The C-terminal region of α -crystallin: involvement in protection against heat-induced denaturation

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Recent studies have demonstrated that the α -crystallins can protect other proteins against heat-induced denaturation and aggregation. To determine the possible involvement of the C-terminal region in this activity, the α -crystallins were subjected to limited tryptic digestion, and the amount of cleavage from the N-terminal and C-terminal regions of the α -A and α -B crystallin chains was assessed using antisera specific for these regions. Limited tryptic digestion resulted in cleavage only from the C-terminal region of α -A crystallin. This trypsin-treated α -A

crystallin preparation showed a decreased ability to protect proteins from heat-induced aggregation using an *in vitro* assay. Together, these results demonstrate that the C-terminal region of α -A crystallin is important for its ability to protect against heat-induced aggregation, which is consistent with the hypothesis that post-translational changes that are known to occur at the C-terminal region may have significant effects on the ability of α -A crystallin to protect against protein denaturation *in vivo*.

INTRODUCTION

The α -crystallins are some of the most abundant proteins of the lens. They consist of two polypeptide chains that are designated α -A and α -B, that exhibit extensive similarity in their sequences [1]. During ageing of the normal lens, the α -crystallins undergo extensive post-translational modification, including deamidation [2], oxidation [3] and cleavage from the C-terminus of the molecule [4–6]. The C-terminal region of the α -A crystallin molecule is also the site of additional covalent modification during the process of cataractogenesis in the human senile lens [7].

There have been numerous efforts to identify functional roles for the large amounts of α -crystallin that are found in the lens other than as a medium for the refraction of incident light. Previous studies have shown sequence similarities between the α -crystallins and the heat-shock proteins [8,9]. Using an *in vitro* assay, it has been demonstrated recently that the α -crystallins can protect other proteins against the effects of heat-induced aggregation [10], and that this protective ability decreases during ageing of the lens [10].

It is possible that this age-related decrease in the ability of α -crystallin to protect against heat-induced aggregation may be causally related to the known *in vivo* modifications of α -crystallin, which include increased cleavage from the C-terminus of the molecule. To directly test whether the C-terminal region of α -crystallin is important in the protective ability of the molecule, we have used limited tryptic digestion to cleave the C-terminal region of native α -A crystallin. The results of our study demonstrate that this region of the molecule is important in the ability of the molecule to protect against heat-induced aggregation.

MATERIALS AND METHODS

Bovine lenses from newborns were obtained from Amtech, Inc. (Tyler, TX, U.S.A.), and were stored at -75°C until use. The

cortex from the decapsulated lenses was homogenized in TSK buffer (0.1 M sodium sulphate, 0.6 M sodium phosphate, pH 7.0), and then centrifuged at 30 000 *g* for 20 min at 4°C . The supernatant was resolved on a TSK 3000SW gel-permeation column using TSK buffer, and the α -crystallin eluting in the void volume was collected, dialysed and lyophilized. Purification of the α -A and α -B chains using a C_4 reverse-phase column was done using the method of Perry and Abraham [12].

Limited tryptic digestion of α -crystallin was done essentially using the procedure of Siezen and Hoenders [13]. The lyophilized α -crystallin was solubilized in 0.01 M NH_4HCO_3 (pH 7.4) to a final concentration of 10 mg/ml and diphenyl carbamyl chloride-treated trypsin (type 11, Sigma Chemical Co.) was added at weight ratios of 1:1000, 1:2000, 1:4000, 1:6000, 1:10000 and 1:15000 (trypsin: α -crystallin). After incubation at 30°C for 10 min, all the samples were treated with soybean trypsin inhibitor (Sigma Chemical Co.) at a weight ratio of 1:5 (trypsin:inhibitor), based upon the reaction using a trypsin: α -crystallin ratio of 1:1000.

SDS/PAGE was done using the method of Laemmli [14], with a 15% (w/v) polyacrylamide gel. After electrophoresis, the resolved proteins were either stained with Coomassie Blue or electrophoretically transferred on to nitrocellulose and probed with antisera and radio-iodinated Protein A [15]. Rabbit polyclonal antisera against synthetic peptides corresponding to the N-terminal (anti- α -A-NT; residues 1–11) and C-terminal (anti- α -A-CT; residues 164–173) regions of human α -A crystallin [16] were raised as previously described [15]. Rabbit polyclonal antisera were also raised to the sequence KEEKPAVTAA PPK(Y), which includes residues 163–175 of the C-terminal region of bovine α -B (anti- α -B-CT) [17]. The last tyrosine residue was added to facilitate coupling to the carrier protein.

