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Cold-stable eye lens crystallins of the Antarctic notothenioid toothfish *Dissostichus mawsoni* Norman

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Summary

The eye lenses of the Antarctic notothenioid fishes that inhabit the perennially freezing Antarctic seawater are transparent at –2°C, whereas the cold-sensitive mammalian and tropical fish lenses display cold-induced cataract at 20°C and 7°C, respectively. No cold-cataract occurs in the giant Antarctic toothfish *Dissostichus mawsoni* lens when cooled to temperatures as low as –12°C, indicating highly cold-stable lens proteins. To investigate this cold stability, we characterised the lens crystallin proteins of the Antarctic toothfish, in parallel with those of the sub-tropical bigeye tuna *Thunnus obesus* and the endothermic cow *Bos taurus,* representing three disparate thermal climes (–2°C, 18°C and 37°C, respectively). Sizing chromatography resolved their lens crystallins into three groups, α/β_H, β and γ, with γ crystallins being the most abundant (>40%) lens proteins in fish, in contrast to the cow lens where they comprise only 19%. The upper thermal stability of these crystallin components correlated with the body temperature of the species. *In vitro* chaperone assays showed that fish α crystallin can protect same-species γ crystallins from heat denaturation, as well as lysozyme from DTT-induced unfolding, and therefore are small Heat Shock Proteins (sHSP) like their mammalian counterparts. Dynamic light scattering measured an increase in size of αγ crystallin mixtures upon heating, which supports formation of the αγ complex as an integral part of the chaperone process. Surprisingly, in cross-species chaperone assays, tuna α crystallins only partly protected toothfish γ crystallins, while cow α crystallins completely failed to protect, indicating partial and no αγ interaction, respectively. Toothfish γ was likely to be the component that failed to interact, as the supernatant from a cow α plus toothfish γ incubation could chaperone cow γ crystallins in a subsequent heat incubation, indicating the presence of uncomplexed cow α. This suggests that the inability of toothfish γ crystallins to fully complex with tuna α, and not at all with the cow α crystallins, may have its basis in adaptive changes in the protein that relate to the extreme cold-stability of the toothfish lens.

Key words: lens crystallins, chaperone, Antarctic toothfish, *Dissostichus mawsoni*, bigeye tuna, cold adaptation, cold cataract, dynamic light scattering, alpha crystallin, gamma crystallin.

Introduction

The fish fauna that inhabits the freezing (–2°C) coastal waters of the Antarctic continent in the Southern Ocean is dominated by members of the endemic teleost suborder Notothenioidei. Their ability to survive in the frigid Antarctic marine environments is due to the evolution of blood-borne antifreeze glycoproteins, which allowed them to avoid freezing (Chen et al., 1997b; Cheng and Chen, 1999; DeVries, 1971) and to undergo subsequent adaptive radiation to become the predominant fish Antarctic fish taxon (Eastman, 1993; Eastman and McCune, 2000). Since cold temperatures depress the rates of biochemical reactions as well as affect the stability of protein structure (Hochachka and Somero, 2002), these fishes also exhibit a suite of biochemical and physiological adaptations to low temperature, including cold-efficient catalytic enzymes (Fields and Somero, 1998), cold-effective protein translocation (Romisch et al., 2003), membrane phospholipid unsaturation (Cossins et al., 2002; Romisch et al., 2003), and tubulins that polymerise at subzero temperatures (Detrich et al., 2000; Williams et al., 1985). An important physiological system in notothenioid fish that has not been characterised in any detail with regards to protein property and function at freezing seawater temperature is the lens. Although studies indicate that the structure of the eye and retina are the same to those of sub-tropical fishes, it is not known whether the lens and their constituent proteins differ from temperate and tropical species (Eastman and Lannoo, 2001, 2003).

The model system for studies of the vertebrate lens has been that of the cow. Bovine lens consists of a small insoluble albuminoid fraction, and a large soluble crystallin fraction (Mörner, 1864), which comprises three groups of proteins: α,

β and γ crystallins (Bloemendal, 1986). In mammals, the most abundant protein is α crystallin, a large oligomeric structure composed of two polypeptides, αA and αB, which belong to the small Heat Shock Protein (sHSP) family with chaperone-like ability to protect unrelated proteins from heat or chemical-induced protein denaturation (Horwitz, 2003; Narberhaus, 2002). The β and γ crystallins are members of the βγ gene superfamily of crystallins, which all possess a Greek-key fold protein motif, but whose non-refractive functions remain undetermined (Liaw et al., 1992; Slingsby and Clout, 1999; Wistow, 1993). Depending on the species, between four and six of the known β and γ crystallin genes are expressed (Chiou et al., 1986; Norledge et al., 1997).

When the cow lens is cooled from the body temperature of 37°C to about 19°C, it begins to develop an opacity, a phenomenon known as cold-cataract, which has been attributed to the cold instability of some of the constituent γ crystallins, resulting in a liquid–liquid phase transition within the lens (Delaye et al., 1982; Gulik-Krzywicki et al., 1984; Norledge et al., 1997; Siezen et al., 1985). Cold-cataract has also been reported in a few other endothermic mammals such as the rat (Zigman and Lerman, 1964, 1965). There are very few studies on the temperature response of the lens crystallins of ectothermic vertebrates, but one study indicates that for the sub-tropical striped mullet *Mugil cephalus*, a cold-insoluble precipitate appears to form when the lens homogenate is cooled from 24°C to 7°C (Ferguson et al., 1971). In marked contrast to these warmer bodied species, polar fishes, particularly the Antarctic notothenioids that live in perennially freezing temperatures of –2°C, maintain complete lens transparency, suggesting a high degree of cold stability of their constituent lens proteins.

In this study, we report the first detailed characterisations of the whole lens and the lens crystallin proteins of the giant Antarctic notothenid fish *Dissostichus mawsoni*, in terms of thermal response, protein composition, and various physical and biochemical properties. For comparison, we also examined the thermal, physical and biochemical properties of the crystallin proteins of the equally large lens of a mesophilic fish, the bigeye tuna *Thunnus obesus* and the endothermic cow *Bos taurus*, which collectively span the vertebrate body temperature range. This comparative analysis has allowed us to gain insights into the adaptive changes in protein properties that may have ensured transparency of the Antarctic notothenioid eye lens in constantly freezing seawater temperatures.

Materials and methods

Lens collection

Lenses of the giant Antarctic toothfish *Dissostichus mawsoni* Norman were collected from live specimens caught in McMurdo Sound, Antarctica; cow *Bos taurus* lenses were obtained from Allen's Farm Quality Meats, Homer, Illinois; lenses of the small tropical marine fish blackbar soldierfish *Myripristis jacobus* were from specimens purchased at a local pet store; and the lenses of bigeye tuna *Thunnus obesus* were a generous gift of Timothy Ke (Luen Thai Fishing Venture, Ltd., Hong Kong and LTFV-USA) from specimens caught off the coast of the Marshall Islands. Some lenses were analysed when fresh, and others were stored frozen at –80°C until analysis.

Cold-cataract formation

The effect of temperature on the transparency of the lens was determined using fresh lenses. Bovine lenses, either within or removed from the eye capsule, were wrapped in plastic and embedded in a bucket of wet ice (0°C). Lenses of the tropical blackbar soldierfish *M. jacobus* were placed in test tubes with a small amount of vitreous humour and incubated at 15°C or in wet ice (0°C). Lenses of the Antarctic toothfish *D. mawsoni* were kept at –2°C (body temperature of toothfish and ambient temperature of Antarctic seawater) or at –12°C for a period of 48 h in a vial of High Temperature Silicon Oil (Aldrich 17,563-3). The appearance of each lens after these various temperature incubations was assessed and photographed with a digital camera (Fig. 1).

Size-exclusion chromatographic separation of the soluble crystallin proteins

Size-exclusion chromatography (SEC) using Sephacryl 200 High Resolution (S200HR) resin (Sigma, St Louis, MO, USA) was performed to fractionate the soluble lens protein components of *D. mawsoni, T. obesus* and *B. taurus*. Fresh or frozen lenses were first solubilised by stirring overnight at 8°C in three volumes of solubilisation buffer (10 mmol l^{-1} potassium phosphate, 100 mmol l^{-1} KCl, 0.05% NaN_3, pH 7.6). The potassium phosphate buffer pH was adjusted as per the method of Gomori (1955) and the complete buffer suction filtered through a Buchner funnel with No. 54 Whatman (hardened) filter paper. Solubilised lens homogenate was centrifuged at 20 198 ***g*** (13 000 revs min^{-1}) in an SS-34 rotor (Sorval RC-5B, Asheville, NC, USA) for 20 min set at 4°C to sediment the insoluble components. Between 0.5 and 1.0 g of soluble lens proteins (supernatant) were loaded (maximum volume 5 ml) on a S200HR column (2.5 cm i.d.×116 cm length) equilibrated with the solubilisation buffer, and protein elution was carried out with the same buffer at room temperature with a flow rate of ~36 ml h^{-1}. Tube fractions (5 ml) were collected, and protein absorbance at 280 nm of each fraction was measured. Integration of the area under protein absorption peaks in the elution chromatogram was performed using the programme ORIGIN 6.0.4 (OriginLab, Northampton, MA, USA).

SDS-polyacrylamide gel electrophoresis and immunoblot detection of α, β and γ crystallin polypeptides

SDS-polyacrylamide gel electrophoresis (SDS-PAGE) was performed using BioRad Mini-PROTEAN 3 system (BioRad Laboratories, Hercules, CA, USA). Crystallin protein (10–20 μg per sample) was loaded on a discontinuous SDS-polyacrylamide gel (4% stacking gel, 15% resolving gel) and

electrophoresed for 1 h and 15 min at a constant voltage of 200 V. Gels were stained with 0.1% Coomassie R-250 in 10% acetic acid, 40% methanol, and destained with 10% acetic acid, 40% methanol.

