Eur. J. Biochem. **268**, 713–721 (2001) © FEBS 2001

Chaperone function of mutant versions of αA- and αB-crystallin prepared to pinpoint chaperone binding sites

Barry K. Derham[1], Martinus A. M. van Boekel[2], Paul J. Muchowski[3], John I. Clark[3], Joseph Horwitz[4], Henry W. Hepburne-Scott[5], W. W. de Jong[2], M. James C. Crabbe[5] and John J. Harding[1]

[1]*Nuffield Laboratory of Ophthalmology, Walton Street, University of Oxford, UK;* [2]*Department of Biochemistry, University of Nijmegen, the Netherlands;* [3]*Department of Biological Structure, University of Washington, Seattle, USA;* [4]*Jules Stein Eye Institute, University of California at Los Angeles School of Medicine, USA;* [5]*School of Biological Sciences, University of Reading, UK*

A major stress protein, α-crystallin, functions as a chaperone. Site-directed mutagenesis has been used to identify regions of the protein necessary for chaperone function. In this work we have taken some of the previously described mutants produced and assessed their chaperone function by both a traditional heat-induced aggregation method at elevated temperature and using enzyme methods at 37 °C. In general the different assays gave parallel results indicating that the same property is being measured. Discrepancies were explicable by the heat lability of some mutants. Most mutants had full chaperone function showing the robust nature of α-crystallin. A mutant corresponding to a minor component of rodent αA-crystallin, αAins-crystallin, had decreased chaperone function. Decreased chaperone function was also found for human Ser139→Arg, Thr144→Arg, Ser59→Ala mutants of αB-crystallin and double mutants Ser45→Ala/Ser59→Ala, Lys103→Leu/His104→Ile, and Glu110→His/His111→Glu. A mutant Phe27→Arg that was the subject of previous controversy was shown to be fully active at physiological temperatures.

Keywords: alpha crystallin; chaperone; mutants; chaperone assays.

Attempts to identify regions of α-crystallin important for chaperone function have included the use of point mutants, prepared by site-directed mutagenesis [1–7]. There are several methods for quantifying chaperone function of proteins. For α-crystallin these have included heat-induced aggregation of β-low crystallin and γ-crystallin at elevated temperatures [8], precipitation of proteins on reduction of disulphide bonds usually at 25 °C [9,10], and inactivation of enzymes by glycation or other chemical modifications at 37 °C [11–14]. Usually these methods give parallel results [3,15,16] supporting the notion that the different assays are measuring essentially the same property. However this area is not without its controversies [3,5]. Furthermore van Boekel *et al*. [17] point out differences in the relative effectiveness of αA-crystallin and αB-crystallin as molecular chaperones at different temperatures.

It has been postulated that α-crystallin consists of three major regions, the putative N-terminal domain (residues 1–63), central region (residues 64–105), and the C-terminal or 'α-crystallin domain' followed by the C-terminal extension (106–173–175 including the extension) [18,19]. Changes in each of the three regions of α-crystallin occur in the different mutants. The N-terminal region is exposed [20,21]. Removal of the N-terminal methionine occurs throughout ageing [22], and truncation of the N-terminus of αA-crystallin and αB-crystallin was found in the water-insoluble fraction of human lens α-crystallin [23].

Mutations within the N-terminal region of the αB-chain (reviewed in [19]) cause major decreases in chaperone function. These mutations all involve conserved Phe residues [24]. There is some controversy over whether changes to Phe24 and 27 in a phenylalanine rich sequence of the αB-chain caused a change in chaperone function or just in the stability of the protein [3,5]. Plater *et al*. [3] suggested that replacement of the hydrophobic Phe24 and 27 by strongly charged arginines caused serious disruption of the adjacent region (residues 28–34). Mutations of Asp2, Phe24 and Phe27 showed similar decreases in chaperone function *in vivo* and *in vitro* [3]. Horwitz *et al*. [5] found normal chaperone activity for Phe27→Arg. This controversy was investigated in the present work.

In the central region, mutation of a conserved Asp69 from αA-crystallin decreases chaperone function [2]. Asp69 is in a highly conserved cluster of charged residues, EVRSDRD(69)K. However, it is not in a region implicated in chaperone function by the other methods such as the localization of the alcohol dehydrogenase binding region, and determination of hydrophobic regions [19]. Substitutions involving a change in charge within the α-crystallins are rare [25]. The dramatic decrease in chaperone function in Asp69→Ser suggests those ionic interactions, as well as the hydrophobic interactions seen in the N-terminus, influence chaperone function. However, as the only chaperone activity assay used was at 58 °C [2], it is possible that the mutation simply destabilized the protein.

Correspondence to J. J. Harding, Nuffield Laboratory of Ophthalmology, University of Oxford, Walton Street, Oxford, OX2 6AW, Great Britain. Fax: + 44 1865 794508, Tel.: + 44 1865 248996, E-mail: john.harding@eye.ox.ac.uk

Abbreviations: sHSP, small heat shock protein.*Enzymes*: malate dehydrogenase from porcine heart (EC 1.1.1.37); glyceraldehyde 3-phosphate dehydrogenase (EC 1.2.1.12).

(Received 7 November 2000, accepted 24 November 2000)

Mutants have been engineered to characterize the 'α-crystallin domain', and the exposed C-terminal extension. The sequential set of 12 cysteine mutants between Tyr109 and Leu120 of αA-crystallin [4], a region of the 'α-crystallin domain' that is conserved among small heat shock proteins (sHSPs), did not alter chaperone function yet caused irregular variations in aggregate size [26]. The C-terminal region of α-crystallin has the greatest homology to other sHSPs, and the mutagenesis studies of Muchowski *et al*. [7] addressed the role of sites in this region that might be important for chaperone function. Mutations in this part of both αA- and αB-crystallin have been associated with human cataract. These mutants, Arg116→Cys of αA- and Arg120→Gly of αB-crystallin, have diminished chaperone function [27–30]. However they also have an abnormal secondary and tertiary structure demonstrable by circular dichroism spectra.

There is little homology between the C-terminal extension of mammalian sHSP25 and α-crystallin [31] and the C-terminal extensions of the αA- and αB-crystallin are very different from each other [32]. ^{1}H-NMR spectroscopy revealed short, very flexible C-terminal extensions of eight (αA) and 10 (αB) amino acids, which adopt little specific conformation in solution [33]. The polar nature of the extensions may enable them to function as solubilizing agents for the α-crystallin and the high molecular mass complex [34]. However, the flexibility and solvent accessibility makes the C-terminal extension susceptible to cleavage [35,36]. The functional importance of the C-terminus was demonstrated when increasing cleavage of 16 terminal amino acids decreased the ability of αA-crystallin to inhibit heat aggregation of proteins [37,38]. Recombinant αA-crystallin with the last 16 amino acids absent had a marked loss of chaperone function and tertiary structure integrity, as well as decreased solubility [39]. Paradoxically, *in vitro* proteolytic removal of the C-terminal Thr171→Lys does not significantly affect αB-crystallin chaperone function. Removal of the last 42 residues of sHSP27 decreased chaperone function [40]. Although the αB-chain may have played the major role in chaperone function *in vivo,* assays *in vitro* indicate that it is no more effective than the αA-chain [17], although their relative functions alter with temperature [41].