To test the ability of α -crystallin preparations to protect against heat-induced aggregation, 20.0 μg of α -crystallin was added to a cuvette containing 0.25 mg of alcohol dehydrogenase (1X crystallized, Sigma Chemical Co.) dissolved in a buffer

Abbreviations used: anti- α -A-NT, antiserum raised against peptide corresponding to the N-terminus of α -A; anti- α -A-CT, antiserum raised against peptide corresponding to the C-terminus of α -A; anti- α -B-CT, antiserum raised against peptide corresponding to the C-terminus of α -B.

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containing 50 mM sodium phosphate and 0.14 M NaCl, pH 7.0. The cuvette was allowed to equilibrate for 5 min at 44.0 °C in the constant-temperature heating block of a Shimadzu UV-160 dual-beam spectrophotometer. The relative scattering at 360 nm due to heat-induced aggregation of alcohol dehydrogenase was measured over a period of 42 min with the spectrophotometer in the absorbance mode.

RESULTS

Figure 1 demonstrates the specificity of the N-terminal and C-terminal sera. Antiserum anti- α -A-NT recognizes both α -A and α -B (lanes a and b). Antiserum anti- α -A-CT is specific for the α -A chain (lanes c and d), and antiserum anti- α -B-CT is specific for the α -B chain (lanes e and f). In agreement with other studies [1], the staining of the gel demonstrates that purified α -A migrates slightly faster than α -B (lanes g and h).

To determine whether trypsin cleaves the N-terminal and/or C-terminal regions of α -crystallin, α -crystallins incubated with or without trypsin were resolved by SDS/PAGE, then transferred on to nitrocellulose and probed with the N-terminal and C-terminal sera. Figure 2 shows that the anti- α -A-NT antiserum binds both to the 20 kDa and to the 18 kDa bands that are present in the tryptic digest (lane b), suggesting that the N-terminus both of the α -A chain and of the α -B chain is resistant to limited tryptic digestion. In contrast, anti- α -A-CT antiserum binds to the 20 kDa (but not the 18 kDa) component of the same digest (lane d), suggesting that the trypsin treatment results in significant cleavage from the C-terminus of the α -A molecule. The probing of the same samples with anti- α -B-CT antiserum demonstrates no apparent changes (lanes e and f), suggesting that the C-terminal region of the α -B molecule is resistant to trypsin treatment under the conditions used in this study.

To confirm quantitatively that no cleavage occurs at the N-terminus either of the 18 kDa or of the 20 kDa components, the total amount of anti- α -A-NT antiserum binding to these bands

Figure 1 Specificity of anti-peptide sera for the subunits of α -crystallin

The α -A and α -B chains were purified as described in the Materials and methods section, and 4 μ g of protein was resolved using 15% (w/v) PAGE, then electrophoretically transferred to nitrocellulose and probed with anti-peptide sera and radio-iodinated protein A. Lanes a, c, e and g are purified α -A; lanes b, d, f and h are purified α -B. Lanes a and b were probed with anti- α -A-NT antiserum; lanes c and d with anti- α -A-CT antiserum; lanes e and f with anti- α -B-CT antiserum; lanes g and h represent the gel stained with Coomassie Blue (CB). The horizontal arrows to the left of the gel designate the migration of molecular-mass markers ovalbumin (43 kDa), carbonic anhydrase (29 kDa) and beta-lactoglobulin (18.4 kDa).

Figure 2 Binding of anti-peptide sera to native α -crystallin either with or without trypsin treatment for 10 min

Each lane contains 4 μ g of α -crystallin, resolved using 15% (w/v) PAGE. Lanes a, c, e and g represent samples not treated with trypsin (—); lanes b, d, f and h represent samples treated with trypsin at a weight ratio of 1:1000 (trypsin: α -crystallin) (+). Lanes a and b were probed with anti- α -A-NT antiserum; lanes c and d with anti- α -A-CT antiserum; lanes e and f with anti- α -B-CT antiserum; lanes g and h represent the gel stained with Coomassie Blue. The horizontal arrows to the left of the gel designate the same molecular-mass markers as in Figure 1. Abbreviations used are as in the legend in Figure 1.

Table 1 Binding of N- and C-terminal antisera to the 20 kDa and 18 kDa components of α -crystallin

The table shows the area (in arbitrary units) of the 20 kDa and 18 kDa bands of α -crystallin. Each figure is the average of triplicate determinations $\pm$ S.D. Edman degradation of both samples showed that the N-terminus was blocked in both cases.