For immunoblotting, SDS-PAGE separated proteins were transferred electrophoretically (BioRad Mini-Trans Blot Electrophoretic Cell) from un-stained gels to nitrocellulose membrane (Hybond ECL, Amersham Biosciences, Corp., Piscataway, NJ, USA) at 90 V for 3.5 h at 8°C. Membranes were incubated for 1 h at room temperature with the blocking solution 1× TBS (20 mmol l^{-1} Tris-HCl, pH 7.5, 150 mmol l^{-1} NaCl) containing 5% non-fat milk powder (Carnation, Nestlé USA Inc., Solon, OH, USA) and 0.05% Tween-20, and then probed with polyclonal rabbit anti-bovine crystallin primary antibodies (gift of Dr J. Sam Zigler, National Eye Institute, NIH, USA) in antibody (Ab) buffer (1× TBS, 5% non-fat milk powder, 0.25% Triton-X100). The anti-bovine crystallin (α, β, γ) antisera were able to react with fish crystallins in this study. Bovine crystallins were detected colorimetrically, while the fish crystallins required more sensitive chemiluminescence detection. For colorimetric detection, the primary Ab stock (1 mg ml^{-1}) was diluted 1:500 for α, β and γ, and for chemiluminescence detection, 1:2000 for α, 1:2500 for β, and 1:5000 for γ crystallin, respectively. Unbound primary Ab was removed by washing in 1× TBS with 0.05% Tween-20. Secondary antibody, horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG (H+L) (1.5 mg ml^{-1}, ZYMAX 81-6120, Zymed, San Francisco, CA, USA) was diluted 1:5000 in Ab buffer for colorimetric detection, and 1:50 000 for chemiluminescence detection. Bovine crystallin blots were incubated in colormetric reagent (BioRad HRP Conjugate Substrate Kit) for approximately 10 min, washed with distilled water and air-dried. Fish crystallin blots were developed with chemiluminescence reagent (Pierce SuperSignal WestPico Chemiluminescent Substrate, Pierce, IL, USA) per manufacturer's instructions and autoradiographed on Kodak BioMax film for 10 s to 5 min, as appropriate.

Determination of upper limits of thermal stability of crystallins

The upper limit of thermal stability or T_S is defined as the maximum temperature that a crystallin fraction can be subjected to without precipitation of the protein. T_S values for the SEC purified α and γ crystallin fractions from toothfish, bigeye tuna and cow were empirically determined to establish the appropriate range of test temperatures for the chaperone-like activity assays (next section). The protein concentrations of the crystallin fractions were calculated from their A_{280} values (cow) using published (Siezen et al., 1986) and our gravimetrically determined extinction coefficients (fish). To determine these gravimetrically, α, β and γ fish crystallin solutions were collected from the SEC separations (separately), diluted down below 1.0 A_{280} units, and the absorbance at 280 nm recorded. Exactly 10 ml was lyophilised for 24 h in a pre-weighed, pre-lyophilised clean glass vial. Vials containing the lyophilised crystallins were weighed immediately on an analytical scale with 0.001 mg resolution. All measurements for fish crystallin were made twice. All extinction coefficients are expressed as A_{280} in 1 cm path length of a 1% solution (Table 1). Samples of each crystallin (800 µl at 1 mg ml^{-1}) were incubated at various temperatures in a UV-transparent polystyrene microcuvette placed in a spectrophotometer equipped with a water-jacketed 7-cuvette holder (Helios Gamma, Spectronic Instruments Inc., Rochester, NY, USA). The incubation temperature was controlled using a circulating water bath (HaakeFS) and the actual sample temperature was monitored using a digital thermocouple thermometer equipped with a fine wire probe (Cole-Parmer, Vernon Hills, IL, USA) in an isothermal adjacent microcuvette containing sample buffer. Heat-induced protein aggregation results in static light scattering that can be followed by monitoring turbidity (increased absorbance) at 360 nm (Jaenicke and Seckler, 1997). A_{360} of the sample was automatically measured at 5 min intervals by a computer interfaced with the spectrophotometer using the program HYPERTERMINAL (Hilgraeve, Monroe, MI, USA). The highest incubation temperature at which A_{360} remained at or very near zero for at least 1 h was taken as the T_S.

Table 1. *A_{280} absorption coefficients at 1% (m/v) for crystallins from different species*

Species	Crystallin fraction	
	α (α/β_H)*	γ
D. mawsoni	18.0	12.5
T. obesus	16.8	11
B. taurus	8.6	20

Crystallin fractions were as described in the Results section.
*Values for α crystallin from fish contain some contributions from the presence of β_H in the size-exclusion chromatography (SEC) fraction.

Assays for chaperone-like activity of α crystallin

Lens α crystallins are known sHSPs and have the chaperone-like ability to protect other proteins from stress-induced denaturation (Horwitz et al., 1998). The chaperone-like function of the fish and bovine α crystallins in protecting against heat-induced aggregation of γ crystallins, and from chemically induced (by DL-dithiothreitol) aggregation of lysozyme was assayed. Since SEC fractionation of crystallins does not achieve perfect separation of the individual classes of crystallins (see Results), the crystallin fractions for these chaperone assays were chosen as follows. The SEC γ crystallin peak from SEC separation of crystallins of all three species (see Results and Fig. 2A–C, peak III) was predominantly γ crystallin, based on immunoblots (see Results and Fig. 3), and thus the five highest A_{280} fractions of this peak were used in the assays. The γ_S fraction (Fig. 2C, peak IIIa) was not used in chaperone assays or dynamic light scattering (DLS) studies. For bovine α crystallin, the first 2–3 fractions of the ascending slope of the SEC peak I (Fig. 2C, peak I) are near homogeneous for α crystallin (see Results), so these were used

for the assays. For fish, none of the fractions from the SEC α crystallin peak (Fig. 2A,B, peak I) are homogeneous for α crystallin, but contain various amounts of β crystallins (see Results). We found that the first 2–3 fractions of the ascending slope of the elution peak have the highest and most consistent chaperone activity, indicating the greatest concentration of α crystallins, and these were selected for all subsequent chaperone assays. Thus, the terms α crystallin and γ crystallin in the described chaperone experiments refer to these particular SEC elution fractions. Because of this protein heterogeneity, the stated concentrations of α crystallin used in the assays represent the upper bound values. In other words, the observed chaperone activity is achieved by α crystallin concentrations at or lower than the stated values. Beta crystallins are not known to be molecular chaperones (or sHSPs) and therefore their presence in the α crystallin fractions is not expected to contribute to the observed chaperone activity in the assays.

Assays of chaperone protection against heat aggregation were performed similar to previously published methods (Horwitz, 1992; Liao et al., 2002). Gamma crystallin (final concentration of 1 mg ml^{-1}) of each species was mixed with different amounts of α crystallin (final concentration of 62.5 μg ml^{-1} to 1.0 mg ml^{-1}) of the same species in a total assay volume of 800 μl. The incubation temperature was 47°C, 55°C and 60°C for toothfish, tuna and cow, respectively, and 5 min A_{360} measurements were carried as described above. T_S of γ crystallin is lower than T_S of α crystallin (see Results), thus no increase in A_{360} at these incubation temperatures was interpreted to be the result of chaperone protection by α crystallin against thermal aggregation of γ crystallin. All assays were repeated three times.

The ability of α crystallin to protect DTT-induced aggregation of lysozyme at 37°C, a common approach used for testing the chaperone-like activity of α crystallins (Abgar et al., 2000; Vanhoudt et al., 2000), was used for the three species in this study. Various amounts (0.2–2.2 mg) of α crystallin fraction were added to 200 μg of chicken egg white lysozyme (EC 3.2.1.17, Sigma) in a final volume of 1 ml. Freshly made 1 mol l^{-1} DTT (Sigma) was added to a final concentration of 20 mmol l^{-1} to initiate lysozyme unfolding, and A_{360} was measured at 5 min intervals. Three replicates of each condition were performed for each species.

Cross-species chaperone assays

To assess whether crystallins from cold-adapted and warm-adapted organisms could interact, α and γ crystallins of toothfish, tuna and cow, representing three disparate thermal environments (polar, –2°C; subtropical, 18°C; endothermal, 37°C), were used in pairwise cross-species chaperone protection assays similar to the same-species assays described above. Pairwise combinations of α and γ crystallin from *D. mawsoni*, *T. obesus* and *B. taurus* at a 1:1 mass ratio (1 mg:1 mg in 0.8 ml), were incubated at the T_S of the α crystallin component, and A_{360} recorded at 5 min intervals. For the combination of the bovine α crystallin and toothfish γ crystallin, an additional two-part assay was performed as follows. Bovine α crystallin (final concentration $\frac{1}{3}$ mg ml^{-1}) and toothfish γ crystallin (final concentration 1 mg ml^{-1}) were incubated for 1 h at 60°C (final volume of 0.8 ml). Aggregated protein that was observed was then sedimented by centrifugation at 21 000 ***g*** for 10 min at 4°C. The supernatant was combined with bovine γ crystallin (final concentration 1 mg ml^{-1}) in a total volume of 1 ml or less, and incubated for a second 1 h period at 60°C. A_{360} was recorded at 5 min intervals for all assays.

Estimation of effective α crystallin size by dynamic light scattering

The effective molecular size in solution of individual crystallins of cow and fish lens, and of interacting αγ-crystallin complexes, were measured by dynamic light scattering (DLS) with the Brookhaven Instruments goniometer system (BI-200SM, Brookhaven Instruments Corp., Holtsville, NY, USA) equipped with a Lexel argon-ion laser (model 95, Cambridge Lasers Lab., Fremont, CA, USA) operating at 514 nm. Individual α and γ crystallin fractions, freshly purified by SEC, were first centrifuged at 18 400 ***g*** at 4°C for 20 min and the supernatant was filtered through 0.050 μm SPI-Pore polycarbonate filters (SPI Supplies, West Chester, PA, USA) to remove dust and other particulates before sizing. We found that the filtration step was necessary to obtain reproducible DLS sizes, especially for α crystallins, which form increasingly larger high molecular mass (>1000 kDa) oligomers with storage time. For unheated samples, the filtered crystallin fraction was sized in a clean DLS cell at room temperature. Heated samples (1 h incubation at the desired temperature) were first cooled to room temperature, centrifuged to pellet any heat-aggregated protein, and the supernatant was transferred to a clean DLS cell and sized. Light intensity at 90° angle was measured by a photomultiplier tube and the output signal was processed by a Brookhaven 9000AT correlator. Crystallin size was calculated by Brookhaven Instruments DLS Software Version 2.17 and expressed as the hydrodynamic diameter in nm.

Results

Lens shape and structure

A schematic of the shape and corresponding photographs of Antarctic toothfish *D. mawsoni* and cow *B. taurus* lens are shown in Fig. 1a–d. Fish lenses are spherical (Fig. 1a) and denser than bovine lens. The lens of a 27 kg Antarctic toothfish is approximately 1.5 cm in diameter and weighs about 3.5 g (Fig. 1b). Lenses of similar-sized bigeye tuna (*T. obesus*) are larger, about 3 cm in diameter and weigh about 10–12 g (not shown). The adult bovine lens is approximately 2 cm in diameter and has a mass of about 4.5 g (Fig. 1c,d). It is not spherical, but biconvex in shape, with the less convex side facing the cornea and the more convex side facing the retina. All lenses are enveloped in a collagen lamellar structure called the capsule (Davson, 1990). In contrast to the dense fish lens, the cow lens is much softer and once decapsulated, its outer cortical layers readily slough off.

