$ folded to a quasi-native state bound to the cofactor (third lane, third arrow from the top) (cf. Melki et al. 1996). Similarly, human $\beta 5$ tubulin, rabbit CCT, and Cof A formed a human $\beta 5$/Cof A product, but one of higher mobility (lane 6, lower arrow). The decreased mobility of the NcTb$\beta 4$/Cof A complex was most likely due to the more basic isoelectric point of NcTb$\beta 4$ tubulin (pI = 4.71) relative to that of human $\beta 5$ tubulin (pI = 3.91). In rabbit reticulocyte lysate, NcTb$\beta 4$ was able to mature to a native conformation such that the folded subunits exchanged with endogenous rabbit β chains to yield labeled, $\alpha\beta$ tubulin heterodimers (Fig. 4, lane 7, asterisk). Additional intermediates that probably corresponded to NcTb$\beta 4$ in association with other cofactors (Tian et al. 1999) were also observed (indicated with the bracket on the right side of the gel). Together, these observations demonstrate that CCT and its co-factors are required to fold NcTb$\beta 4$ tubulin to a native tertiary structure that is competent to form tubulin dimers with heterologous α-chain partners.

Folding activity of Nc CCT: extracts and purified complex

When folding reactions were performed with *N. coriiceps* testis supernatant extracts at 4 and 20°C, the CCT and co-factors in situ were able to fold NcTb$\beta 4$ and human $\beta 5$ tubulins to their native conformations (Fig. 5a, middle and lower arrows, respectively). To characterize the functional properties of purified Nc CCT, we performed reconstituted in vitro folding reactions at 4 and 20°C with denatured NcTb$\beta 4$ and human $\beta 5$ tubulins as substrates. Control reactions with rabbit CCT were run in parallel.

Figure 5b shows that Nc CCT bound each of the tubulins at both temperatures (cf. bands labeled by upper arrow, lanes 3–12, and 15–24). However, Nc CCT did not produce additional complexes corresponding to quasi-native β-tubulin bound to Cof A, in striking contrast to the results obtained with rabbit CCT (Fig. 5b, bands labeled with arrow and bracket, lanes 7 vs. 8, and 9 vs. 10). The same result was obtained when folding of β-tubulins was performed in the absence of Cof A (lanes 3–6), and when the reactions were incubated at 4°C (lane 19 vs. 20 and 21 vs. 22). Two potential explanations might account for these observations: (1) Nc CCT binds the unfolded protein chains non-specifically and sequesters them; or (2) Nc CCT requires homologous *N. coriiceps* Cof A or another factor to produce the quasi-native β-tubulin/Cof A complex. We examined the specificity of binding by incubating Nc or rabbit CCT with denatured human actin. In contrast to the case for tubulins, Nc CCT bound human actin very weakly, whereas rabbit CCT bound the heterologous