α-Crystallins of rodents and a variety of unrelated mammalian species contain a minor product which results from alternative splicing of an additional exon [42] and constitutes 10–20% of the αA-crystallin mRNA [43]. It has 23 amino acids inserted into the connecting segment between residues 63 and 64 at the junction of the putative N-terminal domain and the central region [18]. The αAins-crystallin (196 residues) complex is larger than that of αA-crystallin and more disperse [44].

Sixteen mutant α-crystallins were available for our investigations in Oxford, using two different chaperone assays, enzyme inactivation and heat aggregation assays, to investigate the effect of specific mutations on the chaperone function of α-crystallin. Both assays produce similar results [15,16]. The heat aggregation assay is less physiological than the enzyme inactivation assay but has been more widely used to date. The mutants include αAins-crystallin, and others with changes to the C-terminal extension of αA-crystallin, highly conserved regions of αB-crystallin, and the controversial Phe27→Arg mutation of αB-crystallin. Our aims were to resolve the controversy over one mutant, Phe27→Arg; to gain further insight into which regions of α-crystallin are important for its chaperone function; to determine whether an enzyme inactivation assay gave parallel results to other methods with the mutant α-crystallins; and to achieve a better understanding of the mechanism of the chaperone function of this fascinating small heat shock protein.

MATERIALS AND METHODS

Commercial materials

Malate dehydrogenase from porcine heart (EC 1.1.1.37), glyceraldehyde 3-phosphate dehydrogenase (EC 1.2.1.12) NADH, oxaloacetate, SDS and other chemicals were from Sigma Chemical Co. (Poole, Dorset, UK).

α-Crystallin mutants

Wild-type and mutants of bovine αA-crystallin [2] and rat αAins-crystallin [1] were as described and had been sequenced. The specific C-terminal extension changes are presented in Fig. 1. The human αB-crystallin mutant Phe27→Arg [5], and the other human αB-crystallin mutants, Ser139→Arg, Thr144→Arg, Gly147→Arg, Ser19→Ala, Ser45→Ala, Ser59→Ala and combinations of Ser45→Ala plus Ser59→Ala, and Gly141→Arg plus Ser19→Ala [7] were as described. The cDNAs had been sequenced in both directions. This last mutant was

CHANGE TO *C*-TERMINAL EXTENSION

	170	171	172	173	
	— Ala—	Pro—	Ser —	Ser — OH	Wild-Type
A	— Ala—	Pro—	Ser —	**Lys**—OH	
B	— Ala —	**Leu**—	**Gly**—	**Lys**—**Gly**—OH	
C	— Ala —	**Leu**—	**Asp**—	**Lys**—**Gly**—OH	
D	— Ala —	**Leu**—	**Trp**—	**Lys**—**Gly**—OH	

Fig. 1. Mutations of bovine αA-crystallin (bold). All mutants had similar aggregate size and conformation as the wild-type except the Trp mutant which was smaller and had reduced flexibility of the C-terminal extension.

originally designated as Gly141→Arg [7] but was subsequently found to have the second point mutation (van den IJsell and Quinlan, personal communication). Mouse αB-crystallin mutants, one single Phe118Ala and two double mutants, Lys103→Leu/His104→Ile, and Glu110→His/His111→Glu have been described [6].

The chaperone function of the wild-types and mutants were assessed by the fructose inactivation of malate dehydrogenase assay (see below) and the heat-induced aggregation of β-low crystallin assay (see below) carried out in parallel in Oxford. In addition the αB-crystallin mutants were assayed using the heat-induced aggregation of glyceraldehyde 3-phosphate dehydrogenase (see below).

The purity of the mutants was checked using SDS/PAGE.

SDS/PAGE

SDS-polyacrylamide gel electrophoresis was performed according to Laemmli [45] under reducing conditions using LKB Bromma 2052 Midget Electrophoresis Unit.

Chaperone assays

β-Low crystallin heat-induced aggregation assay. The ability of α-crystallin mutants to prevent the increase in turbidity on heating solutions of β-low crystallin was assessed by the method described by Horwitz [8] and modified by Derham and Harding [15].

Rabbit β-low crystallin (1.87 mg) was dissolved in 6 mL of degassed 0.05 M sodium phosphate buffer, pH 6.7. The β-low crystallin solution was passed through a Gelman 0.2 μM pore-sized sterilized Millipore filter into a sterilized glass container. Taking 0.8 mL of the β-low crystallin solution gave a concentration of 250 $\mu g \cdot mL^{-1}$ of β-low crystallin solution within the 1 mL plastic cuvettes (Whatman). α-Crystallin (1.25 mg) was dissolved in 1 mL of degassed 0.05 M sodium phosphate buffer, pH 6.7. Taking 100 μL of the α-crystallin stock solution and placing it within the 1 mL disposable polystyrene cuvettes 10 mm path length already containing 0.8 mL β-low solution gave a final incubation concentration of 100 $\mu g \cdot mL^{-1}$ (w/w ratios of 1 α-crystallin to 2.5 β-low crystallin). This is the minimum proportion of α-crystallin for protection, in order that any functional impairment of the mutants would be detectable. Two cuvettes containing the control of β-low crystallin and the α-crystallin under observation were examined in every experiment. The cuvette solutions were stirred and placed in the six-cell holder of the Kontron 930 spectrophotometer connected to a heated water bath set to 55 °C within the cuvettes. To prevent evaporation the cuvettes were capped.

The light scattering within the cuvettes resulting from heat-induced aggregate formation was monitored at 360 nm continuously for 60 min. Chaperone function was represented as the percentage of protection afforded by α-crystallin against the scattering produced by the β-low crystallin control aggregation after 60 min. All base lines at time zero were zeroed to that of the control target protein. All assays were repeated in triplicate.

Glyceraldehyde 3-phosphate dehydrogenase heat-induced aggregation assay. This assay monitoring the heat-induced aggregation of the enzyme glyceraldehyde 3-phosphate dehydrogenase closely resembles that described above with β-low crystallin. Glyceraldehyde 3-phosphate dehydrogenase was prepared to give a concentration of 250 $\mu g \cdot mL^{-1}$ within the 1 mL cuvettes, as previously described. The α-crystallin solution was prepared and incubated as described for β-low crystallin above, however, the water bath was set to maintain 37 °C within the cuvettes. The assay was started, monitored and results recorded as described for the β-low heat aggregation assay.