Cold-cataract formation

Fig. 1e–l illustrates the effect of temperatures below normal body temperature on the transparency of the lens of cow and fish. Fig. 1e is a freshly dissected bovine lens at room temperature (~25°C) showing complete transparency. Cold temperature induced opacity or cold-cataract in a cow lens after cooling in ice (0°C) for ~1.5 h (Fig. 1f). When the cooled lens was retrieved for photographing at room temperature, the slightly opaque outer cortex rapidly clarified, while the nucleus remained opaque, as indicated by the arrow (Fig. 1e). The nucleus completely clarified later, after 2 h at room temperature. Lenses from the small tropical (24°C) marine fish, the blackbar soldierfish *Myripristis jacobus* (Fig. 1g–i), are approximately 6 mm in diameter and have the same structural morphology as the *D. mawsoni* and *T. obesus* lenses. Transparency of the lens was maintained on cooling to 15°C (Fig. 1g). A second lens incubated at 0°C (in ice) for 6 h became less transparent (Fig. 1h) than the lens at 15°C. Further incubation at 0°C (48 h total) resulted in further opacity throughout the lens. Retrieval from ice for photography at room temperature partially clarified the cortex, but the nucleus remained opaque, as indicated by the arrow (Fig. 1i). Further equilibration at room temperature for several hours did not clarify the nucleus.

Antarctic toothfish lenses are completely transparent at –2°C, the ambient environmental temperature and normal body temperature of this ectotherm (Fig. 1j). A second lens after 6 h and 48 h incubation at –12°C is shown in Fig. 1k and l, respectively. The lens was immersed in a silicon oil to permit incubation temperature as low as –40°C without the oil freezing or losing optical clarity. In contrast to cold-cataract development in cooled bovine and soldierfish lens, the toothfish lens remained internally clear even after 48 h at –12°C (Fig. 1l), with only a surface veneer of slight opacity, probably due to the reaction of the silicon oil with the outer layers of the collagen capsule. The silicon oil did become slightly translucent itself, but the lens remained clear. Cooling the lens below –12°C was not possible as the lens invariably froze. It can be concluded, however, that cold-cataract formation does not occur in the Antarctic toothfish lens at temperatures as low as –12°C.

SEC fractionation of lens crystallins

Fig. 2 shows that the chromatograms of the crystallins from toothfish, bigeye tuna and cow fractionated on S200HR size-exclusion resin are all similar in pattern. The bovine elution chromatogram (Fig. 2C) is similar to that reported in the literature (Björk, 1961; Bloemendal, 1986; Siezen et al., 1986; van Dam, 1966) using G75 Sephadex and 200HR Sephacryl resins. Prior characterisations of bovine lens showed that the three predominant peaks I, II and III (or IIIa and III) containing primarily α, β and γ crystallin, respectively (Bloemendal, 1986). Using the S200HR resin, the high molecular mass α crystallin and β_H (β_{High}) crystallin both elute in peak I, which is designated as the 'α/β_H' peak in Fig. 2. (The constituents of these elution peaks are given here, with the verification of their specific identities detailed in the next Results section.) Fractionation on Sepharose 6B resin provided better separation of cow α and β_H crystallins (data not shown), but much poorer separation of β_L (β_{Low}) from the γ crystallins for all three species. Therefore, the S200HR resin was used in this study to allow optimal comparisons of SEC chromatograms and subsequent analyses.

Although similar in pattern, the SEC elution profiles of fish crystallins differ from the cow in the relative size of the β and γ peaks. The bovine β crystallins partitioned into β_H (part of peak I, apex and descending slope; Fig. 2C) and a prominent distinct β_L peak (Fig. 2C, peak II). While β_H is present in peak I of tuna and toothfish, there are also distinct small β_H and β_L peaks in the bigeye tuna chromatogram (Fig. 2B, peaks IIa and IIb). The toothfish profile (Fig. 2A) has no β_H peak and only a very minor β_L peak (Fig. 2A, peak II). In contrast, the γ crystallin peak of both fish species (Fig. 2 A, B peak III) is much larger than the bovine γ crystallin peaks (Fig. 2C, peaks IIIa and III combined). Fig. 2C, peak IIIa contains cow γ_S, which elutes ahead of the rest of the cow γ crystallins in peak III.

Estimations of relative protein abundance based on peak areas (Fig. 2) for each species are tabulated in Table 2. Toothfish (52% α/β_H, 5% β_L and 43% γ) is similar to bigeye tuna (47% α/β_H , 6% β_H, 6% β_L and 41% γ) in relative percentage abundance of the three classes of crystallins. Both fish differ from the cow (54% α/β_H, 27% β_L and 19% γ) in having a substantially higher percentage of γ crystallins, over 40% as opposed to 19%, making it the predominant crystallin fraction in the fish lens. Separate estimates of percentage abundance of α and β crystallins are not possible because of their overlapping elution within peak I (Fig. 2A,B), as revealed by SDS-PAGE analysis and immunoblotting with α, β, γ antisera (next section). The SEC γ peaks, peak III of fish and peaks IIIa and III for cow, however, are near homogeneous for γ crystallins, based on immunoblots (next section), and therefore their percentage abundance estimations and comparisons are reliable.

SDS-PAGE and immunoblot analyses of crystallin fractions

The identities of the class of crystallins and the extent of protein heterogeneity in the SEC elution peaks (Fig. 2) were assessed by SDS-PAGE and immunoblots (Fig. 3). The lane numbers in the SDS gels and the immunoblots (western blots) (Fig. 3) correspond to the number of selected fractions from the SEC elution (Fig. 2, labelled next to the peaks). The results of the SEC fractionation, SDS gels and immunoblots collectively show the following. For all three species, the crystallin polypeptides range in size from about 18 kDa to 35 kDa. Rabbit antisera against the three bovine crystallins could crossreact with the respective fish crystallins. SEC separation achieved near-homogeneous γ crystallin fractions in all species (Fig. 3A,E,I, γ fractions), but did not adequately resolve α and β_H crystallins from each other in the two fish species, such that SEC peak I of both fish species encompasses considerable protein and size heterogeneity (Fig. 3A,E,I, α/β_H fractions).

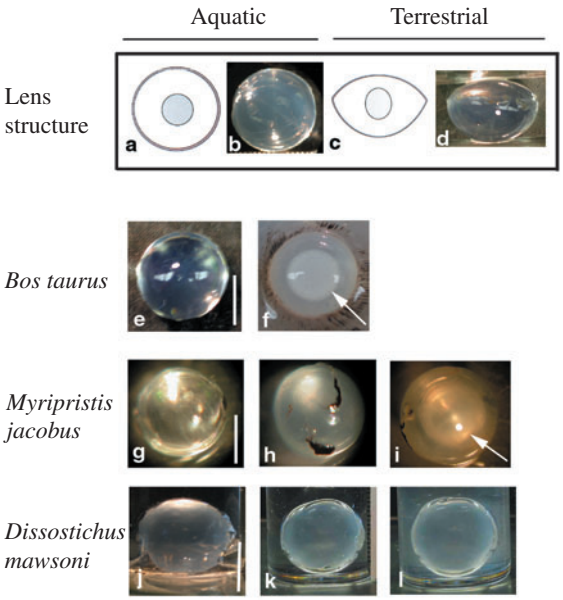

Fig. 1. Lens shapes and cold-cataract cooling experiment on the lenses of three species (*Bos taurus, Myripristis jacobus* and *Dissostichus mawsoni*) from three different physiological temperatures (37°C, 25°C and –2°C, respectively). (a–d) Schematics of the shapes (a,c) and a picture (b,d) of a *D. mawsoni* (a,b) and *B. taurus* (c,d) lens. (e,f) Cold-cataract experiment results showing lenses from the cow, *B. taurus.* (e) A fresh lens at room temperature (25°C); (f) a bovine lens that had been packed in ice for approximately 1.5 h, from which it was removed and allowed to warm. This image was taken during the warming process after the cortex had rapidly clarified and the nucleus was still opaque, showing the cold-cataract (arrow). (g–i) The eye lens from the tropical marine blackbar soldierfish *M. jacobus* held at (g) 15°C for 6 h; (h) 0°C for 6 h; (i) 0°C for 48 h, showing a definite inner nuclear region that is more opaque that the cortex region (arrow). (j–k) Images of the Antarctic toothfish *D. mawsoni* eye lens. (j) Endogenous clear –2°C lens contrasted to a lens held at –12°C for 6 h (k). The still clear toothfish lens after 48 h at –12°C (l) has a thin sheen of opacity. It is important to note that the opacity is restricted to the surface and not to the inner portions of the lens, as in the cow (f) and the soldierfish (i). Scale bars, 1.2 cm (e), 0.4 cm (g), 1.0 cm (j).

More specifically, the selected fractions 1–5 from the bovine peak I (Fig. 2C) contain αA and αB crystallins at ~21 and 23 kDa, and an additional ~18 kDa band for fractions 1–4 (Fig. 3I, lanes 1–5), which is probably an αA crystallin truncation product previously reported in the literature (Augusteyn et al., 1992; Horwitz et al., 1998). These protein bands are all immunopositive to anti-bovine α crystallin polyclonal antibodies in the immunoblot (Fig. 3J, lanes 1–5), and of these five fractions, the first two (indicated with arrow at peak I, Fig. 2C) are predominantly α crystallin, as indicated by strong anti-α immunopositive bands (Fig. 3J, lanes 1,2), and little or no immunopositivity to anti-β (Fig. 3K, lanes 1,2). The subsequent fractions 3–5 of bovine SEC peak I (Fig. 3I, lanes 3–5), all contain β_H crystallins, as indicated by the two sets of anti-β crystallin immunopositive bands close to the 31.1 kDa and 28.4 kDa and markers in the immunoblot (Fig. 3K, lanes 3–5).