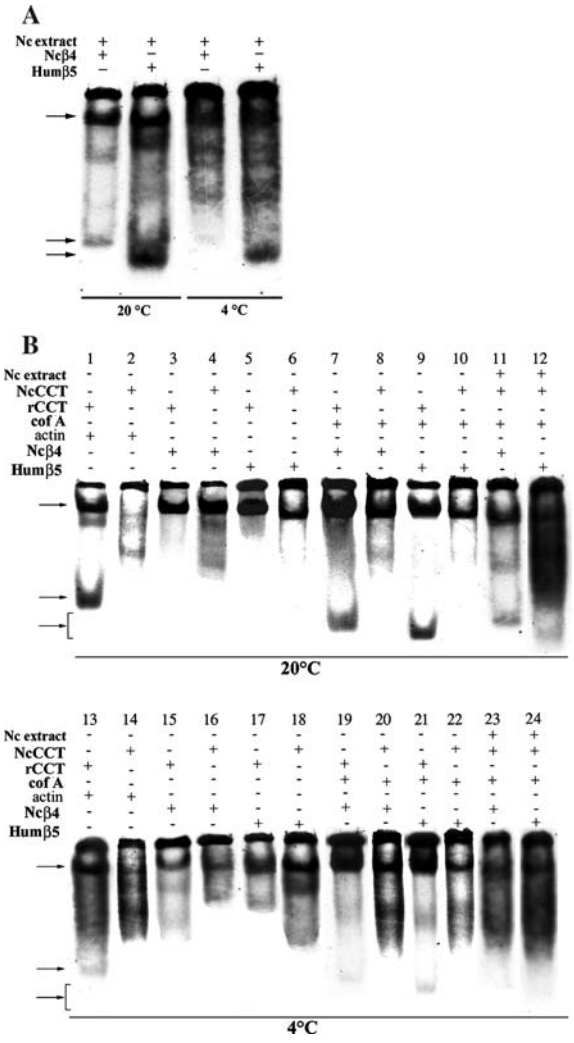

Fig. 5 *N. coriiceps* CCT: Functional Properties. **a** In vitro folding of denatured NcTb$\beta 4$ and Hum $\beta 5$ tubulins by clarified *N. coriiceps* testis extracts, at 20 and 4°C. The *upper arrowhead* indicates the location of NcTb$\beta 4$/CCT and Hum $\beta 5$/CCT complexes; the *middle and lower arrowheads* show the locations of folded NcTb$\beta 4$ and Hum $\beta 5$ (i.e., NcTb$\beta 4$/α-tubulin and Hum $\beta 5$/α-tubulin dimers), respectively. **b** Folding activity of purified Nc CCT. In vitro folding reactions containing labeled denatured actin (*lanes 1*, *2*, *13*, and *14*), NcTb$\beta 4$ (Nc$\beta 4$, *lanes 3*, *4*, *7*, *8*, *11*, *15*, *16*, *19*, *20*, and *23*) or human $\beta 5$ (Hum $\beta 5$, *lanes 5*, *6*, *9*, *10*, *12*, *17*, *18*, *21*, *22*, and *24*) tubulins in the presence (+) or the absence (−) of purified CCT from rabbit (rCCT) or *N. coriiceps* (Nc CCT) at 20 and 4°C. Rabbit Cof A was present as indicated. The *upper*, *middle*, and *lower arrows* indicate the location of actin–CCT or β-tubulin–CCT complexes, folded actin, and NcTb$\beta 4$/Cof A or Hum $\beta 5$/Cof A complexes, respectively

substrate strongly (compare the bands denoted by the upper arrow in lanes 2 and 1, and 13 and 14, respectively). We conclude that Nc CCT does not form non-specific complexes with unfolded proteins but rather is likely to require a factor that is absent from the reconstituted folding reactions to release folded products.

To test this hypothesis we supplemented the folding assays in lanes 8, 10, 20 and 22 with testis extracts. The results are presented in lanes 11, 12, 23 and 24 in Fig. 5b.

In the reactions performed a 20°C, we obtained an additional complex corresponding to quasi-native β-tubulin bound to Cof A (lanes 11 and 12), as fishes such as *Danio rerio* have in their genome a gene homologous to mouse cofactor A (GenBankTM database accession number BC046032, 75.9% of identity).

A trigger factor may be required to release folded tubulin from the Nc CCT/β-tubulin complex

Clearly, folding reactions reconstituted in vitro from purified Nc CCT and rabbit Cof A lacked some component that is essential to release folded β-tubulin from the chaperonin. In light of our understanding of the mechanism of binding and release of proteins by CCT, we propose that this "trigger" factor is either a nucleotide exchange factor that facilitates target release by conformational changes associated with ATP hydrolysis and ADP exchange or a specific target protein release factor that reduces target affinity for CCT. The first possibility was tested by measurement of the ATPase activity of Nc CCT in the absence (▲) or with the presence of added unfolded tubulin (Δ) at 30°C (Fig. 6a). In both cases, the kinetics of ATP hydrolysis were identical and biphasic, with an initial burst of ATPase activity (of unknown origin) followed by a slower, steady-state rate that matched that of rabbit CCT in the presence (□) or absence (■) of unfolded tubulin. The specific ATPase activity of Nc CCT is 1–2 min–1, similar to the ATPase activity calculated for rabbit CCT (Melki and Cowan 1994). The capacity of Nc CCT to hydrolyze ATP indicates that the complex retains activity and is unlikely to require an ATP/ADP exchange factor to facilitate its folding activity.

The second possibility, that a release factor is required for Nc CCT function, was evaluated by challenging NcTbβ4/Nc CCT complexes to exchange their tubulin chains with other empty (i.e., target protein-free) chaperonins at 20°C. The NcTbβ4/Nc CCT complex (2.4 μM) was incubated at intervals from 12 s to 45 min in the presence of ATP (1 mM) and 2.5-fold molar excesses of either Cpn60 (6 μM), a GroEL-like chaperone, or rabbit CCT (6 μM) (Fig. 6b, c). Although Cpn60 and rabbit CCT were able to bind NcTbβ4 tubulin under these conditions (see control reactions), the Nc CCT-bound NcTbβ4 chain did not exchange with either of the competitor chaperonins within the time period of the experiment. The ability of Nc CCT in testis extracts to facilitate the formation of tubulin dimers containing