Malate dehydrogenase inactivation assay. The ability of α-crystallin to preserve the activity of malate dehydrogenase (MDH) during incubation with fructose was assessed by the method previously described [13,15,16]. The enzyme activity of MDH was determined by monitoring the decrease in absorbance at 340 nm of NADH as it is oxidized by oxaloacetate using a Kontron 930 spectrophotometer.

Solutions were prepared in 0.05 M potassium phosphate buffer, pH 7.5 and sterilized through 0.2-μm pore-size Millipore filter except malate dehydrogenase (shaken before use) solution, which was prepared in 50 mM potassium phosphate buffer, pH 7.0. Firstly, 2.84 mL of the above buffer (preheated to 37 °C) was mixed with 30 μL of MDH solution (7.5×10^{-5} mol), 100 μL of oxaloacetate ($2 mg \cdot mL^{-1}$) and 30 μL of NADH ($10 mg \cdot mL^{-1}$) in the spectrophotometer at 37 °C in a quartz cuvette. All solutions were shaken before use. Oxaloacetate and NADH were stored on ice and in darkness to prevent degradation. The mixture was stirred rapidly and the decrease in absorbance was monitored against a buffer blank. The on-board computer of the spectrophotometer calculated the linear rate of NADH oxidation between 0.5 and 1.5 min.

For glycation of malate dehydrogenase, the enzyme was incubated in 0.05 M potassium phosphate buffer, pH 7 with 5 mM fructose. Stock solutions of the incubation solution with and without the sugars were made and separated into individual 1.5 mL sterilized vials with rubber tops through a sterilized 0.2-μm pore-sized sterilized Millipore filter. The vials were placed in a small plastic holder half-filled with water, in a shaking water bath at 37 °C. Zero time readings were taken in triplicate from single vials and further vials were assayed at 6 h. All assays were on individual vials.

α-Crystallin protects MDH against glycation-induced inactivation [13]. α-Crystallin was dissolved in 0.05 M potassium phosphate buffer, pH 7.0 and then added to a solution of MDH to give an α-crystallin concentration of $0.12 mg \cdot mL^{-1}$ in the incubation. This is the minimum proportion of α-crystallin to give protection. The incubation solutions were made up in 1.5 mL sterile glass vials as above. The vials were placed in a shaking water bath at 37 °C. Zero time readings were taken in triplicate from single vials and further vials were assayed at 0 and 6 h. Specific incubations of MDH and α-crystallin together with and without fructose were produced. Chaperone function was represented as a percentage of activity remaining compared to the corresponding α-crystallin incubation. All readings were repeated in triplicate and all assays were repeated in triplicate. All assays were on individual vials.

RESULTS AND DISCUSSION

It has been proposed that the structures of α-crystallin, and other sHSPs, are organized into N- and C-terminal domains, linked by a smaller central domain, corresponding to the exon positions each consisting of two structurally related motifs followed by a remaining variable C-terminal extension sequence [18,32]. The mutations investigated in this paper encompass all three regions of α-crystallin. The Ser to Arg mutations were in the 'N-terminal domain' whereas the mutations in the conserved region 139–147 are in the putative 'α-crystallin domain' and the C-terminal extension mutants are located immediately after the 'α-crystallin domain'.

The aggregation sizes of the recombinant preparations were slightly smaller than that of α-crystallin isolated from calf lens water-soluble fraction. Near- and far-UV CD of individual recombinant α-crystallins [49] differ from those of isolated mammalian α-crystallin. This may be due to post-translational modifications not present in recombinant proteins. The CD of the recombinant arginine mutants associated with cataract differed even from recombinant wild-type α-crystallins [27,28].

Purity of mutants

The purity of the recombinant αA- and αB-crystallins was investigated using SDS/PAGE. All six αA-crystallins and the αB-crystallins were very pure (no extra bands present) and their position on the gels resolved around 20 kDa except for αAins-crystallin, which ran more slowly on account of the extra 23 amino acids (Fig. 2).

Chaperone function of αA- and αAins-crystallin mutants

Wild-type αA-crystallin caused almost total suppression of aggregation of β-low crystallin and protection from enzyme inactivation (Fig. 3). αAins-crystallin displays a diminished chaperone activity of approximately 50% using the two different assays (Fig. 3) as reported previously [1], supporting the idea that the different assays are measuring essentially the same property. The C-terminal extension mutant Ser173→Lys (mutant A), which increases the positivity of the αA-crystallin C-terminal extension, completely protected against aggregation of α-low crystallin and enzyme inactivation (Fig. 3). Likewise, replacement of the last three residues Pro-Ser-Ser with Leu-Gly-Lys-Gly (mutant B), which increases length and positive charge, maintains normal chaperone ability (Fig. 3). When the last three residues were replaced with Leu-Asp-Lys-Gly (mutant C), which has the same net charge as the wild-type αA-crystallin but has increased extension polarity, there was no loss in chaperone ability (Fig. 3). These results are in parallel with those of Smulders *et al.* [50], which confirms the role of α-crystallin as a molecular chaperone and indicates that different chaperone assays are measuring essentially the same function.

The introduction of a Trp residue into the C-terminal extension (mutant D) transforms it from predominantly hydrophilic to very hydrophobic in nature. This mutant gave complete protection against the glycation-induced inactivation of malate dehydrogenase at 37 °C, but negligible protection against aggregation of β-low crystallin at 55 °C. When the Trp mutant was assayed by heat aggregation of β-low crystallin at 55 °C virtually all chaperone function was lost (Fig. 3D), which reflects the result obtained using the insulin assay at 40 °C [50]. However, when the Trp mutant was assayed under the more physiological conditions of the enzyme inactivation assay, full chaperone function was maintained (Fig. 3D). There is only a 3 °C difference between the insulin and enzyme