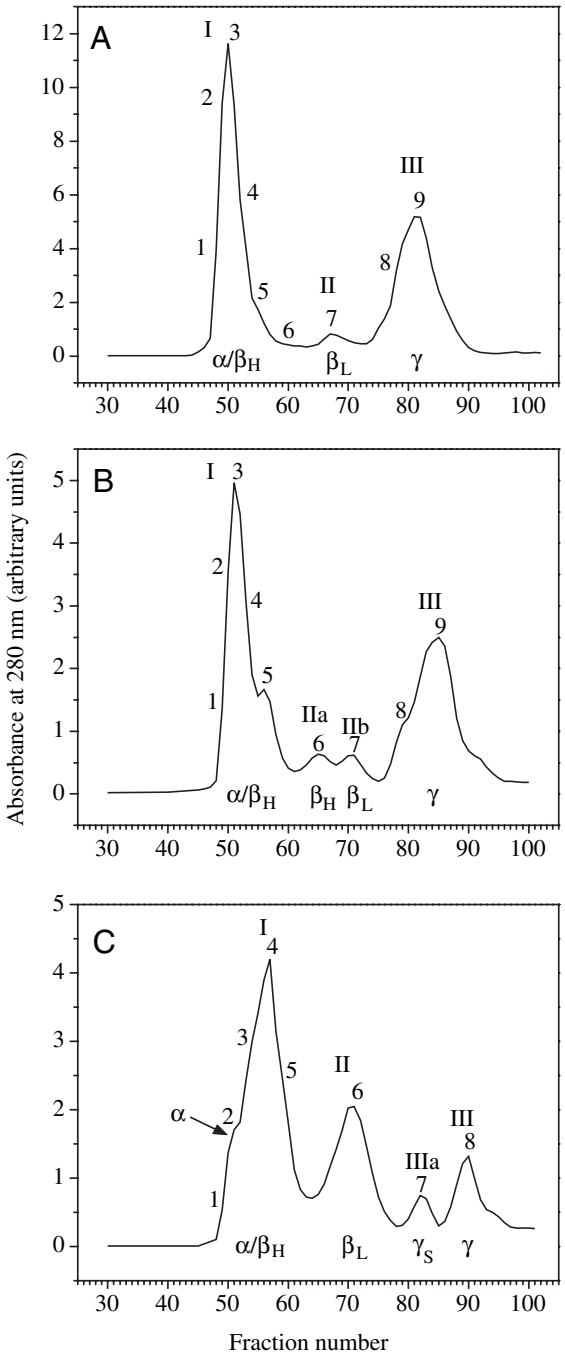

Fig. 2. Size-exclusion chromatography using Sephacryl 200 High Resolution resin of crystallins from three species, *D. mawsoni* (A), *T. obesus* (B) and *B. taurus* (C). Peaks are labelled as α/β_H (peak I), β_H (peak IIa), β_L (peaks II or IIb), or γ_S and γ (peaks IIIa and III). Numbers labelled on each individual peak correspond to the lanes of the SDS-PAGE and immunoblots presented in Fig. 3. Fraction volume sizes are 5 ml each.

The SEC peak I fractions of the two fish (Fig. 2A,B) contain substantially more protein heterogeneity on SDS-PAGE (Fig. 3A,E, lanes 1–5) than their bovine counterpart and none of the selected fractions examined is homogeneous for α crystallin. Chemiluminescence immunodetection using anti-

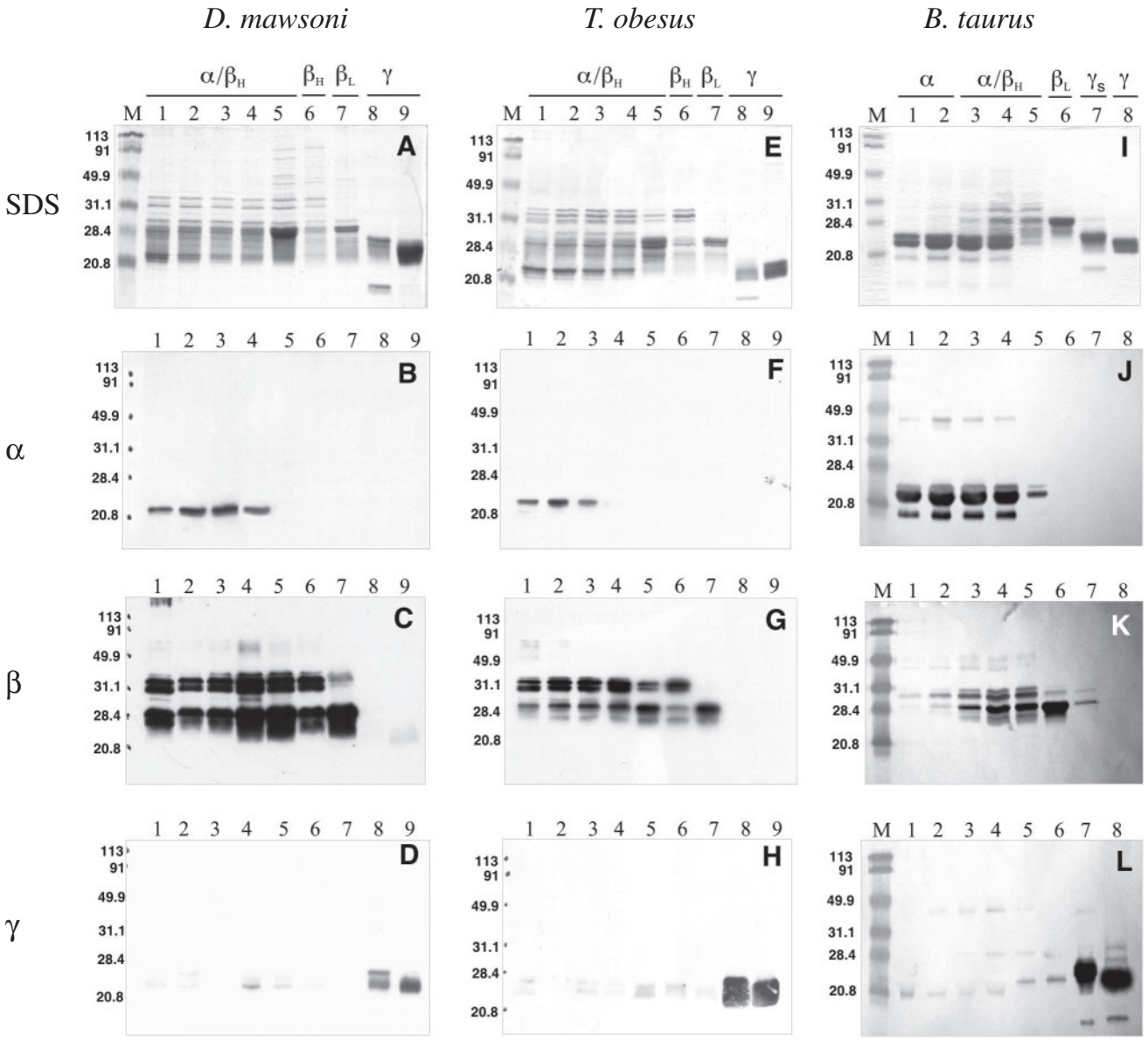

Fig. 3. SDS-PAGE (A,E,I) and immunoblot analysis (B–D, F–H, J–L) of SEC elution profiles of crystallins from *D. mawsoni* (A–D), *T. obesus* (E–H) and *B. taurus* (I–L). The primary antibodies used for detection are denoted on the left. Molecular mass markers (Lane M; kDa) are denoted on the left of each panel. Lane numbers correspond to the locations of the numbered positions on Fig. 2. for each species. All immunoblots are independent replicas of SDS-PAGE presented in Fig. 3A,E,I.

bovine α crystallin polyclonal antibodies show α crystallins of ~21 kDa in peak I fractions 1–4 for toothfish (Fig. 3B, lanes 1–4), and peak I fractions 1-4 for bigeye tuna (Fig. 3F, lanes 1–4, signal weak for lane 4). All five selected fractions of SEC peak I of both fish species contain two sets of anti-β immunoreactive bands, close to the 28.4 kDa and 31.1 kDa markers (Fig. 3B, F, lanes 1–5). These sizes are consistent with the reported values (26–35 kDa) for mammalian β crystallins (Slingsby and Clout, 1999) and known fish β crystallins (Wistow, 1995; Zigler and Sidbury, 1976). The presence of the ~31.1 kDa immunopositive bands indicates that β_H coeluted with α crystallin in peak I of fish. For both fish and cow, some fractions of the α/β_H peak contain several weakly staining, large-sized protein bands, close to the 49.9 kDa marker (and above in the toothfish) in the SDS gel (Fig. 3A,E,I). These ~49.9 kDa bands are likely oligomeric β or α/β aggregates since they are weakly immunoreactive to anti-β (Fig.2C,G,K), and to anti-α (Fig. 2J). The bands greater than 49.9 kDa of the toothfish (Fig. 3A) are not immunoreactive to any antisera, and thus their identity remains to be investigated.

For both fish and cow, the SEC peak II (peaks IIa and IIb for bigeye tuna) contains only β crystallin. SEC peak II of toothfish and cow, and peak IIb of the bigeye tuna (Fig. 2A–C) appears homogeneous for β_L in immunoblots (Fig. 3C,G, lane 7, and K, lane 6).

For all three species, SEC peak III (Fig. 2) is homogeneous

Table 2. *Relative proportions of crystallins determined from integration of peaks from the SEC elution profiles*

Species	Crystallin (%)			
	α (α/β_H)	β_H	β_L	γ
D. mawsoni	52	–	5	43
T. obesus	47	6	6	41
B. taurus	54	–	27	19*

Percentages represent the fraction of total areas under all peaks for a given species (Fig. 2).

*For the cow, γ_S and γ peak areas were combined.

for γ crystallins on immunoprobing (Fig. 3D,H, lanes 8,9, L, lane 8). In the case of the cow, γ_S (Fig. 3L, lane 7), formerly β_S in older literature, eluted as a discrete peak ahead of the other γ crystallins (Fig. 2C, peaks IIIa and III). Because the SEC γ crystallin fractions do not have α or β crystallins within them, their relative percentage abundance in the lens, estimated on the basis of protein elution peak areas (Table 2), is reliable. Gamma crystallins constitute the predominant crystallin in fish lens (43% of toothfish crystallins, 41% of bigeye tuna crystallins), which is over twofold more abundant than its bovine counterpart (19% of total crystallins).

Upper limit of temperature stability (T_S) of α and γ crystallin from D. mawsoni, T. obesus *and* B. taurus

Fig. 4 shows A_{360} *versus* time for a series of incubations at increasing temperatures for toothfish α crystallin and γ crystallin. The highest temperature at which the A_{360} remained at or near zero for at least 1 h was defined as the T_S value, and was found to be 47°C and 33°C for toothfish α and γ crystallins, respectively. The T_S value for the α and γ crystallins of bigeye tuna and cow were similarly determined. T_S values of the α crystallins of bigeye tuna and cow were 55°C and 68°C, respectively, and T_S of their γ crystallins were 39°C and 50°C respectively (Table 3). Thus the γ crystallins are much more heat-labile relative to α crystallins, by 14°C, 16°C and 18°C for toothfish, tuna and cow, respectively. Additionally, there was a direct correlation between the T_S of the crystallins and the body temperature of each animal, highest for the endothermic cow (mean body temperature 38.3°C; Piccione et al., 2003), followed by the sub-tropical ectothermic bigeye tuna (mean body temperature ~18°C; Holland et al., 1992), and lowest for the ectothermic Antarctic toothfish, which inhabits the perennially freezing seawater of the Southern Ocean (mean body temperature –1.9°C; Hunt et al., 2003).