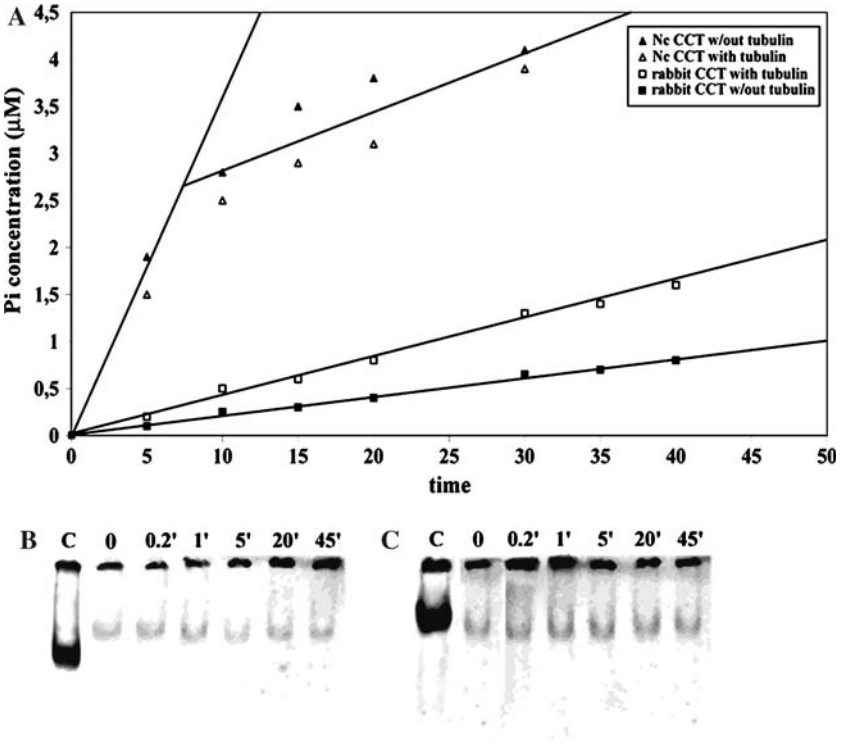

Fig. 6 *N. coriiceps* CCT: ATPase activity and substrate exchange reactions. **a** Measurement of the ATPase activity of Nc CCT, at 30°C. The kinetics of ATP hydrolysis by Nc CCT in the presence (Δ) or the absence (▲) of unfolded β-tubulin and that of rabbit CCT in the presence (□) or absence (■) of β5-tubulin were measured by extraction of acid labile phosphate. **b**, **c** Exchange reactions. The NcTbβ4/Nc CCT complex was incubated with Cpn60 (6 μM) (**b**) and rabbit CCT (6 μM), 20°C (**c**). Aliquots were withdrawn from the reactions at the times indicated, frozen on dry ice, and analyzed by non-denaturing SDS polyacrylamide gel electrophoresis. Time 0 shows the NcTbβ4/Nc CCT complex before incubation with Cpn60 or rabbit CCT. Control reactions, in which rabbit CCT or Cpn60 were incubated with denatured NcTbβ4, were electrophoresed in parallel to show the positions of the NcTbβ4/Cpn60 and NcTbβ4/CCT complexes, respectively

NcTbβ4 (Fig. 5a) and the inability of Nc CCT-bound NcTbβ4 tubulin to exchange into other chaperonins in purified, reconstituted systems, taken together, support the hypothesis that a target release factor is needed to dissociate the NcTbβ4 chain from Nc CCT. Determination of the nature of this release factor must await further experimentation.

Sequence analysis of *N. coriiceps* CCT β and θ subunits

In addition to the putative release factor, the functionality of Nc CCT at low temperature almost certainly reflects the primary structures of its constituent subunits. As a first step toward elucidation of the structure/function relationships of *N. coriiceps* CCT, we have cloned and sequenced cDNAs that encode the β and θ subunits. A structure-based alignment of the two subunits from *N. coriiceps*, two fishes that live at a different temperature, e.g., zebrafish and *Tetraodon nigroviridis*, and mouse, with thermosome subunits is presented in Fig. 7. Thermosome is the archaeal chaperonin, homologous to the eukaryotic CCT (Ditzel et al. 1998). Residue substitutions unique to the *N. coriiceps* subunits are shown in red. The sequence identity of the *N. coriiceps* β subunit to these reference sequences was 89, 91, and 86%, respectively, and the identity of the Antarctic fish θ chain to its referents was 87, 86, and 81%. Clearly, the *N. coriiceps* CCT subunits have diverged substantially from those of other vertebrates, including two representative teleost fishes.

Four categories of residue substitutions were found in the two *N. coriiceps* CCT subunits: (1) Pro → Ala replacements (e.g., equatorial residue 429 and 144 of the β and θ subunit, respectively); (2) charged residue → Ala substitutions (positions 154 and 177 of the β subunit and 141 of the θ chain); (3) bulky/polar residues → Gly/Ala [position 474 of β, and 201 of θ (plus Gly insertions at positions 475 and 538 of β and θ, respectively)]; and (4) Leu/Ile → Val (positions 86, 448 and 508 of θ). The first three categories would be expected to increase intramolecular flexibility within the CCT complex, as has been observed for cold-adapted variants of several proteins (Fields and Somero 1998; Alimenti et al. 2003; Pucciarelli et al. 2005). Furthermore, these substitutions are similar to those that transform mesophilic subtilisin-like proteases into cold-active variants (Wintrode et al. 2000). The fourth group, Leu/Ile → Val, would decrease hydrophobicity within the equatorial domain of the θ subunit.

Figure 8 places the amino acid sequence changes of the *N. coriiceps* β and θ subunits in the context of their three-dimensional structures, which have been modeled on the thermosome subunits. Based on the structure of the thermosome particle (Ditzel et al. 1998), β-chain substitutions E/D58Q, T/A/S60L and D177A and θ-chain replacements I84V (vertebrate residue(s)/position/Nc subunit residue) were located in the regions involved in *intra-ring* contacts, and the chemistry of these substitutions may reduce intrachain hydrogen bonding and salt-bridge formation (Feller and Gerday 1997) while modestly increasing hydrophobicity, with the net effect being an increase in molecular flexibility. The substitutions P429A, N/I/T474G, and (-)475G (Gly insertion) in CCT β and A451S in CCT θ were located in regions that are directly involved in, or close to, *inter-ring* contacts between CCT subunits. These, too, would be expected to introduce structural flexibility, possibly facilitating intersubunit conformational adjustments associated with the CCT folding cycle and/or decreasing the strength of the *inter-ring* association. A potentially significant residue change was found in the θ-chain "flip loop," which occupies the triphosphate-binding site in the absence of nucleotide: Val 86, may favor the conformational change of the subunit's "flip loop" from a relaxed structure to a helical state upon ATP binding (Ditzel et al. 1998). When compared to the tertiary structure of the apical domain of the CCT γ subunit (Pappenberger et al. 2002), the substrate-binding surfaces of β and θ contained substitutions that would likely alter the non-covalent interactions between the chaperonin and target proteins. For example, θ-chain Ile 231, located in the loop between strands S9 and S10, increased the hydrophobicity of the substrate-binding cleft, and Asp 309 of the θ subunit replaced a basic residue. One may conjecture that these changes alter the affinity of Nc CCT for target proteins such that binding is facilitated at low temperature.