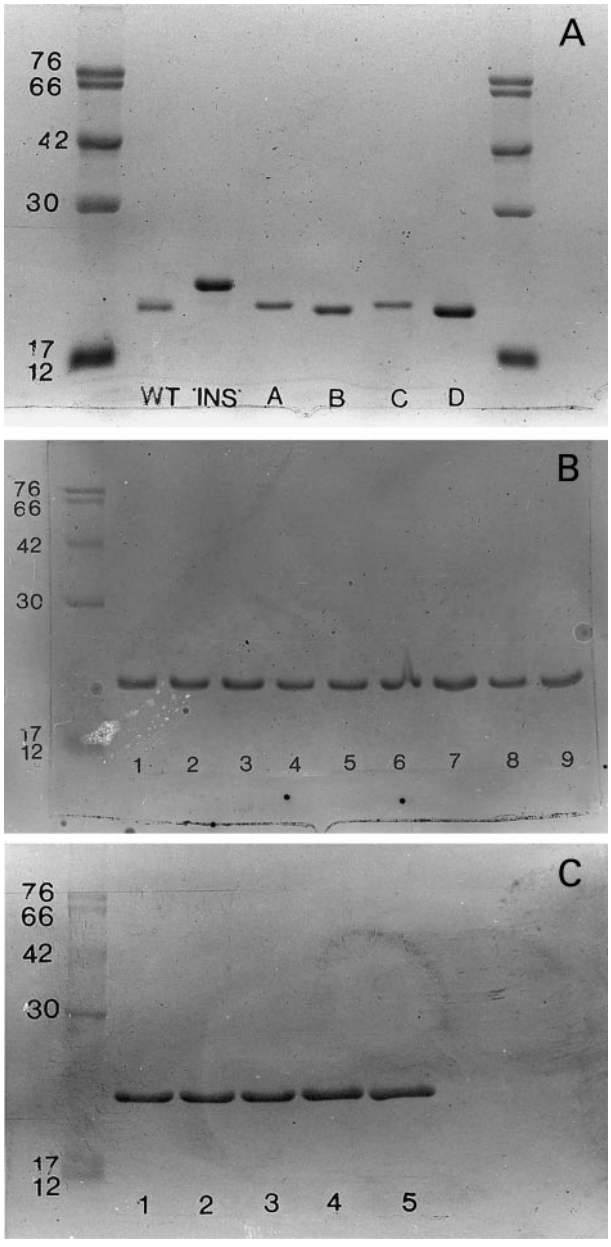

Fig. 2. SDS/PAGE of mutants and wild-types of α-crystallin. (A) Bovine αA-crystallin and rat αAins-crystallin (refer to Fig. 1 for full explanation of mutations). (B) Human αB-crystallin; (1) Wild-type (2) Ser139→Arg (3) Gly141→Arg + Ser19→Ala (4) Thr144→Arg (5) Gly147→Arg (6) Ser19→Ala (7) Ser45→Ala (8) Ser59→Ala (9) Ser45→Ala plus Ser59→Ala; (C) human αB-crystallin mutant (1) Wild-type (2) Phe27→Arg; murine αB-crystallin (3) Phe118→Ala, and two double mutants (4) Lys103→Leu/His104→Ile (5) Glu110→His/His111→Glu.

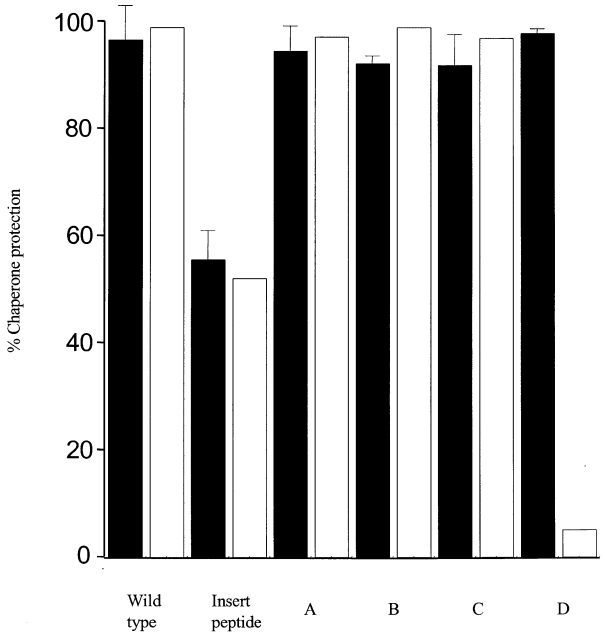

Fig. 3. The chaperone function of wild-type αA-crystallin, αAins-crystallin and four C-terminal mutants of αA-crystallin assessed by two methods. ■ Protection of malate dehydrogenase activity α-crystallin following fructation at 37 °C. □ Protection of heat-induced aggregation of β-low crystallin at 55 °C by α-crystallin. Refer to Fig. 1 for full explanation of the specific mutations of the C-terminal extension.

inactivation assay though there is a huge difference in chaperone function when assayed. However, between these temperatures there is a change in far-UV circular dichroism (secondary structure) of the Trp mutant [50]. This suggests a pivotal change in the environment of the Trp, which may account for the differences between the chaperone assays. This also highlights the caution that must be applied to results of chaperone assays at raised temperatures and the benefits of the enzyme inactivation assay at 37 °C. ^{1}H-NMR spectroscopy of the Ala-Leu-Trp-Lys-Gly mutant revealed fewer cross-peaks than the Ala-Leu-Asp-Lys-Gly control [50]. The Trp mutant had cross-peaks present for the first part of the C-terminal extension (Glu165-Ala170) but not for the remainder. Therefore the terminal sequence of the C-terminal extension in the Trp mutant has reduced flexibility compared with the entire C-terminal extension in the Asp mutant or wild-type. Tryptophan fluorescence spectra indicate that the extra Trp is almost fully exposed to the solvent [50]. Immobilization of the C-terminal extension may account for why the Trp mutant has a lower mass than that of the wild-type and the other mutants and may explain why it is heat-labile. The strongly hydrated polar extension may compensate for the relatively high surface hydrophobicity of the complexes formed between αA-crystallin and unfolded substrates and keep the complex in solution. When an unfolding target protein binds to an αA-crystallin aggregate there is an increase in surface hydrophobicity. However, when the C-terminal extension is immobilized or removed, although the aggregate is soluble, there comes a point when the restricted polar extension cannot compensate for the increase in hydrophobicity and the complex becomes insoluble. The presence of a hydrated and flexible extension is more important for solubility and chaperone activities than its precise amino-acid sequence [50].

Chaperone function of αB-crystallin mutants

Wild-type human αB-crystallin produced approximately 80% chaperone function of wild-type αA-crystallin using both the enzyme inactivation assay ($P = 0.0046$) and the heat aggregation assay ($P < 0.001$) (Figs 3 and 4) consistent with previous studies [17]. However, Datta and Rao [41] recently demonstrated that their relative function is temperature-dependent so that at 35 °C αB-crystallin is the superior chaperone.

All the human αB-crystallin mutants were expressed in the soluble fraction and all purified proteins eluted from a size-exclusion column at a size nearly identical to wild-type αB-crystallin suggesting that the residues mutated do not have a dominant role in intersubunit binding [17]. The αB-crystallin residues were mutated to Arg, which is a very polar, positively charged amino acid and has a long side chain. The residues Ser139, Gly141, Thr144 and Gly147 are located in a highly conserved region of αB-crystallin, the most highly conserved region of the small heat shock proteins. Although the sequence has not been implicated with any specific function, the changes seen when the residues are sequentially mutated indicates this area might have a role in chaperone function.