Chaperone-like activity of α crystallin from D. mawsoni, T. obesus *and* B. taurus

In the absence of α crystallin, heat aggregation of Antarctic toothfish γ crystallin occurred rapidly, after 10 min at 47°C. Turbidity as measured by A_{360} increased sharply to 0.8 in the first 30 min, and reached a plateau of 1.0 in the next 30 min (Fig. 5A). At mass ratios of 1:1 and 1:2 of α:γ crystallin,

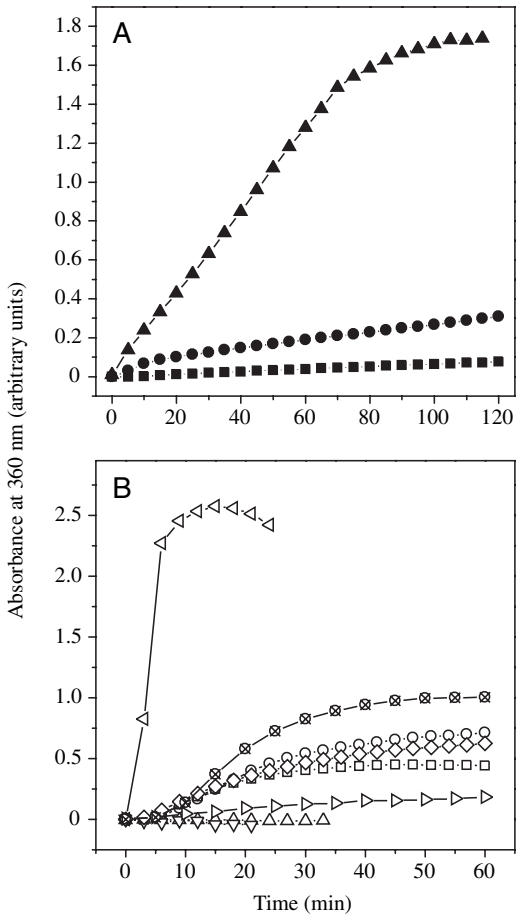

Fig. 4. Determination of the upper limit of thermal stability T_S for the (A) α and (B) γ crystallins from *D. mawsoni*, measured as the change in turbidity (A_{360}) over time at various incubation temperatures. Concentration of crystallins was 1 mg ml^{-1} in all assays. (A) Upper limit of T_S for α crystallin. At temperatures above 47°C, turbidity increases with time. α at 47°C (■); 55°C (●); 60°C (▲). (B) Upper limit of T_S for γ crystallin. γ at 30°C (▽); 33°C (△); 35°C(▷); 42.5°C (□); 44°C (◇); 45°C (○); 47°C (⊗); 57.5°C (◁). The T_S was determined as 47°C for the α and 33°C for the γ crystallin.

aggregation of the γ crystallin was prevented during the 1 h assay time (Fig. 5A). Protection decreased as α crystallin concentration was decreased, but even with 1:16 ratio of α to γ crystallin, there was still significant protection compared to unchaperoned γ crystallin (Fig. 5A).

For comparison, chaperone assays were performed using α crystallin of the bigeye tuna *T. obesus* and the cow *B. taurus* and same-species γ crystallin, at 55°C for bigeye tuna and at 60°C for the cow (Fig. 5B,C). By itself, γ crystallin from either species increased in turbidity rapidly on heating, while mass α to γ ratios of 1:1, 1:2 and 1:4 displayed effective protection of γ crystallin (Fig. 5B,C). At the lower α to γ mass ratios of 1:8 and 1:16, very effective chaperone protection of γ crystallin was observed in the case of the tuna, while much less chaperone protection was observed in the case of cow (Fig. 5A–C).

Table 3. *Empirically determined upper limit of thermal stability for α and γ crystallins from S200HR size fractionations*

Species	T_S (°C)	
	α crystallin	γ crystallin
D. mawsoni	47	33
T. obesus	55	39
B. taurus	68	50

T_S, thermal stability.
For S200HR size fractionations, see Fig. 2.

Chaperone protection of lysozyme by α crystallin from D. mawsoni*,* T. obesus *and* B. taurus

In the absence of α crystallin, lysozyme rapidly unfolded in 20 mmol l^{-1} DTT at 37°C, with A_{360} kinetics similar to heat denaturation, plateauing at A_{360}~1.1 over 1 h (Fig. 5D–F). Protection against DTT-induced denaturation of lysozyme by α crystallin (calculated by dividing the reduction in A_{360} between the unchaperoned and chaperoned assays by the A_{360} of the unchaperoned assay at the 1 h end point) increased with increasing amounts of α crystallin added. A 10:1 mass ratio of *D. mawsoni* α crystallin to lysozyme resulted in 79% protection, 66% at a 5:1 mass ratio, and 25% at a 2.5:1 mass ratio (Fig. 5D). For *T. obesus* α crystallin, an 11:1 mass ratio provided 88% protection, 75% at a 7.5:1 mass ratio, but only 9% at a 5:1 mass ratio of *T. obesus* α crystallin to lysozyme (Fig. 5E). The *B. taurus* α crystallin exhibited much greater chaperone ability than either fish, achieving 95% protection of lysozyme at a mass ratio of only 3:1 α crystallin to lysozyme (Fig. 5F).

Dynamic light scattering (DLS) sizing of α and γ crystallins

To determine whether the chaperone effect is related to a stable interaction between the α and γ crystallins, the sizes of individual crystallins, and of α and γ together before and after incubation at the T_S of the α crystallin of each species, were measured by DLS (Table 4). The hydrodynamic diameters of α crystallin at room temperature were 27.6 nm, 29.1 nm and 31.7 nm for toothfish, bigeye tuna and cow, respectively, while that of γ crystallin was about 4–5 nm for all three species. The major bovine α crystallin fraction from Sepharose 6B fractionation (data not shown) was found to be 19.4 nm in diameter, which is smaller than the 31.7 nm of the S200HR bovine α crystallin fraction. This discrepancy is probably due to the presence of residual high molecular mass aggregate in the S200HR α crystallin fraction, which partitioned as a small peak in Sepharose 6B elution ahead of the major α peak (data not shown). Except for the cow, the α crystallin increased in size after 1 h incubation at its T_S, consistent with 'activation' of the α crystallin chaperone complex (Putilina et al., 2003), and perhaps also due to association of α with the β crystallin present in the heterogeneous α crystallin fraction used. A mixture of γ and α crystallin (1:1 mass ratio) at room temperature has a similar size to α crystallin alone, probably because there is no molecular interaction between γ and α crystallin before heating, and thus the DLS size of the mixture essentially reflects that of the much bigger α crystallins. After a 1 h incubation at the respective α crystallin T_S, the hydrodynamic diameter of the of γ and α crystallin mixture increased by 94% (27.4–53.4 nm) for *D. mawsoni*, 142% (24.9–60.3 nm) for *T. obesus*, and 55% increase (from 30.3 nm to 47.2 nm) for *B. taurus* (Table 4), indicative of molecular association of the two crystallins forming a larger size complex.

Cross-species chaperone protection of γ crystallin by α crystallin

The ability of α crystallin of each species to protect the γ crystallin of a different species from heat aggregation was evaluated (Fig. 6). The chaperone assays were carried out at the T_S of the α crystallin, for pairwise combinations in which the γ crystallin has a lower T_S than that of the α crystallin. The combination of toothfish α plus cow γ at 47°C, though not meaningful as a chaperone assay since cow γ with a T_S of 50°C is more heat stable, was included as a 'non-interacting' control. Protection was observed for bigeye tuna α crystallin plus bovine γ at 55°C, toothfish α crystallin plus tuna γ at 47°C, and bovine α plus tuna γ at 68°C, with A_{360} reaching only ~0.05, 0.10 and 0.25 (respectively) at the 1 h end point. Tuna α crystallin did not completely protect toothfish γ at 55°C, and an A_{360} of 0.75 was reached at 1 h; however, the slope of the turbidity increase was much less steep (~tenfold) than that of toothfish γ alone at 55°C, indicative of partial interaction of the two crystallins leading to partial protection. The most notable and unexpected result is that cow α crystallin offered no protection at all to toothfish γ at 68°C, resulting in a rate of turbidity increase identical to that of toothfish γ alone at 55°C, indicating that all or most of the toothfish γ crystallins had precipitated (Fig. 6).

DLS measurements of heated heterologous αγ combinations showed that chaperone protection was associated with a size increase of the crystallins, indicating the formation of a αγ complex (Table 5). Assuming the pre-incubation DLS size of the pairwise α+γ combinations is similar to that of the α component, ~20–29 nm (Table 4), the post-incubation DLS size of the cross-species α+γ complex increased significantly, to ~43–73 nm for the combinations that exhibited chaperone protection (Table 5). In contrast, the hydrodynamic diameter of the heated cow α and toothfish γ combination (the supernatant after removal of protein aggregates) was the smallest, at 38.5 nm, indicating the least extent of interaction.