Discussion

In this report, we describe the purification of the chaperonin CCT from a cold-living Antarctic fish, *N. coriiceps*, and characterize partially its functional and structural properties. We find that Nc CCT in testis extracts was able to fold denatured β-tubulins from homologous (*N. coriiceps*) or heterologous (human) sources to yield the protein in its native conformation. Purified Nc CCT bound denatured β-tubulin but, when reconstituted with rabbit Cof A, failed to release the protein to yield the tubulin/cofactor intermediate. Challenge of the NcTbβ4/Nc CCT complex with 2.5-fold greater concentrations of Cpn60 or rabbit CCT did not yield target exchange to the empty chaperonins, which suggests that the affinity of Nc CCT for its targets is high. The most plausible explanations of these results are that Nc CCT (1) either requires a novel release or trigger factor, or (2) its homologous Cof A (either of which were present in testis extracts but lost during purification of CCT) to displace target proteins so they may proceed through the remainder of the folding cycle. Finally, we found that the amino acid sequences of the CCT β and θ chains contained residue substitutions in the equatorial, apical, and intermediate domains that are consistent with increased subunit flexibility, which is a characteristic property of most cold-adapted proteins (Fields and Somero 1998; Detrich et al. 2000; Feller and Gerday 1997).

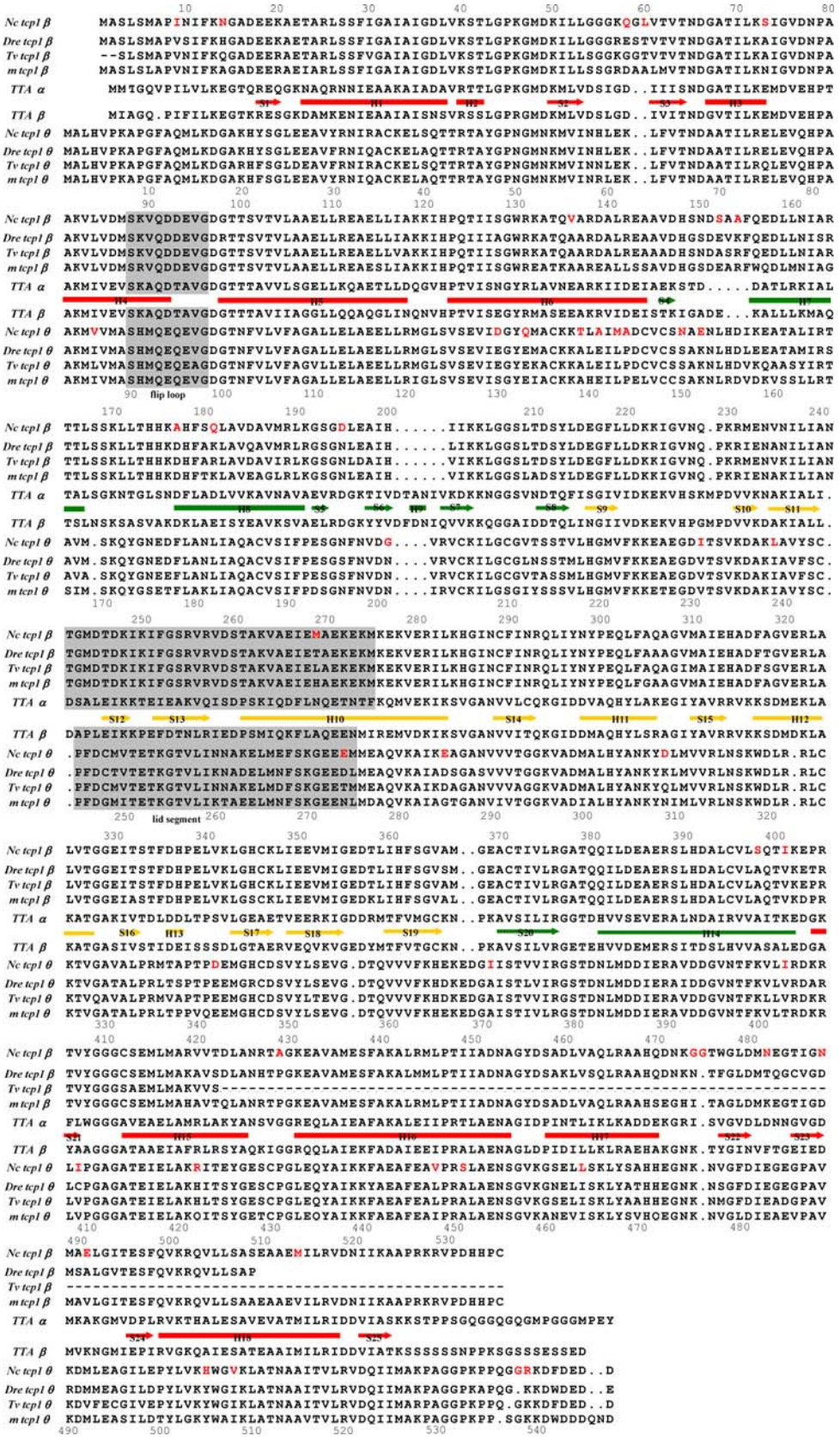