Human αB-crystallin mutant Ser139Arg showed approximately 60% chaperone function when assayed by both methods, approximately 20% decrease from the

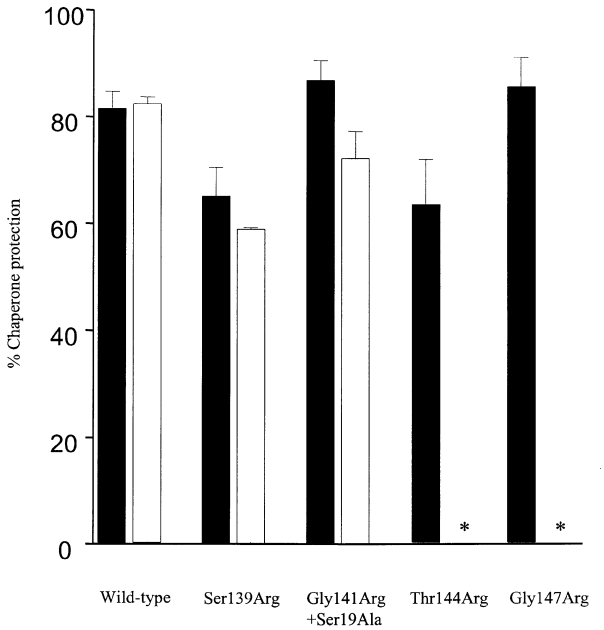

Fig. 4. The chaperone ability of human αB-crystallin mutants assessed by two methods. ■ Protection of malate dehydrogenase by α-crystallin following fructation at 37 °C. □ Protection of heat-induced aggregation of β-low crystallin at 55 °C by α-crystallin. * Indicates proteins fell out of solution.

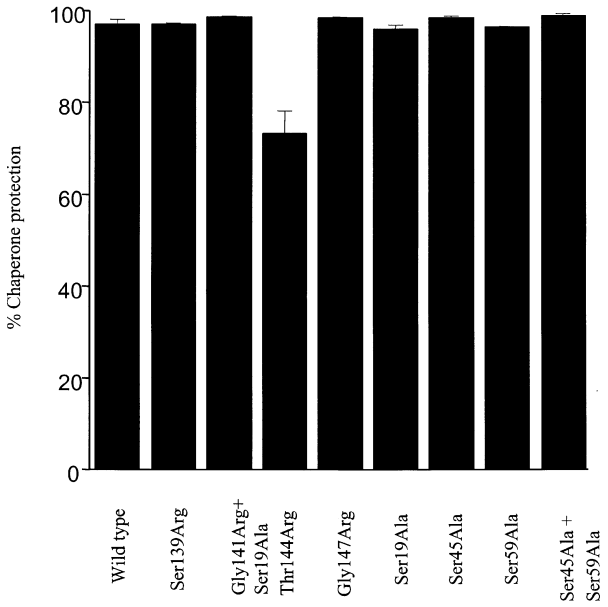

Fig. 5. The chaperone ability of human αB-crystallin mutants assessed by the protection of heat-induced aggregation of glyceraldehyde 3-phosphate dehydrogenase at 37 °C.

control ($P = 0.025$ for enzyme assay, E, and $P < 0.001$ for heat aggregation assay, H).

The mutant Thr144→Arg showed approximately 60% chaperone function when assayed by enzyme inactivation ($P = 0.07$) (Fig. 4), and Gly147→Arg showed approximately 80% chaperone function when assayed by enzyme inactivation ($P = 0.93$) (Fig. 4), neither significantly different from recombinant wild-type human αB-crystallin. However, when assayed using the heat denaturation assay, the proteins precipitated. This suggests that the mutations cause the proteins to become heat-labile.

All αB-crystallin mutants were assayed by the spontaneous aggregation of glyceraldehyde 3-phosphate dehydrogenase at 37 °C. Using these more physiological conditions, all αB-crystallin mutants assessed showed full chaperone function except mutant Thr144→Arg which showed approximately 70% ($P < 0.001$) (Fig. 5), similar to the protection afforded against the inactivation of malate dehydrogenase (Fig. 4), and in the aggregation of alcohol dehydrogenase reported previously [7]. The Thr144→Arg mutation therefore caused the greatest change in the function of αB-crystallin.

All the single mutations involving Ser to Ala caused negligible reductions in chaperone function (Fig. 6) suggesting a very limited role in chaperone function. Compared to the wild-type human αB-crystallin the single mutants were not statistically significantly different: Ser19→Ala ($P = 0.054$ for E and $P = 0.09$ for H), Ser45→Ala ($P = 0.096$ for E and $P = 0.97$ for H), except for Ser59→Ala ($P = 0.029$ for E and $P = 0.0013$ for H) using both assay methods. The chaperone function is reduced to approximately 50% when two serines, Ser45→Ala and Ser59→Ala were substituted ($P = 0.078$ for E and $P < 0.001$ for H), which suggests a compounding effect of mutations upon the decrease of chaperone function. Replacement of the Ser residues does not cause a change in quaternary structure of αB-crystallin as determined by size-exclusion chromatography [7]. The mutation of a serine plus change of the small Gly residue to the large side-chain of Arg caused no significant decrease in chaperone function (Fig. 5), which is surprising considering the drastic change in size and charge. All three serines are phosphorylation sites [46–48].

The double mutant Gly141→Arg/Ser19→Ala showed chaperone function similar to wild-type αB-crystallin when using the enzyme inactivation assay ($P = 0.35$) and slightly impaired function when using the heat aggregation assay ($P = 0.04$) (Fig. 4). All wild-type and human mutant αB-crystallins, with the exception of Thr144→Arg and Gly147→Arg, show similar results with all three types of assay thereby indicating a similar protective role for α-crystallin.

Plater *et al.* [3] showed that the single point mutation Phe27→Arg in murine αB-crystallin completely abolished its chaperone function when assayed by the heat aggregation of γ-crystallin at 66 °C and by the reduction of insulin B-chain method at room temperature. However, Horwitz *et al.* [5] contended that this mutation, albeit in the human protein, caused no change to chaperone function when assayed by the reduction of insulin method at 25 °C or by the heat aggregation of α-lactalbumin at 37 °C. To arbitrate between the two opposing findings the mutant was assayed by both the heat aggregation method and enzyme inactivation method. Mutation of a single phenylalanine residue, Phe27→Arg, caused differing results depending on the type of assay (Fig. 7). Using the heat aggregation assay the Phe27→Arg mutant showed a decrease in chaperone function of approximately 50% compared to the wild-type αB-crystallin ($P = 0.001$). However, when assayed under more physiological conditions, enzyme inactivation at 37 °C, the chaperone function of the Phe27→Arg mutant

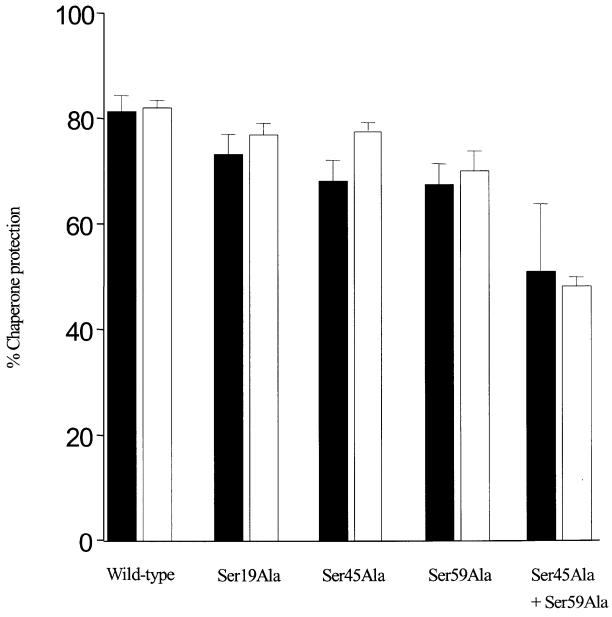

Fig. 6. The chaperone ability of human αB-crystallin mutants assessed by two methods. ■ Protection of malate dehydrogenase activity by α-crystallin following fructation at 37 °C. □ Protection of heat-induced aggregation of β-low crystallin at 55 °C by α-crystallin.