Antiserum used	No trypsin digestion	Trypsin: α -crystallin (1:1000)
Anti- α -A-NT	171.2 $\pm$ 18.5	165.9 $\pm$ 31.3
Anti- α -A-CT	129.3 $\pm$ 2.5	42.3 $\pm$ 5.9*
Anti- α -B-CT	145.0 $\pm$ 39.5	142.3 $\pm$ 11.2

*Statistically significant with respect to undigested sample, $P < 0.001$.

was determined by densitometry. Table 1 shows that there is no significant difference in the total amount of anti- α -A-NT antiserum recognizing the 20 kDa plus the 18 kDa components of α -crystallin, with or without treatment with trypsin. Sequence analysis by Edman degradation of both samples demonstrated blocked N-termini (results not shown), further confirming that no detectable cleavage occurred at the N-terminus either of the α -A or of the α -B molecules.

In contrast, Table 1 shows that after 10 min of trypsin treatment, the binding of anti- α -A-CT antiserum to the 20 kDa component is significantly less than the binding of the same antiserum to the sample without trypsin treatment. The binding of anti- α -B-CT antiserum is unchanged between the two samples. These results confirm quantitatively that, under the conditions used in this study, cleavage is preferentially from the C-terminus of the α -A molecule, which is consistent with the results of Siezen and Hoenders [13].

To assess the effects of this cleavage on the ability of α -crystallin to protect against heat-induced aggregation, the α -crystallin samples, with or without trypsin treatment, were tested for their ability to protect the enzyme alcohol dehydrogenase

Figure 3 Effect of trypsin treatment on the ability of α -crystallin to protect against heat-induced aggregation

See the Materials and methods section for details of the assay used. Curve a, alcohol dehydrogenase; b, alcohol dehydrogenase + α -crystallin (trypsin: α -crystallin ratio 1:1000); c, alcohol dehydrogenase + α -crystallin (without trypsin treatment); d, trypsin + either trypsin inhibitor or α -A peptide corresponding to residues 164–173.

Table 2 Correlation between anti-C-terminus region binding and the ability of α -crystallin to protect against denaturation

The figures for anti-CT binding represent the area (in arbitrary units) of the 20 kDa band of α -crystallin after trypsin treatment compared with the area of α -crystallin without trypsin treatment. Each figure is the average of triplicate determinations $\pm$ S.D. The figures for inhibition of protein denaturation represent the change in absorbance at 360 nm, measured for alcohol dehydrogenase in the presence of trypsin-treated α -crystallin and shown as a percentage of the change in absorbance for an equivalent amount of α -crystallin which had not been treated with trypsin. Each figure is the average of triplicate determinations $\pm$ S.D.

Trypsin: α -crystallin ratio	Anti-CT binding (%)	Inhibition of protein denaturation (%)
No trypsin	100	100
1:1000	32.8 $\pm$ 4.8	7.7 $\pm$ 5.9
1:2000	48.7 $\pm$ 1.1	11.2 $\pm$ 3.0
1:4000	54.5 $\pm$ 3.0	15.4 $\pm$ 4.0
1:6000	65.8 $\pm$ 1.1	25.0 $\pm$ 1.6
1:10000	73.8 $\pm$ 17.8	45.7 $\pm$ 10.0
1:15000	87.3 $\pm$ 6.8	74.7 $\pm$ 7.5

against aggregation due to heating at 44 °C. Figure 3 shows a typical trace of relative scattering measured at 360 nm. Although both α -crystallin samples could to some extent protect alcohol dehydrogenase against denaturation, trypsin treatment decreased this protective ability. Control experiments indicated that trypsin, either plus trypsin inhibitor, or plus the peptide of α -A consisting of residues 164–173 that is expected to be released on trypsin treatment, did not show any detectable heat-induced aggregation. Furthermore, digested α -crystallin incubated in the absence of alcohol dehydrogenase did not result in any measurable aggregation (results not shown).

If cleavage from the C-terminal region of α -A crystallin did reduce the ability of α -A crystallin to protect against heat-induced denaturation, there should be a correlation between the loss of anti-CT binding and the ability to inhibit denaturation. Table 2 shows that increasing the dilutions of trypsin that were used in the digestion resulted in an increase in anti-CT binding, with a concomitant increase in the ability of the α -crystallin sample to protect against denaturation. These results clearly

demonstrate that increasing cleavage from the C-terminal region decreases the protective activity of α -crystallin.

DISCUSSION

Although many studies have characterized covalent changes in the α -crystallins during the ageing and cataractogenic processes, it has been very difficult to correlate these changes with a functional property that could directly affect the transparent properties of the lens. With the recent discovery that the α -crystallins are functionally related to the heat-shock proteins [10,11], it is now possible to test the hypothesis that these covalent changes in α -crystallin may directly affect the ability of α -crystallin molecules to protect other lens proteins against denaturation.

A well-characterized modification of α -crystallin is cleavage from the C-terminus of the molecule. During ageing, this process occurs both in the α -A and in the α -B chains [4–6]. Previous studies using anti-peptide antibodies have demonstrated that the covalent modification at the C-terminal region of α -A is accelerated in human cataractous lenses [7], suggesting that this process may play an aetiological role in the development of lens opacity.