Fig. 7 Sequences of CCT β and θ from *N. coriiceps*. The amino acid sequences of the CCT β and θ subunits of *N. coriiceps* (*Nc tcp1* β and *Nc tcp1* θ) are aligned relative to those of *Danio rerio* (*D.re tcp1* β and *D.re tcp1* θ, acc. nos. AF506226 and AAH50492, respectively), *Tetraodon nigroviridis* (*Tn tcp1* β and *Tn tcp1* θ, acc. nos. CAF90004 and CAG00493), and mouse (*m tcp1* β and *m tcp1* θ, acc. nos. P80314 and NP033970). Predicted secondary structural elements correspond to those of the thermosome subunits (TTA α and TTA β) (Ditzel et al. 1998), and the colors indicate the three domains of each subunit: equatorial (*red*), intermediate (*green*), and apical (*yellow*). Residue substitutions of the Nc CCT β and θ subunits that differ with respect to the reference sequences are shown in *red*. Sequence elements that form the "flip loop" and the helical protrusion of the lid segment are highlighted in *gray*

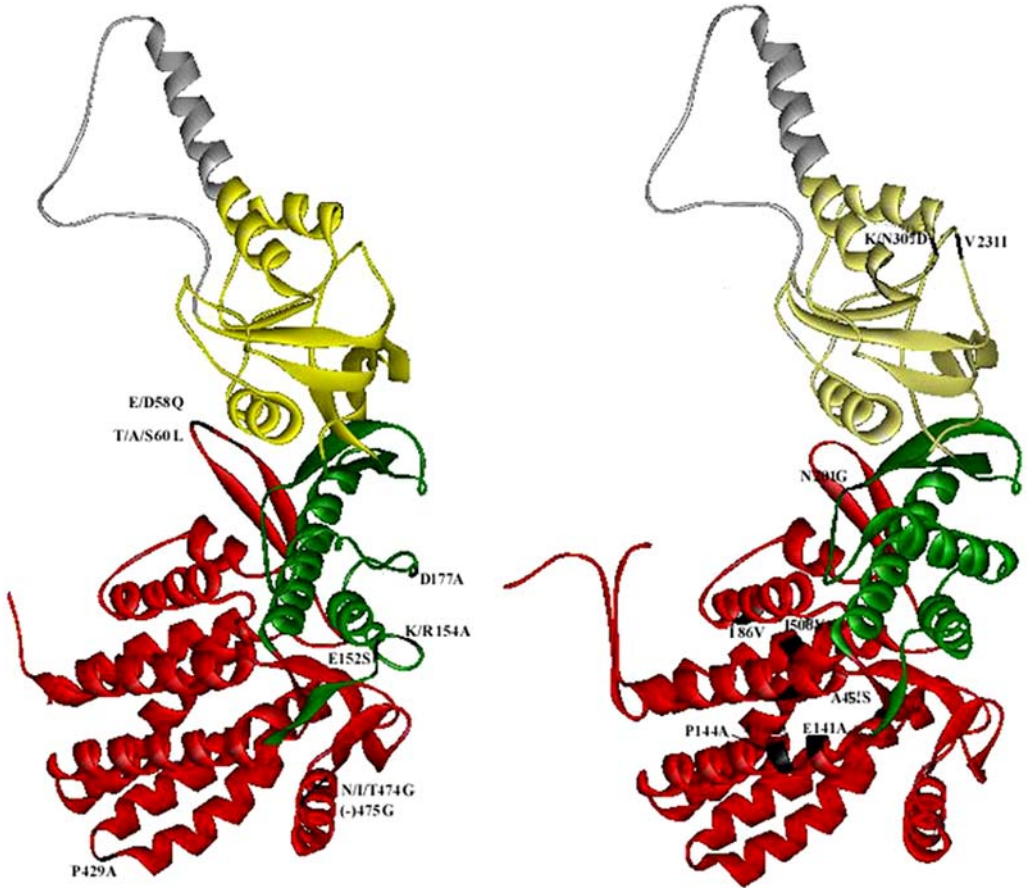

Fig. 8 Tertiary structures and residue substitutions of the Nc CCT β and θ subunits. The three structural domains of the β (*left panel*) and the θ (*right panel*) chains are colored according to the tertiary structure of the thermosome subunits (Ditzel et al. 1998): *red* corresponds to the equatorial domain, *green* the intermediate, and *yellow* the apical. The helical protrusion of the apical domain is shown in *gray*. Substitutions in the Nc CCT β and θ subunits (see Fig. 7) are labeled in *black*, where each *number* refers to the sequence position that is interested by the substitution and the *letters* that precede and follow the number stand for amino acids present in the reference sequences and in Nc *CCT* β and θ respectively. The figure was prepared with DS ViewerPro5.0