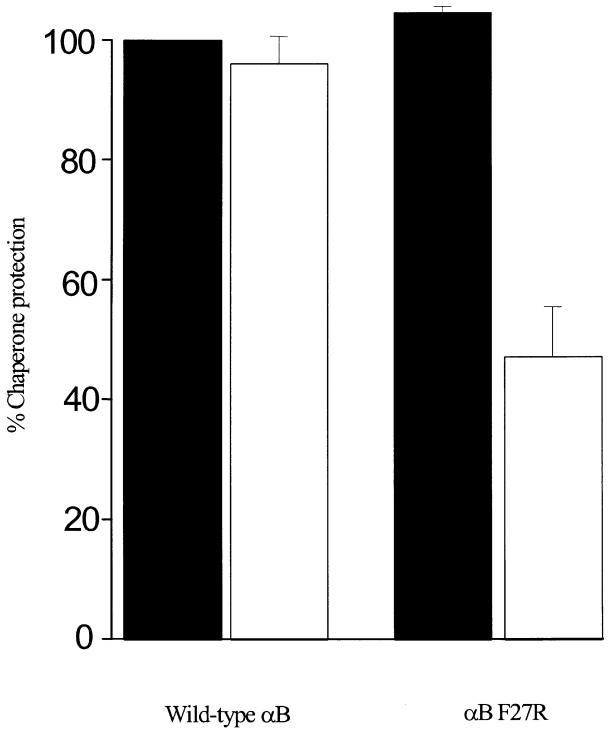

Fig. 7. The chaperone ability of recombinant human αB-crystallin and Phe27→Arg mutant assessed by two methods. ■ Protection of malate dehydrogenase activity by α-crystallin following fructation at 37 °C. □ Protection of heat-induced aggregation of β-low crystallin at 55 °C by α-crystallin.

was equivalent to the chaperone function of the wild-type αB-crystallin (Fig. 7). This partly explains the discrepancy between the results of the two other groups [3,5]. In agreement with Horwitz *et al.* [5] the Phe27→Arg mutant maintained full chaperone function when assayed under physiological conditions at 37 °C (Fig. 7). It appears that at high temperature the mutation Phe27→Arg has an effect on the heat stability of human αB-crystallin. Clearly, use of assays at elevated temperature is unwise when using mutants of unknown stability. There is the possibility that the difference in findings between these two authors may be due to the source of the recombinant αB-crystallin; Plater *et al.* [3] used murine αB-crystallin, whereas Horwitz *et al.* [5] used human αB-crystallin. The four differences between the corresponding amino-acid sequences may cause the contrast in findings.

The double-mutated residues Lys103→Leu/His104→Ile, Glu110→His/His111→Glu and the single mutation Phe118→Ala of murine αB-crystallin produced very similar decreases in chaperone function when assayed by both the heat aggregation and enzyme inactivation assays (Fig. 8). When assayed by the heat aggregation method these mutants showed approximately 40% chaperone function whereas when assayed by the enzyme inactivation method they showed approximately 70% chaperone function, Lys103→Leu/His104→Ile ($P = 0.013$ for E and $P < 0.001$ for H), Phe118→Ala ($P < 0.001$ for E and $P < 0.001$ for H) and Glu110→His/His111→Glu ($P = 0.004$ for E and $P < 0.001$ for H).

The double mutation Lys103→Leu/His104→Ile, which involves both a change in charge and hydrophobicity, lies in a very conserved region of αB-crystallin, which has been identified as a binding site of alcohol dehydrogenase during chaperoning [51]. The decrease in chaperone function, as determined by both assays, suggests that this area may be important for chaperone ability and that the mutation does not affect chaperone function simply by making αB-crystallin more heat labile (Fig. 8). The Phe118→Ala mutation is in a region that is very conserved and has been suggested to be involved with subunit contact [4], but previous findings show that this mutation of the αB-crystallin chain had little effect on chaperone function [6]. The results presented here show a decrease in chaperone function. This shows the effect of replacing a highly hydrophobic aromatic residue with a small weakly hydrophobic residue. The difference between the two assays may again indicate instability of the mutant. Mutants Arg116→Cys of αA- and Arg120→Gly of αB-crystallin in this region had diminished chaperone activity and altered structure [27–30]. Arg116 of αA- and Arg120 of αB-crystallin are corresponding residues.

The double mutation Glu110→His and His111→Glu, a reversal between amino acids having basic and acidic side chains, again lies in a very conserved region, which may be involved with subunit contact [4]. The decrease in chaperone function is very similar to that seen with the previous two mutants and suggests that this conserved area, the region from Lys103 to His111 of αB-crystallin, may be important for chaperone ability since both chaperone assays revealed decreases in function when assaying the mutant.

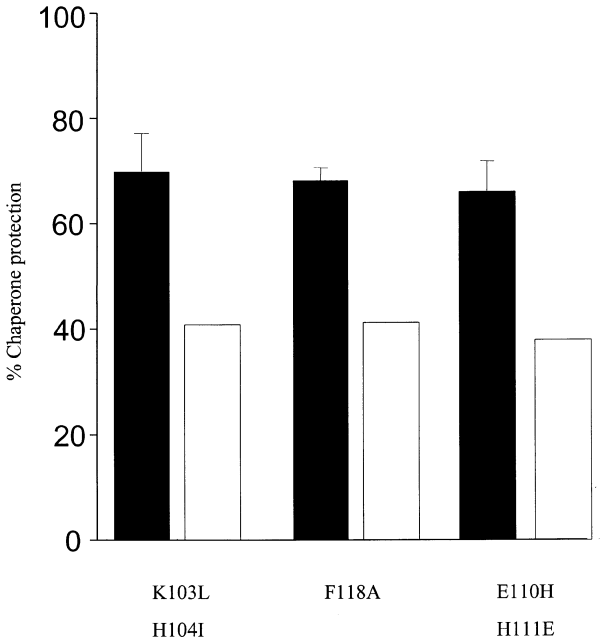

Fig. 8. The chaperone ability of recombinant murine αB-crystallin mutants, both single and double mutations. ■ Protection of malate dehydrogenase activity by α-crystallin following fructation at 37 °C. ■ Protection of heat-induced aggregation of β-low crystallin at 55 °C by α-crystallin.