The extent of non-interaction of cow α with toothfish γ was assessed in a two-part chaperone assay (Fig. 7). Fig. 7A shows the A_{360} of a 1 h incubation of bovine α plus toothfish γ at 60°C, and the control incubation of bovine α plus bovine γ, both at mass ratio $\frac{1}{3}$ mg α to 1 mg γ in 1 ml final volume ($\frac{1}{3}$ mg of cow α crystallin is the estimated minimal amount that would completely protect 1 mg of bovine γ crystallin; Fig. 5). The 60°C incubation of bovine α crystallin plus toothfish γ crystallin resulted in rapid turbidity increase, with the same

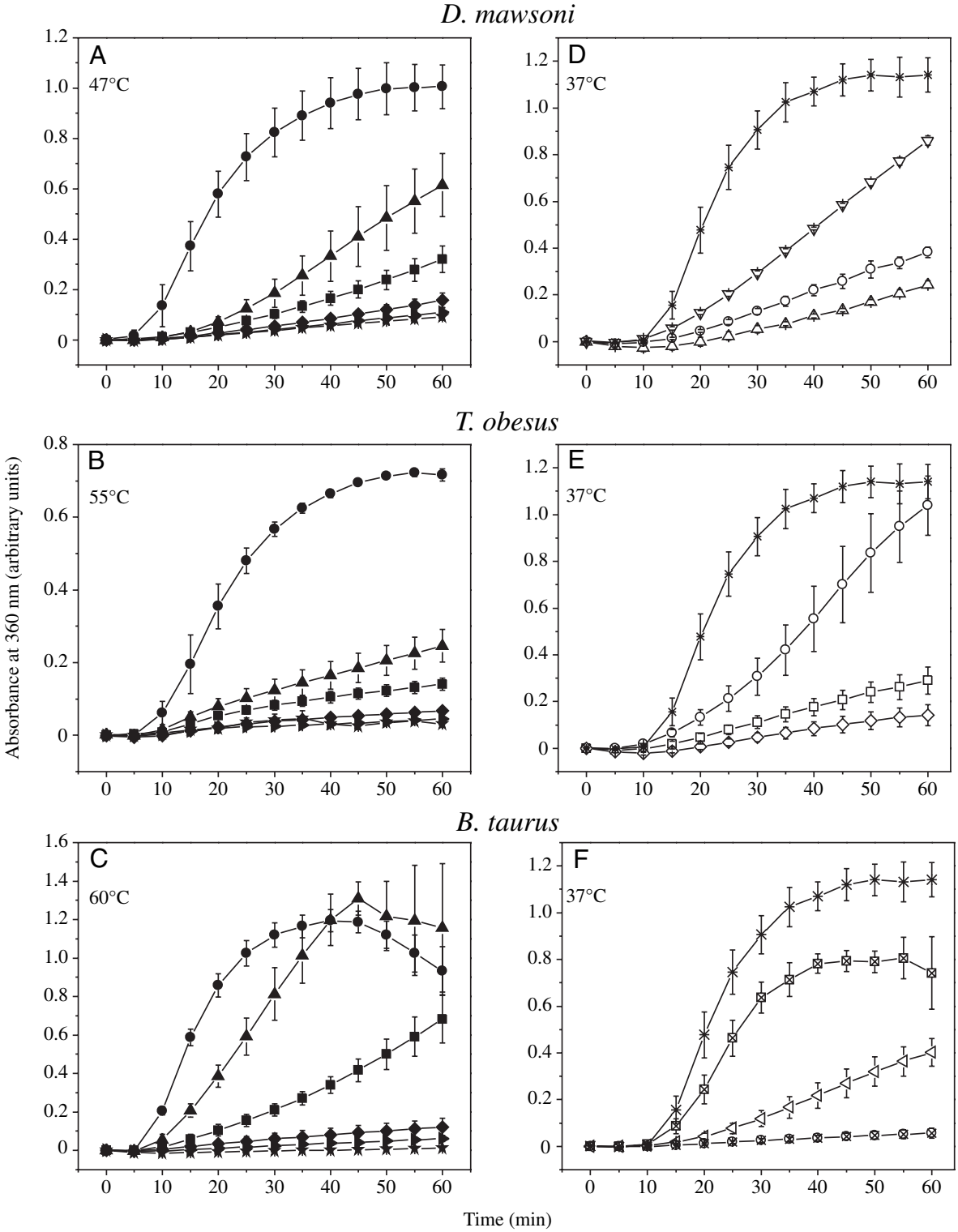

Fig. 5. Chaperone protection by α crystallin of heat-aggregated γ crystallin (A–C) along with protection of DTT-denatured lysozyme (D–F). (A,D) *D. mawsoni*; (B,E) *T. obesus*; (C,F) *B. taurus*. Temperatures for the assay are indicated in each panel. Values are means ± S.E.M. (*N*=3). In A–C the α crystallin and γ crystallin are both from the same species, with fractional amounts of α crystallin added based on a 1:1 mass ratio of α to γ crystallin at a final assay concentration of 1 mg ml^{-1} for both crystallins (i.e. ∗ in A–C). In D–F the amount of α crystallin added was based on a 1:1 mass ratio of α crystallin to lysozyme, where in all assays (D–F) there was 0.2 mg ml^{-1} of lysozyme containing 20 mmol l^{-1} DTT. For A–C: ● γ; ▲ 1:16 α:γ; ■ 1:8 α:γ; ◆ 1:4 α:γ; ▶ 1:2 α:γ; ∗ α:γ. For D–F: ∗ 0:1 α:lyso; ⊕ 1:1 α:lyso; ◁ 2:1 α:lyso; ⊗ 3:1 α:lyso; ▽ 2.5:1 α:lyso; ○ 5:1 α:lyso; □ 7.5:1 α:lyso; △ 10:1 α:lyso; ◇ 11:1 α:lyso.

Table 4. *Hydrodynamic diameters of α and γ crystallins determined by dynamic light scattering*

	Species crystallin diameter (nm)					
	D. mawsoni		*T. obesus*		*B. taurus*	
Crystallin	25°C	47°C	25°C	55°C	25°C	68°C
α	27.6	45.7	29.1	40.5	31.7	22.0
γ	4.0	ppt	4.8	ppt	5.0	ppt
α+γ	27.4	53.4	24.9	60.3	30.3	47.2

All measurements were taken at 25°C before and after a 1 h incubation period at the indicated temperatures.

Single crystallin proteins were measured at 1 mg ml^{-1}. Combined α and γ crystallin measurements made at a final concentration of 1 mg ml^{-1} for each crystallin.

ppt, precipitated.

Table 5. *Cross-species mixtures of α and γ crystallins*

Incubation temperature (°C)	1:1 mass mixture of crystallins (1 mg ml^{-1})		Hydrodynamic diameter (nm)
	α crystallin	γ crystallin	
68	*B. taurus*	*D. mawsoni*	38.5
55	*T. obesus*	*D. mawsoni*	72.4
47	*D. mawsoni*	*T. obesus*	50.3
68	*B. taurus*	*T. obesus*	43.4
47	*D. mawsoni*	*B. taurus*	51.5
55	*T. obesus*	*B. taurus*	73.2

Final concentration for each crystallin was 1 mg ml^{-1}.

All hydrodynamic measurements were made by dynamic light scattering (DLS) at 25°C after incubation for 1 h at the T_S for each fish's α crystallin (47°C for *D. mawsoni* ; 55°C for *T. obesus* and 68°C for *B. taurus* α crystallin).

kinetics and peak A_{360} as the 68°C incubation (Fig. 6), while bovine γ was fully protected by bovine α, as expected (Fig. 7A). After centrifugation, additional bovine γ crystallin was added to the supernatant of both incubations to the same final concentration (1 mg ml^{-1}) as the first 1 h incubation, for a second 1 h incubation at 60°C (Fig. 7B). The supernatant from the cow α crystallin plus toothfish γ crystallin incubation showed substantial chaperone protection of added cow γ crystallin in the second hour, reaching an A_{360} of only 0.486. This result indicates that most of the cow α crystallin did not complex with toothfish γ crystallin during the first 1 h incubation, and therefore was free to interact with the added cow γ crystallin in the second 1 h incubation. In contrast, the supernatant from cow α crystallin plus cow γ crystallin provided no protection of added cow γ crystallin in the second hour, reaching an A_{360} of 1.5 at 50 min. This indicates that little or no free cow α crystallin was available to chaperone the added cow γ crystallin, probably because most or all of the cow α crystallin was saturated or complexed to cow γ crystallin during the first 1 h incubation.

Discussion

The giant notothenioid fish *Dissostichus mawsoni* Antarctic toothfish is endemic to the Antarctic region of the Southern Ocean, where water temperatures are perennially at or near the freezing point of seawater (–2°C), the coldest known extreme for marine ectotherms. Its eye lens is transparent at this freezing temperature, indicating that the lens and its protein constituents are highly cold-stable. The whole lens cooling experiments presented in this study revealed that the toothfish lens remained clear even when cooled to temperatures as low as –12°C (Fig. 1j–l). This low temperature stability is in contrast to the endothermic mammalian lens, which undergoes rapid cold-induced lens opacity (cold-cataract) below 20°C, as was shown for the bovine lens in this study (Fig. 1e,f) and by others (Banh and Sivak, 2004; Clark and Benedek, 1980a; Delaye et al., 1982). Investigations of the phenomenon with intact teleost fish lenses have been sparse, limited to temperate and subtropical species (Hikida and Iwata, 1985, 1986; Loewenstein and Bettelheim, 1979). We have demonstrated that the cold stability of the Antarctic toothfish lens is far

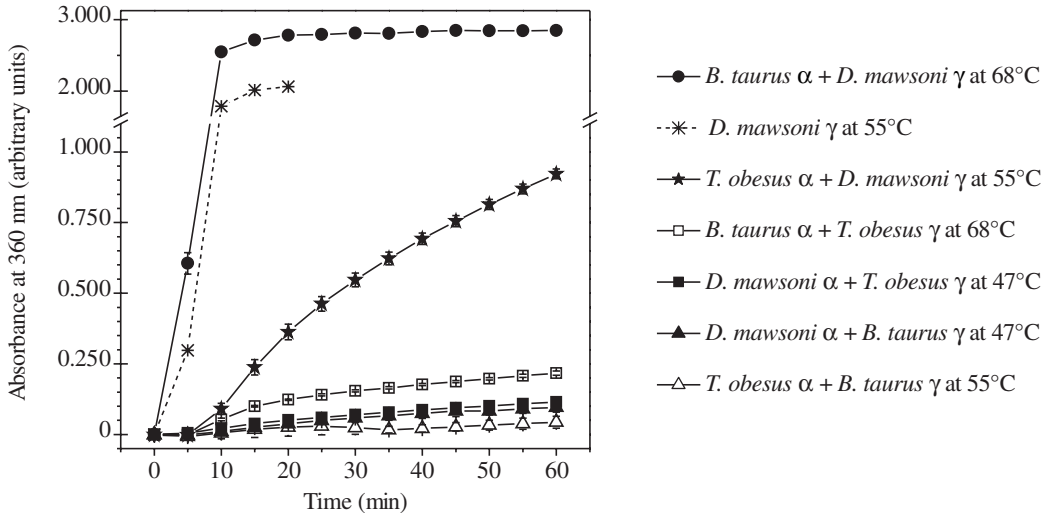

Fig. 6. Cross-species chaperone protection assay of γ crystallin by α crystallin from the three species *D. mawsoni*, *T. obesus* and *B. taurus*. Chaperone assay temperature was at T_S for the α crystallin in the assay. Final concentration of both α and γ crystallin in the assay was 1 mg ml^{-1}. Combinations of α and γ crystallin are indicated. Values are means ± S.E.M.; bars are obscured by symbols (*N*=3).