Chaperonin-mediated protein folding in an extreme cold environment

The suite of amino acid substitutions observed in the CCT β and θ chains are consistent with the evolution of polypeptide flexibility to facilitate efficient folding activity in the cold. For example, increased mobility in the CCT equatorial domain, which contains the ATP binding site and is involved in the intra- and inter-ring contacts, may reduce the activation energy for the structural rearrangements that underlie allosteric communication between subunits during folding. Some of these residue changes might also decrease the association constant for ring–ring interaction, thus explaining the observation of both single- and double-ring CCT particles. Greater flexibility in the apical domain, which is involved in recognition of non-native substrates, may facilitate the clockwise rotation of the helical protrusion that closes the folding chamber around the target protein. Those substitutions present in the intermediate domain may enhance its performance as the hinge that connects the equatorial and apical domains. Why, then, is the apparent affinity of Nc CCT for NcTbβ4 tubulin in vitro so large and, with the exception of testis extracts, the yield of folded product negligible? One possibility is that the CCT apical domain/target interactions are quite strong due to residue changes in the apical substrate-binding cleft and perhaps in the target itself. (Only two of the eight CCT subunits have been sequenced, so our understanding of apical domain/target association in this system is incomplete.) Thus, the presence of homologous Cof A in the folding reaction may be critical to release a folded product. Alternatively, we propose that a critical cofactor, perhaps novel to cold-adapted notothenioid fishes, may be necessary to release the target protein from Nc CCT. Possible cofactor candidates include (1) Hop/p60 (Gebauer et al. 1998), which has been shown to interact with CCT and to increase the rate of ATP dissociation from CCT; (2) phosducin-like proteins, which in several recent papers have been shown to act as bona fide CCT co-factor proteins (McLaughlin et al. 2002; Martin-Benito et al. 2004; Stirling et al. 2006); (3) GimC/prefoldin (Vainberg et al. 1998), which increases by 5-fold the rate of actin

release from CCT and is essential for yeast cell growth at low temperatures (Geissler et al. 1998; Siegert et al. 2000). It is worth noting that GimC/prefoldin is the most likely candidate for facilitating the release of substrates from NcCCT. Indeed, Siegert et al. 2000 have shown in vivo that CCT–actin complexes are longer lived in yeast lacking a functional prefoldin complex, which support the notion that prefoldin facilitates the efficient transit of substrate through the CCT system into a folded state. Actually, fishes such as *Danio rerio* have in their genome two genes homologous to mouse prefoldin 2 and 5 (GenBank^{TM} database accession numbers NP_001003754 and XP_695628, respectively, 61.7 and 76.5% of identity, respectively)

A second possibility posits that the failure of Nc CCT to release folded product results from binding of actin or tubulin chains in a conformation that is not permissive for further folding. In this case, a novel co-chaperone may be necessary to convert the CCT-bound target to a conformation conducive to folding by the chaperonin complex. Fractionation of the *N. coriiceps* testis extract and reconstitution of a folding-competent system in vitro will be necessary to test these possibilities, thus expanding our knowledge of assisted protein folding in cold-living ectotherms.

Protein flexibility and function at low temperature: a two-edged sword?

The importance of polypeptide flexibility to the efficient function of psychrophilic proteins has emerged as a consensus hypothesis among polar biochemists and physiologists (NRC 2003). In general, increased flexibility appears to be mediated by the substitution and/or incorporation of structure-destabilizing amino acids and by decreases in the number and strength of weak intramolecular bonds in these proteins. Yet flexibility also increases the likelihood that the proteins of marine ectotherms may undergo denaturation in response to cold as a stressor (cf. Place et al. 2004; Place and Hofmann 2005, and references therein). Thus, positive selection for protein flexibility in the chronically cold-living Antarctic notothenioid fishes and the concomitant threat of cold-induced protein denaturation may have driven the evolutionary conversion of the ''inducible' *hsp70* gene to a constitutive role in vivo. We propose that the CCT of notothenioids has evolved its novel properties due to similar countervailing evolutionary pressures. Might it be possible that β-tubulin or other CCT targets have co-evolved to bind tightly to Nc CCT to sequester the latter, thus protecting it from cold denaturation and proteosome-mediated degradation? If so, the concerted evolution of a release factor or co-chaperone would be necessary to maintain the functionality of the flexible, cold-adapted CCT complex.

Acknowledgements We acknowledge with gratitude the excellent logistic support provided to our Antarctic field research program, performed at Palmer Station and on the seas of the Palmer Archipelago, by the staff of the Office of Polar Programs of the National Science Foundation, by the personnel of Antarctic Support Associates and Raytheon Polar Services Company, and by the captain and crew of the R/V Laurence M. Gould. We thank Patricia Singer (University of Maine DNA Sequencing Facility) for her excellent performance of the automated DNA sequencing. This work was supported by National Science Foundation grants OPP-9815381, OPP-0089451, OPP-0336932, and the Association pour la Recherche sur le Cancer.