The correlation between both forms of assay reported here supports the idea that both measure similar chaperone properties of α-crystallin. Presumably the proteins are partially unfolded in both types of assay revealing hydrophobic surfaces that require the protective attentions of a molecular chaperone. The exact nature of the interaction between α-crystallin and unfolding proteins may only be revealed when a detailed structure of the protein has been obtained. The correspondence between assays is lost when the structure of α-crystallin is altered at raised temperatures, or the protein precipitates. Assay of mutant proteins of unknown stability at elevated temperatures can produce flawed results.

Considering the number of conserved residues that have been replaced, site directed mutagenesis has not been very successful at finding potential chaperone binding sites. Physical methods appear to be more effective. The chaperone function of α-crystallin is very resistant to single amino acid changes, as was found for post-translational modifications [19] and emphasizes that α-crystallin is a very tough protein. However, the results on mutants indicate the possible involvement of residues Ser45, Ser59, Ser139, Thr144, K103, H104, E110, H111 and F118 of αB-crystallin in chaperone function. Clearly there are multiple sites for binding target proteins.

ACKNOWLEDGEMENTS

We are grateful to Research into Ageing and the Knoop Trust for financial support; and to Drs Roy Quinlan and Paul van den IJssel (University of Dundee) for the revised sequence of the Gly141Arg/Ser19Ala mutant.

REFERENCES

1. Smulders, R.H., van Geel, I.G., Gerards, W.L., Bloemendal, H. & de Jong, W.W. (1995) Reduced chaperone-like activity of αA(ins)-crystallin, an alternative splicing product containing a large insert peptide. *J. Biol. Chem.* **270**, 13916–13924.
2. Smulders, R.H., Merck, K.B., Aendekerk, J., Horwitz, J., Takemoto, L., Slingsby, C., Bloemendal, H. & de Jong, W.W. (1995) The mutation Asp69→Ser affects the chaperone-like activity of αA-crystallin. *Eur. J. Biochem.* **232**, 834–838.
3. Plater, M.L., Goode, D. & Crabbe, M.J. (1996) Effects of site-directed mutations on the chaperone-like activity of αB-crystallin. *J. Biol. Chem.* **271**, 28558–28566.
4. Berengian, A.R., Bova, M.P. & Mchaourab, H.S. (1997) Structure and function of the conserved domain in αA-crystallin. Site-directed spin labelling identifies a β-strand located near a subunit interface. *Biochemistry* **36**, 9951–9957.
5. Horwitz, J., Bova, M., Hunag, Q.L., Ding, H., Yaron, O. & Lowman, S. (1998) Mutation of αB-crystallin: effect on chaperone-like activity. *Int. J. Biol. Macromol.* **22**, 263–269.
6. Hepburne-Scott, H.W. & Crabbe, M.J.C. (1999) Maintenance of chaperone-like activity despite mutations in a conserved region of murine lens αB crystalline. *Mol. Vis.* **5**, 15–19.
7. Muchowski, P.J., Wu, G.J., Liang, J.J., Adman, E.T. & Clark, J.I. (1999) Site-directed mutations within the core 'alpha-crystallin' domain of the small heat-shock protein, human alphaB-crystallin, decrease molecular chaperone functions. *J. Mol. Biol.* **289**, 397–411.
8. Horwitz, J. (1992) α-Crystallin can function as a molecular chaperone. *Proc. Natl Acad. Sci. USA* **89**, 10449–10453.
9. Farahbakhsh, Z.T., Huang, Q.L., Ding, L.L., Altenbach, C., Steinhoff, H.J., Horwitz, J. & Hubbell, W.L. (1995) Interaction of α-crystallin with spin-labelled peptides. *Biochemistry* **34**, 509–516.
10. Das, K.P. & Surewicz, W.K. (1995) Temperature-induced exposure of hydrophobic surfaces and its effect on the chaperone activity of α-crystallin. *FEBS Lett.* **369**, 321–325.
11. Ganea, E. & Harding, J.J. (1995) Molecular chaperones protect against glycation-induced inactivation of glucose 6-phosphate dehydrogenase. *Eur. J. Biochem.* **231**, 181–185.
12. Ganea, E. & Harding, J.J. (1996) Inhibition of 6-phosphogluconate dehydrogenase by carbamylation and protection by α-crystallin, a chaperone-like protein. *Biochem. Biophys. Res. Commun.* **222**, 626–631.
13. Heath, M.M., Rixon, K.C. & Harding, J.J. (1996) Glycation-induced inactivation of malate dehydrogenase protection by aspirin and a lens molecular chaperone, α-crystallin. *Biochim. Biophys. Acta.* **1315**, 176–184.
14. Hook, D.W.A. & Harding, J.J. (1996) α-Crystallin acting as a molecular chaperone protects catalase against steroid-induced inactivation. *FEBS Lett.* **382**, 281–284.
15. Derham, B.K. & Harding, J.J. (1997) The effects of ageing on the chaperone-like function of rabbit alpha-crystallin, comparing three methods of assay. *Biochim. Biophys. Acta.* **1336**, 187–194.
16. Derham, B.K. & Harding, J.J. (1997) Effect of ageing on the chaperone-like function of human α-crystallin assessed by three methods. *Biochem. J.* **328**, 763–768.
17. van Boekel, M.A., Hoogakker, S.E., Harding, J.J. & de Jong, W.W. (1996) The influence of some post-translational modifications on the chaperone-like activity of α-crystallin. *Ophthalmic Res.* **28** (Suppl. 1), 32–38.
18. Wistow, G. (1985) Domain structure and evolution in α-crystallins and small heat-shock proteins. *FEBS Lett.* **181**, 1–6.
19. Derham, B.K. & Harding, J.J. (1999) α-Crystallin as a molecular chaperone. *Prog. Ret. Eye Res.* **18**, 463–509.
20. Boyle, D. & Takemoto, L. (1994) Immunolocalisation of the C-terminal and *N*-terminal regions of αA- and αB-crystallins. *Curr. Eye Res.* **13**, 497–504.
21. Smith, J.B., Liu, Y. & Smith, D.L. (1996) Identification of possible regions of chaperone activity in lens α-crystallin. *Exp. Eye Res.* **63**, 125–128.
22. Kamei, A., Iwase, H. & Masuda, K. (1997) Cleavage of amino acid residue(s) from the *N*-terminal region of alpha A- and alpha B-crystallins in human crystalline lens during aging. *Biochem. Biophys. Res. Commun* **231**, 373–378.
23. Lund, A.L., Smith, J.B. & Smith, D.L. (1996) Modifications of the water-insoluble human lens alpha-crystallins. *Exp. Eye Res.* **63**, 661–672.
24. de Jong, W.W., Leunissen, J.A. & Voorter, C.E. (1993) Evolution of the α-crystallin/small heat-shock protein family. *Mol. Biol. Evol.* **10**, 103–126.
25. de Jong, W.W., Zweers, A., Versteeg, M. & Nuy Terwindt, E.C. (1984) Primary structures of the alpha-crystallin A chains of twenty-eight mammalian species, chicken and frog. *Eur. J. Biochem.* **141**, 131–140.
26. Caspers, G.J., Leunissen, J.A. & de Jong, W.W. (1995) The expanding small heat-shock protein family, and structure predictions of the conserved 'α-crystallin domain'. *J. Mol. Evol.* **40**, 238–248.
27. Bova, M.P., Yaron, O., Huang, Q., Ding, L., Haley, D.A., Stewart, P.L. & Horwitz, J. (1999) Mutation R120G in αB-crystallin, which is linked to desmin-related myopathy, results in an irregular structure and defective chaperone-like function. *Proc. Natl Acad. Sci. USA* **96**, 6137–6142.
28. Kumar, L.V.S., Ramakrishna, T. & Rao, C.H. (1999) Structural and functional consequences of the mutation of a conserved arginine residue in alpha A- and alpha B-crystallins. *J. Biol. Chem.* **274**, 24137–24141.