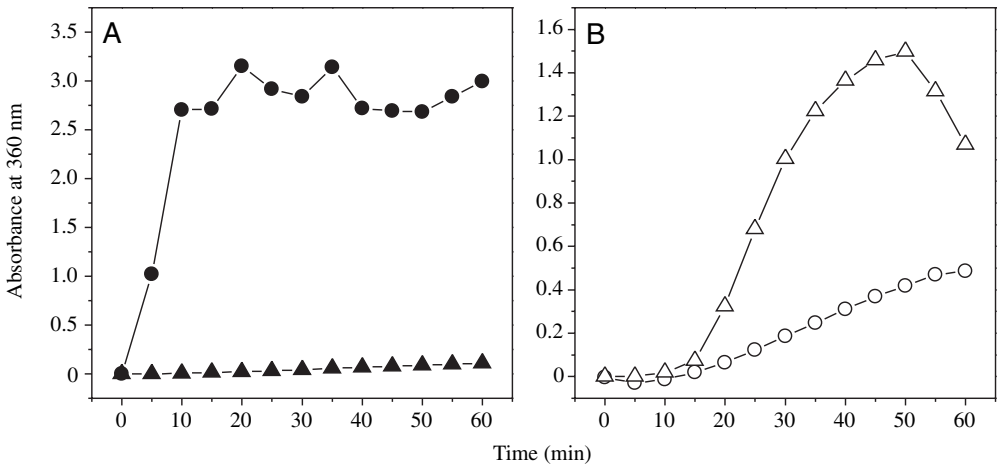

Fig. 7. Functional assessment of *B. taurus* α crystallin after 1 h incubation at 60°C in the presence of *D. mawsoni* γ crystallin. (A) Experimental $\frac{1}{3}$ mg *B. taurus* α crystallin + 1 mg *D. mawsoni* γ crystallin (●) and control reaction of $\frac{1}{3}$ mg *B. taurus* α crystallin + 1 mg *B. taurus* γ crystallin (▲). After 1 h samples were removed from the cuvette and any precipitate was removed by centrifugation at 21 000 ***g*** at 4°C in a benchtop microcentrifuge. (B) Supernatants (~750 µl) were then transferred to a new cuvette and a further 1 mg of *B. taurus* γ crystallin was added. *B. taurus* γ crystallin was concentrated such that the total volume in the second assay (after addition of the *B. taurus* γ crystallin) was not greater than 1 ml. Final composition of assay was (*B. taurus* α + *D. mawsoni* γ) supernatant + *B. taurus* γ crystallin (○) for the experimental and (*B. taurus* α + *B. taurus* γ) supernatant + *B. taurus* γ crystallin (△). Second assay incubation was for 1 h at 60°C.

greater when compared to the lens of other ectothermic teleost fish of warmer clime such as the tropical blackbar soldierfish, *M. jacobus*, which had a body temperature 24°C prior to experimentation (Fig. 1g–i). Though soldierfish lens develops irreversible cold-cataract, it is less cold-sensitive than bovine lens as prolonged exposure (⩾24 h) at 0°C was required, as opposed to the rapid onset of bovine cold-cataract at much higher temperature (Delaye et al., 1982). Thus, there is a direct correlation between body temperature and lens cold-stability (low temperature limit of lens clarity), with the cryophilic toothfish having the most cold-stable lens.

Cold-cataract formation in bovine lens is attributed to a liquid–liquid phase separation of one or more of the cold-labile γ crystallin isoforms (Broide et al., 1991; Siezen et al., 1985). Fish lenses are much denser than mammalian (bovine) lenses (Jagger and Sands, 1996, 1999; Pierscionek and Augusteyn, 1995), thus a plausible hypothesis might be that the greater cold stability of fish lens could be due to protein density-constrained mobility of the crystallin molecules, reducing the propensity for the kind of cold-induced structural rearrangement of the small γ crystallins that occurs in the much softer cow lens, leading to cold-cataract (Gulik-Krzywicki et al., 1984). However, while the increased density could account for the slower onset of the cold-cataract in the blackbar soldierfish, the toothfish lens is as compact and protein-dense as other teleost fish lens (slightly less dense than tuna), but is far more cold stable (Ferguson et al., 1971; Smith, 1972). Therefore, the basis of cold stability in ectothermic fish lens is likely to lie in the adaptive protein properties of the constituent crystallins for function in lower habitat temperatures (synonymous with body temperatures for ectotherms) than the warm-bodied mammals. The extraordinary cold stability of Antarctic toothfish lens exemplifies the preservation of normal protein function at the coldest known extreme of marine ectothermic vertebrate life, the freezing point of seawater. Moreover, initial results showing that the toothfish γ crystallin fraction at concentration of 58 mg ml^{-1} remains clear to temperatures as low as –10°C, which is 14°C lower than similarly concentrated cow γ crystallin fraction (Siezen et al., 1985), further implicates the cold-adapted γ crystallins as the basis of toothfish whole lens cold stability *in vivo*.

In order to examine the thermal, biochemical and physical properties of the individual classes of lens crystallins of Antarctic toothfish, we first fractionated whole lens homogenate by Sephacryl 200HR size-exclusion chromatography (SEC) (Fig. 2). This was also performed for the subtropical bigeye tuna *T. obesus* and the endothermic cow in parallel, to allow comparison of the relationship between crystallin protein properties and organismal body temperature. The three species have lenses of comparable size, and collectively they span the range of vertebrate body temperatures – the very cold-bodied toothfish (–2°C), the cool-bodied bigeye tuna (18°C), and the warm-bodied cow (37°C).

Incomplete SEC separation of individual classes of fish crystallin, especially between α and β crystallin, is a commonly reported difficulty (Chiou et al., 1987; Posner et al., 1999; Wistow, 1995), which also occurred for Antarctic

Table 6. *Temperature stability of the* D. mawsoni *γ crystallin(s)*

Temperature (°C)	A_{280}	Estimated mass (mg)	% reduction from initial value
4	4.56	3.65	0
47	1.05	0.84	77
55	0.62	0.50	86
60	0.20	0.16	96
70	0.04	0.03	99

The γ crystallin was incubated at the indicated temperature for 2 h, centrifuged and the A_{280} was read. Estimated masses are based on absorption coefficients (Table 1).

toothfish and bigeye tuna in this study (Figs 2, 3). Sepharose 6B, which provided good separations of bovine crystallins (Horwitz et al., 1998), is even less adequate than Sephacryl 200HR for fish crystallins separation (data not shown). While S200HR could not achieve complete separation of α crystallin from β crystallins, we documented in detail the identity of the crystallins in fractions spanning the heterogeneous α/β peak as well as in the β and γ elution peaks, with good resolution SDS gels and immunoblots (Figs 2, 3). This allowed us to perform the downstream experiments and interpret the results in a logical manner. The S200HR SEC produced near-homogeneous separation of the γ crystallin for all three species (Figs 2, 3), which permitted a reliable estimation of their percent abundance in the lens (Table 2). Gamma crystallins of the toothfish and bigeye tuna comprise over 40% of the lens crystallins, and are therefore the predominant class of crystallin in the fish lens. This relative abundance is comparable to the reported 52% in carp *Cyprinus carpio* lens (Chiou et al., 1987) and the 35.3% in bluefish *Pomatomas saltatrix* (Zigler and Sidbury, 1976). The percentage abundance of γ crystallins in toothfish (43%) and bigeye tuna (41%) lens is twofold higher than in the bovine (19%) lens (Table 2). The predominance of γ crystallin in fish lens and the predicted ability of γ crystallins to pack at high density (Summers et al., 1986) is consistent with the necessary refractive index differential required in an aquatic medium where little to no refractive benefit occurs between the water and the fish cornea, unlike in the terrestrial mammals that benefit from a substantial refractive index change between air and cornea (Jagger and Sands, 1996; Wistow, 1993; Wolken, 1995).

Examination of the thermal response of individual crystallin (α and γ) from the three species showed that γ crystallin is a more heat-labile component relative to α, and there is a direct correlation between organismal body temperature and heat stability (T_S) of the γ crystallins (γ fraction is near-homogeneous), with bovine being the most heat stable, followed by the tuna, and toothfish the least heat stable (Table 3). While the T_S of the α crystallin fraction of the two fish may be influenced to some extent by the presence of some β_H (Figs 2, 3), the clear relationship between T_S and organismal body temperature for the γ crystallins from these species support the increasing and disparate T_S values of α from toothfish, tuna and cow (47°C, 55°C, 68°C) as reflecting the same relationship. The thermal stabilities of the individual crystallin components agreed with the findings in our whole lens cold stability experiments, with low organismal body temperature correlated with the greatest cold stability (Fig. 1). The T_S values for the α crystallins of the three species are 31–50°C higher than the body temperature, and T_S of the γ crystallins are 13–35°C higher. Our heat tolerance findings of the individual crystallin classes are consistent with a previous whole lens study that showed lens transparency was maintained at temperatures up to 20°C higher than the normal body temperature of a number of vertebrate animals (McFall-Ngai and Horwitz, 1990).

Vertebrate α crystallin is a small heat shock protein (Horwitz, 1992; Ingolia and Craig, 1982), thus a common approach in the studies of lens crystallin properties is to examine the thermal response of the different crystallins, and the chaperone-like protection by α crystallins of the heat-labile crystallin components, in assessing the mechanism of molecular interaction between them. Chaperone assays in the bovine system have focused on protection of the β crystallin fractions, the most abundant non-chaperone crystallin in cow lens (Augusteyn et al., 2002; Horwitz, 1992). In teleost fish lens, the γ crystallins are the major heat-labile constituents (Fig. 2, Table 2). Sequence similarity between fish and mammalian α crystallins indicated that the former are also sHSPs and would have chaperone-like activity (Posner et al., 1999; Runkle et al., 2002), which has indeed shown to be so for recombinant αA crystallin from zebrafish in chaperone assays with DTT-denatured lysozyme (Posner, 2003), and for recombinant αB from carp with DTT-denatured insulin (Yu et al., 2004). Here we demonstrate that native α crystallin isolated from the lens of two other fish, Antarctic toothfish and bigeye tuna, are also effective molecular chaperones, with the ability to protect the same species heat-labile γ crystallins from aggregation induced by heat (Fig. 5A–C), and chemical denaturation of non-lens protein (lysozyme) by DL-dithiothreitol (Fig. 5D–F). The relative effectiveness of the α crystallins of the two fish species as a chaperone may not be directly compared to each other or to the cow, because of uncertainty over the actual amounts of pure α crystallin in the SEC α crystallin fraction used in these assays, the presence of some β_H crystallin in these fractions that may also interact with the α crystallin during heat or DTT exposure reducing the amount of α crystallin available for binding to the test protein, and the different species-specific assay temperatures (T_S) and thermal properties of the test γ crystallin. Notwithstanding, the chaperone-like nature of native fish α crystallins with the hallmark ability to prevent stress-induced aggregation of unrelated proteins (Narberhaus, 2002) was clearly established.