References

Alimenti C, Ortenzi C, Carratore V, Luporini P (2003) Structural characterization of En-1, a cold-adapted protein pheromone isolated from the Antarctic ciliate *Euplotes nobilii*. Biochimica Biophysica Acta 1621:17–21

Billger M, Wallin M, Williams RC Jr, Detrich HW III (1994) Dynamic instability of microtubules from cold-living fishes. Cell Motil Cytoskeleton 28:327–332

Detrich HW, Parker SK, Williams RC, Nogales E, Downing KH (2000) Cold adaptation of microtubule assembly and dynamics: structural interpretation of primary Sequence changes present in the α- and β-tubulins of Antarctic fishes. J. Biol Che. 275:37038–37048

DeWitt HH (1971) Coastal and deep-water benthic fishes of the Antarctic. In: Bushnell VC (ed) Antarctic Map Folio Series, Folio 15. American Geographical Society, New York, pp 1–10

Ditzel L, Lowe J, Stock D, Stetter KO, Huber H, Huber R, Steinbacher S (1998) Crystal structure of the thermosome, the archaeal chaperonin and homolog of CCT. Cell 93:125–138

Eastman JT (1993) Antarctic fish biology: evolution in a unique environment. Academic, San Diego, p 322

Feller G, Gerday C (1997) Psychrophilic enzymes: molecular basis of cold adaptation. Cell Mol Life Sci 53:830–841

Fields PA, Somero GN (1998) Hot spots in cold adaptation: localized increases in conformational flexibility in lactate dehydrogenase A4 orthologs of Antarctic notothenioid fishes. Procl Natl Acad Sci USA 95:11476–11481

Frydman J, Nimmesgern E, Erdjument-Bromage H, Wall JS, Tempst P, Hartl FU (1992) Function in protein folding of TRiC, a cytosolic ring complex containing TCP-1 and structurally related subunits. EMBO J 11:4767–4778

Gao Y, Thomas JO, Chow RL, Lee GH, Cowan NJ (1992) A cytoplasmic chaperonin that catalyzes beta actin folding. Cell 69:1044–1050

Gao Y, Vainberg IE, Chow RL, Cowan NJ (1993) Two cofactors and cytoplasmic chaperonin are required for the folding of alpha- and beta-tubulin. Mol Cell Biol 13:2488–2485

Gao Y, Melki R, Walden PD, Lewis SA, Ampe C, Rommelaere H, Vandekerckhove J, Cowan NJ (1994) A novel cochaperonin that modulates the ATPase activity of cytoplasmic chaperonin J Cell Biol 125:989–996

Gebauer M, Melki R, Gehring U (1998) The chaperone cofactor Hop/p60 interacts with the cytosolyc chaperonin-containing TCP-1 and affects its nucleotide exchange and protein folding activities. J Biol Chem 273:29475–29480

Geissler S, Siegers K, Schiebel E (1998) A novel protein complex promoting formation of functional alpha and gamma-tubulin. EMBO J 17:952–966

Guex N, Peitsch MC (1997) SWISS-MODEL and the Swiss-Pdb-Viewer: An environment for comparative protein modelling. Electrophoresis 18:2714–2723

Hartl FU (1996) Molecular chaperones in cellular protein folding. Nature 381:571–579

Haschemeyer AEV (1983) A comparative study of protein synthesis in nototheniids and icefish at Palmer Station, Antarctica. Comp Biochem Physiol B 76:541–543

Hennig L (1999) WinGene/WinPep: user-friendly software for the analysis of aminoacid sequences. BioTechniques 26:1170–1172

Kubota H, Hynes G, Willison K (1995) Multisubunit machinery assisting in protein folding and assembly in the eukaryotic cytosol. Eur J Biochem 230:3–16

Kusmierczyk AR, Martin J (2001) Chaperonins-keeping a lid on folding proteins. FEBS Lett 505:343–347

Laemmli UK (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 15:680–685

Llorca OE, McCormack A, Hynes G, Grantham J, Cordell J, Carrascosa JL, Willison KR, Fernandez JJ, Valpuesta JM (1999) Eukaryotic type II chaperonin CCT interacts with actin through specific subunits. Nature 412:693–696

Llorca O, Martin-Benito J, Ritco-Vonsovici M, Willison KR, Carrascosa JL, Valpuesta JM (2000) Analysis of the interaction between the eukaryotic chaperonin CCT and its substrates actin and tubulin. EMBO J. 15:5971–5979

Llorca O, Martin-Benito J, Ritco-Vonsovici M, Grantham J, Hynes GM, Willison KR, Carrascosa JL, Valpuesta JM (2001a) Eukaryotic chaperonin CCT stabilizes actin and tubulin folding intermediates in open quasi-native conformations. EMBO J 20:4165–4175

Llorca O, Martin-Benito J, Gomez-Puertas P, Ritco-Vonsovici M, Willison KR, Carrascosa JL, Valpuesta JM (2001b) Analysis of the interaction between the eukaryotic chaperonin CCT and its substrates actin and tubulin. J Struct Biol 135:205–218

Marsh AG, Maxson RE Jr, Manahan DT (2001) High macromolecular synthesis with low metabolic cost in Antarctic sea urchin embryos. Science 291:1950–1952

Martin-Benito J, Bertrand S, Hu T, Ludtke PJ, McLaughlin JN, Willardson BM, Carrascosa JL, Valpuesta JM (2004) Structure of the complex between the cytosolic chaperonin CCT and phosducin-like protein. Proc Natl Acad Sci USA 101(50):17410–17745

McLaughlin JN, Thulin CD, Hart SJ, Resing KA, Ahn NG, Willardson BM (2002) Regulatory interaction of phosducin-like protein with the cytosolic chaperonin complex. Proc Natl Acad Sci USA 99(12):7962–7967

Melki R (2001) Nucleotide-dependent conformational changes of the chaperonin containing tcp-1. J Struct Biol 135:170–175