29. Perng, M.D., Muchowski, P.J., van den Ijsel, P., Wu, G.J., Hutcheson, A.M., Clark, J.I. & Quinlan, R.A. (1999) The cardiomyopathy and lens cataract mutation in alpha B-crystallin alters its protein structure, chaperone activity, and interaction with intermediate filaments *in vitro*. *J. Biol. Chem.* **274**, 33235–33243.
30. Shroff, N.P., Cherian-Shaw, M., Bera, S. & Abraham, E.C. (1999) Mutation of R116C results in highly oligomerized alpha A-crystalline with modified structure and defective chaperone-like function. *Biochemistry* **39**, 1420–1426.
31. Gaestel, M., Schroder, W., Benndorf, R., Lippmann, C., Buchner, K., Hucho, F., Erdmann, V.A. & Bielka, H. (1991) Identification of the phosphorylation sites of the murine small heat shock protein HSP25. *J. Biol. Chem.* **266**, 14721–14724.
32. de Jong, W.W., Leunissen, J.A., Leenen, P.J., Zweers, A. & Versteeg, M. (1988) Dogfish α-crystallin sequences. Comparison with small heat shock proteins and *Schistosoma* egg antigen. *J. Biol. Chem.* **263**, 5141–5149.
33. Carver, J.A., Aquilina, J.A., Truscott, R.J. & Ralston, G.B. (1992) Identification by ^{1}H NMR spectroscopy of flexible C-terminal extensions in bovine lens α-crystallin. *FEBS Lett.* **311**, 143–149.
34. Carver, J.A., Nicholls, K.A., Aquilina, J.A. & Truscott, R.J. (1996) Age-related changes in bovine α-crystallin and high-molecular-weight protein. *Exp. Eye Res.* **63**, 639–647.
35. Siezen, R.J. & Hoenders, H.J. (1979) The quaternary structure of bovine α-crystallin. Surface probing by limited proteolysis *in vitro*. *Eur. J. Biochem.* **96**, 431–440.
36. Yoshida, H., Yumoto, N., Tsukahara, I. & Murachi, T. (1986) The degradation of α-crystallin at its carboxyl-terminal portion by calpain in bovine lens. *Invest. Ophthalmol. Vis. Sci.* **27**, 1269–1273.
37. Takemoto, L. (1994) Release of α-A sequence 158–173 correlates with a decrease in the molecular chaperone properties of native α-crystallin. *Exp. Eye Res.* **59**, 239–242.
38. Takemoto, L., Emmons, T. & Horwitz, J. (1993) The C-terminal region of α-crystallin: involvement in protection against heat-induced denaturation. *Biochem. J.* **294**, 435–438.
39. Andley, U.P., Mathur, S., Griest, T.A. & Petrash, J.M. (1996) Cloning, expression, and chaperone-like activity of human αA-crystallin. *J. Biol. Chem.* **271**, 31973–31980.
40. Mehlen, P., Briolay, J., Smith, L., Diaz latoud, C., Fabre, N., Pauli, D. & Arrigo, A.P. (1993) Analysis of the resistance to heat and hydrogen peroxide stresses in COS cells transiently expressing wild type or deletion mutants of the *Drosophila* 27-kDa heat-shock protein. *Eur. J. Biochem.* **215**, 277–284.
41. Datta, S.A. & Rao, C.H. (1999) Differential temperature-dependent chaperone-like activity of αA- and αB-crystallin bovine homoaggregates. *J. Biol. Chem.* **274**, 34773–34778.
42. de Jong, W.W., Cohen, L.H., Leunissen, J.A. & Zweers, A. (1980) Internally elongated rodent α-crystallin A chain: resulting from incomplete RNA splicing? *Biochem. Biophys. Res. Commun.* **96**, 648–655.
43. King, C.R. & Piatigorsky, J. (1983) Alternative RNA splicing of the murine alpha A-crystallin gene: protein-coding information within an intron. *Cell* **32**, 707–712.
44. Hendriks, W., de Sanders, J., Leij, L., Ramaekers, F., Bloemendal, H. & de Jong, W.W. (1988) Monoclonal antibodies reveal evolutionary conservation of alternative splicing of the alpha A-crystallin primary transcript. *Eur. J. Biochem.* **174**, 133–137.
45. Laemmli, U.K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227**, 680–685.
46. Chiesa, R., Gawinowicz Kolks, M.A., Kleiman, N.J. & Spector, A. (1987) The phosphorylation sites of the B2 chain of bovine alpha-crystallin. *Biochem. Biophys. Res. Commun.* **144**, 1340–1347.
47. Voorter, C.E., de Haard Hoekman, W.A., Roersma, E.S., Meyer, H.E., Bloemendal, H. & de Jong, W.W. (1989) The *in vivo* phosphorylation sites of bovine alpha B-crystallin. *FEBS Lett.* **259**, 50–52.
48. Smith, J.B., Sun, Y., Smith, D.L. & Green, B. (1992) Identification of the posttranslational modifications of bovine lens αB-crystallins by mass spectrometry. *Protein Sci.* **1**, 601–608.
49. Sun, T.X., Das, B.K. & Liang, J.J. (1997) Conformational and functional differences between recombinant human lens alphaA- and alphaB-crystallin. *J. Biol. Chem.* **272**, 6220–6225.
50. Smulders, R.H.P.H., Carver, J.A., Lindner, R.A., van Boekel, M.A.M., Bloemendal, H. & de Jong, W.W. (1996) Immobilisation of the C-terminal extension of bovine αA-crystallin reduces chaperone-like activity. *J. Biol. Chem.* **271**, 29060–29066.
51. Sharma, K.K., Kaur, H. & Kester, K. (1997) Functional elements in molecular chaperone α-crystallin: identification of binding sites in αB-crystallin. *Biochem. Biophys. Res. Commun.* **239**, 217–222.