Chaperone-like heat protection assays are commonly carried out at high *in vitro* temperatures (Augusteyn et al., 2002; Horwitz, 1992; Liao et al., 2002). While such temperatures are not physiological, the chaperone protection has its mechanistic basis in molecular interactions between α crystallins and labile molecules, which can be manifest upon heating, and thus this experimental approach is instructive for understanding inter-crystallin molecular interactions *in vivo*. The chaperone-like ability of α crystallin was deduced to require a heat-activated structural modification that was necessary for association with other crystallins, based on the pronounced size increase observed in bovine α crystallins at 60°C, with a further increase at 66°C, by small angle X-ray scattering (SAXS; Putilina et al., 2003). Our DLS measurements showed that the fish α crystallins heated at their respective T_S for 1 h resulted in a larger hydrodynamic diameter (Table 4), consistent with heat-activated formation of a larger oligomeric structure. The fish α crystallin fractions contained various amounts of β crystallins; thus the heat-induced size increase could be partly due to formation of an α/β heterocomplex. However, the

further increase in size after incubation of α crystallin fraction with the same-species γ crystallin (Table 4) supports the molecular association of γ crystallin molecules with a heat-activated α crystallin oligomeric structure, relevant to the observed chaperone activity. In the bovine system, a similar increase in α crystallin size when heated in the presence of heat-labile β_L crystallin has recently been reported when measured by SAXS (Krivandin et al., 2004).

There was an apparent anomalous decrease of the cow α crystallin DLS size of 31.7 nm to 22.0 nm upon heating (Table 4). The large size of 31.7 nm of unheated cow α crystallin can be attributed to the presence of a small amount of large α crystallin oligomers in the S200HR SEC α crystallin fraction. The presence of this large α crystallin component was confirmed by Sepharose 6B chromatography of bovine lens homogenate, in which it eluted as a small distinct peak, ahead of the dominant α crystallin peak that contains the remaining and more abundant smaller α crystallins (data not shown) that gave a 19.4 nm on DLS sizing. The DLS signal of a heterogeneous size mixture tends to be dominated by that of the large size component even if it is present in smaller amounts, thus the presence of the smaller α crystallins in the S200HR α crystallin fraction was masked by that of the much larger α oligomers. Upon heating, the large α oligomers likely increased rapidly in size, and sedimented when the sample was spun after heating and prior to DLS sizing, leaving the soluble 'activated' α crystallin that gave the 22.0 nm size. The 19.4 nm bovine α crystallin from the major α crystallin peak of the Sepharose 6B separation probably represents the true 'unactivated' α crystallin prior to heating, and agrees more closely with previously reported results (Abgar et al., 2001; Putilina et al., 2003; Vanhoudt et al., 2000).

The cross-species chaperone heat protection assays to evaluate the ability of warm-adapted crystallins to interact with cold-adapted crystallins produced an important and unexpected result; the total lack of protection of toothfish γ crystallin by cow α crystallin, which is very likely to be related to adaptive changes in protein properties that evolved in disparate thermal environments (Fig. 6). For the combinations of cow α plus tuna γ at 68°C, tuna α plus cow γ at 55°C, and toothfish α plus tuna γ at 47°C, the α component could fully or nearly fully protect the heat-labile γ component, regardless of the body temperatures of the species from which the α crystallin was derived, and despite the disparity between the body temperatures of the species pair. This attests to the small heat shock protein ancestry of the vertebrate α crystallins (de Jong et al., 1993), with their intrinsic chaperone-like ability to provide stress protection of other protein molecules regardless of ectothermic or endothermic organismal origin. However, while tuna α could fully protect cow γ, it only partly protected toothfish γ crystallin, and the cow α crystallin completely failed to protect the toothfish γ (Fig. 6). Since both cow and tuna α crystallins have proven chaperone-like function, the lack of protection of the toothfish γ crystallin is likely a result of particular biochemical properties of the latter. Conceivably, the changes in the γ crystallins that have occurred in the frigid Antarctic environment over evolutionary time to maintain the toothfish lens transparency may be the basis for the observed weak interaction with the α crystallin from the warmer bodied bigeye tuna, and complete non-interaction with the α crystallin from the warm-bodied cow.

That the non-protection of toothfish γ crystallin by cow α is a result of non-interaction between the two crystallins was supported by the results of the two-part chaperone assay (Fig. 7). In the first 1 h incubation, the kinetics of the turbidity (A_{360}) increase (Fig. 7A) were the same as observed when heating toothfish γ crystallins alone (Fig. 6), indicating that the heat-precipitated protein was predominantly toothfish γ crystallins, presumably due to their failure to associate with cow α crystallin to gain protection. The reasonable expectation then, is that after removing the heat-precipitated protein the supernatant should consist primarily of functionally intact cow α crystallin, which was confirmed by the significant protection of added cow γ crystallins for an additional hour of heat incubation (Fig. 7B). Thus the non-protection in the first hour of incubation must indeed be due to non-interaction between the two crystallin components. To ensure that any protection in the second 1 h test incubation is not due to excess cow α crystallins, in the first 1 h incubation we only used $\frac{1}{3}$ mg of cow α crystallins, which is estimated as the minimal amount of cow α crystallins that will provide full protection of 1 mg of cow or tuna γ crystallins (Fig. 5). The supernatant from the first 1 h incubation of control cow α + γ crystallin did not protect added cow γ crystallin in the second 1 h incubation (Fig. 7B), which confirmed that the $\frac{1}{3}$ mg cow α crystallins was indeed the appropriate minimum, and that they must have fully complexed to the bovine γ in the first 1 h incubation.

The protection by the supernatant from the cow α plus toothfish γ incubation of added cow γ crystallin in the second 1 h test incubation is not complete, for two reasons. It is difficult to determine the amount of cow α crystallins remaining in the supernatant to accurately achieve the minimum α to γ mass ratio of $\frac{1}{3}$:1. Secondly, a portion of the cow α crystallins may have been complexed with some toothfish γ crystallins that are stable at 60°C in the first 1 h incubation. Stepwise incubation of a toothfish γ crystallin sample showed that the majority of the isoforms (77%) were heat-labile at 47°C, but a small percentage (4.4%) remained soluble at 60°C (Table 6), and presumably these could complex with cow α crystallins in the first 1 h incubation. Regardless, the results of the two-part incubation assay strongly support the majority of Antarctic toothfish γ crystallins being sufficiently biochemically different that they do not associate with cow α crystallins, resulting in the lack of chaperone protection when heat stressed.

The structural basis for the non-interaction between the majority of toothfish γ crystallin polypeptides with cow α crystallins, or weak interaction with tuna α crystallins, is currently unknown. It is conceivable that the pertinent structural properties of toothfish γ crystallin responsible for this lack of interaction with α crystallins of other warmer-bodied species may be the same ones that contribute to the

extraordinary cold stability of the Antarctic toothfish lens, such that it does not suffer cold-cataract even at –12°C (and probably lower). The Antarctic toothfish has an estimated evolutionary age of 7–14 million years (Chen et al., 1997a), and its lens has clearly evolved to preserve transparency at the freezing Antarctic seawater temperature of –2°C. Because of the important role of γ crystallins in teleost fish lens in achieving the high protein density and refractive index gradient required for aquatic light diffraction, it is reasonable to expect that key structural adaptations would occur in the γ components of the lens when subjected to environmental selection. In addition, studies of the bovine system have conclusively demonstrated that it is one or more of the γ crystallins that display a cold-lability and are responsible for mammalian whole lens cold-cataract (Broide et al., 1991; Clark and Benedek, 1980b; Siezen et al., 1985; Thomson et al., 1987). The adaptive amino acid changes that have occurred in the Antarctic toothfish γ crystallins may be ascertained by cDNA cloning for comparative sequence analyses with γ crystallins of different taxa (work in progress). It has been demonstrated that non-polar or so-called 'hydrophobic' residues are more easily solvated by water at low temperatures, which diminishes the importance of these 'hydrophobic' interactions as the predominant intramolecular stabilising factor at low temperatures (Privalov, 1990; Tsai et al., 2002). Previous reports indicate that the association of protein subunits in the polymerisation of actin filaments in the relatives of the Antarctic toothfish, *Pagothenia borchgrevinki* and *Gymnodraco acuticeps*, as well as in the stabilisation of myosin in the related *Notothenia rossii*, are found to rely less on hydrophobic interactions (Hochachka and Somero, 2002; Johnston et al., 1975). Moreover, chaperone function of the bovine α crystallin is believed to involve complexing with the unfolding labile protein largely through hydrophobic interactions (Raman and Rao, 1997). Thus, an evolutionary decrease in the hydrophobicity of the toothfish γ crystallins for adaptation in the cold would be consistent with diminished interaction of toothfish γ crystallin with the α crystallins of other species.

A reduced hydrophobicity in the toothfish γ crystallin, however, has structural implications for toothfish α crystallin if the primary basis for α chaperone function is *via* hydrophobic interactions with the labile protein. Because attempts to identify chaperone essential aspects from mammalian α crystallin polypeptides have been less than convincing, suggesting that the chaperone process is multifaceted and complex and not dependent on one 'key' element (Derham et al., 2001; Kokke et al., 2001; Pasta et al., 2002, 2003; Posner, 2003), the specific effect of reduced hydrophobicity remains to be determined. In other words, the toothfish α crystallin may have co-evolved to achieve the structural requisites for interacting with the less hydrophobic toothfish γ crystallin. It is not known if there is reduced hydrophobic residue content in all the toothfish γ crystallins, but this can be ascertained by comparative sequence analyses with homologues from different ectothermic and endothermic taxa. Any resultant higher order structural characteristics of the crystallin proteins that may be responsible for cold stability are testable with comparative tertiary and quarternary structure analyses of native and site-directed mutants.

An alternative to the hypothesis of adaptive changes in the toothfish γ crystallin proteins as the primary basis for whole lens cold stability is the possibility that toothfish lens is largely composed of a homologue of the bovine γ_S crystallin, which has recently been shown to be extremely cold-stable, with a theoretical liquid–liquid phase separation temperature of –28°C (Annunziata et al., 2003). However, our SEC fractionation of fish lens homogenates did not show an elution peak corresponding to the bovine γ_S (Fig. 2). While fish γ_S may have co-eluted in the only and prominent γ crystallin peak, our ongoing work, including ion-exchange separation, cDNA cloning and sequencing, and two-dimensional PAGE analysis of the toothfish γ crystallin peak, has indicated a richer diversity of unique individual γ isoforms present in the toothfish lens than in bovine lens, which is inconsistent with the predominance of a single isoform. Continued structural elucidations of the toothfish γ crystallins will help provide insights into the remarkable cold stability of the toothfish lens, which may in turn enhance our understanding of the cold sensitivity of their mammalian counterparts.

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