Melki R, Cowan NJ (1994) Facilitated folding of actins and tubulins occurs via a nucleotide-dependent interaction between cytoplasmic chaperonin and distinctive folding intermediates. Mol Cell Biol. 14:2895–2904

Melki R, Carlier MF, Pantaloni D (1990) Direct evidence for GTP and GDP-Pi intermediates in microtubule assembly. Biochemistry 29:8921–8932

Melki R, Rommelaere H, Leguy R, Vandekerckhove J, Ampe C (1996) Cofactor A is a molecular chaperone required for beta-tubulin folding: functional and structural characterization. Biochemistry 35:10432–10445

Melki R, Batelier G, Soulie S, Williams Jr RC (1997) Cytoplasmic chaperonin containing TCP-1: structural and functional characterization. Biochemistry 36:5817–5826

NRC (National Research Council) (2003) Frontiers in polar biology in the genomic era. National Academy Press, Washington

Pappenberger G, Wilsher JA, Roe SM, Counsell DJ, Willison KR, Pearl LH (2002) Crystal structure of the CCTgamma apical domain: implications for substrate binding to the eukaryotic cytosolic chaperonin. J Mol. Biol. 318:1367–1379

Peck LS, Clark MS, Clarke A, Cockell CS, Convey P, Detrich HW III, Fraser KPP, Johnston IA, Methe BA, Murray AE, Römisch K, Rogers AD (2004) Polar Biol 28:351–365

Peitsch MC (1995) Protein modeling by E-mail. Biotechnology 13:658–660

Place SP, Hofmann GE (2005) Constitutive expression of a stress-inducible heat shock protein gene, hsp70, in phylogenetically distant Antarctic fish. Pol Biol 28:261–267

Place SP, Zippay ML, Hofmann GE (2004) Comparison of Hsc70 orthologues from polar and temperate notothenioid fishes: differences in the prevention of aggregation and refolding of denatured proteins Am. J Physiol Regul Integr Comp Physiol 287:R429–R436

Privalov PL (1990) Cold denaturation of proteins. Crit Rev Biochem Mol Biol 25:281–305

Pucciarelli S, Ballarini P, Miceli C (1997) Cold-adapted microtubules: characterization of tubulin posttranslational modifications in the Antarctic ciliate *Euplotes focardii*. Cell Motil Cytoskeleton 38:329–341

Pucciarelli S, Miceli C, Melki R (2002) Heterologous expression and folding analysis of a beta-tubulin isotype from the Antarctic ciliate *Euplotes focardii*. Eur J Biochem 269:6271–6277

Pucciarelli S, Marziale F, Di Giuseppe G, Barchetta S, Miceli C (2005) Ribosomal cold-adaptation: characterization of the genes encoding the acidic ribosomal P0 and P2 proteins from the Antarctic ciliate *Euplotes focardii*. Gene 360(2):103–110

Schwede T, Kopp J, Guex N, Peitsch MC (2003) SWISS-MODEL: an automated protein homology-modeling server. Nucleic Acids Res 31:3381–3385

Siegert R, Leroux MR, Scheufler C, Hartl FU, Moarefi I (2000) Structure of the molecular chaperone prefoldin: unique interaction of multiple coiled coil tentacles with unfolded proteins. Cell. 103(4):621–632

Spiess C, Meyer AS, Reissmann S, Frydman J (2004) Mechanism of the eukaryotic chaperonin: protein folding in the chamber of secrets. Trends Cell Biol 14:598–604

Stafford WF III (1992) Boundary analysis in sedimentation transport experiments: a procedure for obtaining sedimentation coefficient distributions using the time derivative of the concentration profile. Anal Biochem. 203:295–301

Stirling PC, Cuellar J, Alfaro GA, El Khadali F, Beh CT, Valpuesta JM, Melki R, Leroux MR (2006) PhLP3 modulates CCT-mediated actin and tubulin folding via ternary complexes with substrates. J Biol Chem 281(11):7012–7021

Swezey RR, Somero GN (1982) Polymerization thermodynamics and structural stabilities of skeletal muscle actins from vertebrates adapted to different temperatures and hydrostatic pressures. Biochemistry 21:4496–4503

Tian G, Bhamidipati A, Cowan NJ, Lewis SA (1999) Tubulin folding cofactors as GTPase-activating proteins. GTP hydrolysis and the assembly of the α/β tubulin heterodimer. J Biol Chem 274:24154–24158

Vainberg IE, Lewis SA, Rommelaere H, Ampe C, Vandekerckhove J, Klein HL, Cowan NJ (1998) Prefoldin, a chaperone that delivers unfolded proteins to cytosolic chaperonin. Cell 93:863–873

Valpuesta JM, Martin-Benito J, Gomez-Puertas P, Carrascosa JL, Willison KR (2002) Structure and function of a protein folding machine: the eukaryotic cytosolic chaperonin CCT FEBS Lett. 529:11–16

Viitanen PV, Lorimer GH, Seetharam R, Gupta RS, Oppenheim J, Thomas JO, Cowan NJ (1992) Mammalian mitochondrial chaperonin 60 functions as a single toroidal ring J. Biol Chem 267:695–698

Wintrode PL, Miyazaki K, Arnold FH (2000) Cold adaptation of a mesophilic subtilisin-like protease by laboratory evolution J. Biol Chem 275:31635–31640