To test whether the C-terminal region is indeed of functional importance, in the present study we have used limited tryptic digestion of native α -crystallin to preferentially cleave the C-terminus of the α -A chains. We have used the conditions that were described by Siezen and Hoenders [13], who reported cleavage principally from the C-terminus of the α -A molecule when it was treated with trypsin for 10 min at 30 °C with a weight ratio of 1:1000 (trypsin: α -crystallin). In addition, however, we quantitatively assessed the degree of cleavage using antisera. The anti- α -A-NT antiserum raised against the expected N-terminal tryptic peptide of α -A crystallin is also specific for the α -B chain, which is consistent with their close similarities in sequence [16,18]. Based on the results that are shown in Table 1, there is no statistical difference in the total binding of this antiserum to α -crystallin samples either with or without trypsin treatment, demonstrating that, within the experimental error of this study, there was no significant cleavage from the N-terminus either of the α -A or of the α -B molecules. This conclusion was

confirmed by Edman degradation, which showed a blocked N-terminus both for the α -A and for the α -B chains.

Quantitative analysis using antiserum raised to the expected C-terminal tryptic peptide of α -A crystallin indicates that the major site of trypsin cleavage is rather the C-terminal region of this molecule, demonstrating that this part of the molecule is involved in the ability of α -A crystallin to protect against heat denaturation. The exact extent of trypsin cleavage from the C-terminus cannot be assessed, but from the studies of Siezen and Hoenders [13], the major sites of cleavage probably involve residues 158–173 of the α -A chain.

Nonetheless, the results of Table 2 clearly demonstrate that increased cleavage from the C-terminal region decreases the ability of the α -A molecule to inhibit heat-induced denaturation and aggregation. Although there is a correlation between the percentage inhibition of protein denaturation and the percentage of antiserum binding, this relationship is not linear. These results suggest that the protection by α -crystallin against heat-induced aggregation is a complex process, involving multiple steps. Among these steps is the interaction of α -crystallin with the partially denatured protein, the rates of alcohol dehydrogenase denaturation and the subsequent association into aggregates that are large enough to be scattered by incident light.

Based on this and previous studies, the α -A molecule can now be divided into at least two functionally distinct regions. The N-terminal domain is relatively hydrophobic and appears to mediate interactions between α -crystallin and the lens membrane [19], as well as interactions between individual α -crystallin monomeric units to form high-molecular-mass, oligomeric complexes [20]. From the present paper, the C-terminal domain appears to be important in the ability of the molecule to protect against heat-induced denaturation and aggregation.

In vitro studies of other molecular chaperones have demonstrated that they prevent further loss of native structure by directly binding to partially denatured proteins [21]. In agreement with this finding, we have recently demonstrated that the α -crystallins preferentially bind to denatured forms of γ -crystallin (S. Gopalakrishnan, D. Boyle and L. Takemoto, unpublished work). It is possible that the C-terminal region of α -A is directly involved in the binding process. Alternatively, the C-terminal region may interact with other parts of the α -A and/or α -B chains, resulting in a configuration that directly binds to and stabilizes other lens proteins against further denaturation.

Previous studies have also demonstrated that both the α -A and the α -B chains possess the ability to protect against heat-induced denaturation [10]. To obtain the purified α -A and α -B chains, the native α -crystallin aggregate had to be first denatured, and then the individual subunits purified and subsequently renatured. A comparison of the individual α -A and α -B chains with an equal amount of native undenatured α -crystallin demonstrated a quantitative difference in the ability of these preparations to protect against heat-induced aggregation (results not shown).

This quantitative difference could be due to incomplete renaturation of the individual chains. Alternatively, the three-dimensional structure of the pure α -A and α -B preparations may not be identical to their structure in the native aggregates that are obtained from the lens. In the present study, we have therefore only included data obtained from tryptic cleavage of the native α -crystallin aggregate, which we believe is more relevant to the physiological processes that may be occurring in the lens.

From the results of the present paper, we conclude that the C-terminal region of the native α -A crystallin molecule is important in the ability of this protein to protect other polypeptides against heat-induced aggregation. This conclusion is consistent with sequence similarities within this region between the α -crystallins and the heat-shock proteins [8,9]. The results that we have obtained *in vitro* can be directly extrapolated to the lens, where α -crystallin is a major constituent of fibre cells that contain polypeptides with extremely low rates of turnover. These polypeptides are therefore exposed for many decades to the physical and chemical insults present in the ageing lens. The known *in vivo* modifications to the C-terminal region of α -crystallin may therefore decrease the ability of this molecule to protect other lens proteins against the denaturation process, resulting in an increase in aggregation and an eventual opacification of the lens.

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