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DIFFERENCES IN SUSCEPTIBILITY BETWEEN CRYSTALLINS AND NON-LENTICULAR PROTEINS TO COPPER AND H_2O_2-MEDIATED PEPTIDE BOND CLEAVAGE

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The relative susceptibilities of lenticular proteins (α, β and γ-crystallins) and a number of proteins of non-lenticular origin, to hydroxyl radical-mediated peptide bond cleavage were compared. The non-lenticular proteins (bovine serum albumin, ovalbumin, alcohol dehydrogenase, lysozyme, thyroglobulin, β-amylase, haemoglobin and carbonic anhydrase) were readily cleaved into acid-soluble fragments following 5 hours treatment with copper ions and hydrogen peroxide. In contrast the crystallins were almost totally unaffected by similar treatment. When α-crystallin was pre-treated with acid or cleaved into large fragments with cyanogen bromide it became susceptible to hydroxyl radical attack, yet heating the protein did not diminish its resistance. It is suggested that the resistance of α-crystallin to the copper/peroxide-mediated fragmentation may be dependent on the conformation of the protein.

KEY WORDS: Oxygen free radicals, crystallin, polypeptide fragmentation.

INTRODUCTION

Oxidative damage to cellular macromolecules is recognised to be an important component in the aetiology of many disease states.[1-3] In the lens, oxidative events are deleterious to the biochemical architecture of the tissue and play a key role in cataractogenesis – alterations occur in the conformation and solubility of the major lenticular proteins (crystallins) leading ultimately to their aggregation and the production of light-scattering species.[4] One major post-translational modification implicated in destabilization of native crystallin is protein fragmentation. Indeed, crystallin fragments are a feature of ageing[5-7] and cataractous[8-10] lenses.

The mechanisms whereby crystallin fragments are generated in the lens have been the subject of some speculation. Evidence suggests,[11-13] however, the possible involvement of the highly reactive hydroxyl radical in polypeptide cleavage. Such reactive oxygen species may arise in the lens through aberrations in the anti-oxidant and free-radical scavenging systems of the tissue. Indeed the lens normally possesses an active and substantial anti-oxidative protective system which includes a high reduced-glutathione concentration, catalase, glutathione peroxidase, superoxide dismutase and ascorbate. In the aged lens however (and therefore most evident in the lens nucleus or core, the site of the oldest tissue due to the unique physiology of the lens), this protective system is less effective.[15-19]

The high concentrations of ascorbate in the lens and aqueous humour have been suggested to contribute to hydrogen peroxide production in these tissues.[20,21] Indeed, elevated lenticular H_2O_2 is a feature of the cataractous lens.[13,20] In addition, certain transition metals, in particular copper, have been measured in elevated concentrations in senile cataractous lenses.[22,23] Copper ions can react with H_2O_2 via a Fenton-type reaction[21] to generate hydroxyl radicals which been demonstrated to cause fragmentation of proteins such as bovine serum albumin (BSA) and α-crystallin.[14,25–28] However, BSA and crystallins possess very different biological roles and *in vivo* life spans. In fact, the crystallins of the lens core are required to survive for as long as the organism; they are synthesized *in utero* and are not subject to replacement as the cells of the lens core lack nuclei and protein synthetic apparatus. Because of the differences in turnover rate and the fact that most tissues of the body are continuously subject to some degree of free radical attack, it was considered of interest to compare the relative susceptibility of various crystallins and proteins of non-lenticular origin to free radical induced cleavage.

MATERIALS

Bovine HMW α-crystallin, bovine β_H-crystallin, bovine β_L-crystallin, bovine serum albumin, chicken ovalbumin, chicken egg lysozyme, bovine haemoglobin, bovine carbonic anhydrase, yeast alcohol dehydrogenase, sweet potato β-amylase and bovine thyroglobulin were obtained from Sigma Chemical Company Ltd., Poole, Dorset, U.K. Bovine eyes from newly slaughtered cattle were a kind gift from Chitty's Abattoir, Guildford, Surrey, U.K. LKB Ultrogel AcA22 gel filtration medium was obtained from Pharmacia Ltd., Milton Keynes, Buckinghamshire, U.K. Bicinchinoic acid (BCA) protein assay reagent was purchased from Pierce (U.K.) Ltd., Cambridge, U.K.

METHODS

Preparation of α-crystallin

Lenses, dissected from bovine eyes, were decapsulated and the cortical regions dissolved at 4°C by agitation in 20 mM Tris. HCl, pH7.5, containing 1.0 mM EDTA. Centrifugation of the cortical fraction was then performed at 10,000 g for 20 min at 4°C, before application of the supernatant to an Ultrogel AcA22 column (100 × 2 cm). Elution of α-crystallin from the column was performed according to the gel-filtration method of Siezen *et al.*[9] and pooled fractions containing the protein were dialysed against 2 × 500 volumes of distilled water before lyophilization.

Preparation of γ-crystallin

γ-Crystallin was prepared from a 10,000 g supernatant fraction of bovine lens cortical homogenate by elution on a Sephadex G75 column (100 × 2 cm), according to the method of Björk.[29] Pooled fractions containing the protein were dialysed against 2 × 500 volumes of distilled water before lyophilization.

Cyanogen Bromide (CNBr) cleavage of α-Crystallin

Under aqueous acidic conditions cyanogen bromide cleaves peptide bonds specifically at methionine residues. α-Crystallin CNBr-peptides were prepared by incubation of the protein at 20 mg/ml (dissolved in 70% (w/v) formic acid) with excess solid CNBr (1.0 g/ml). Following vigorous agitation and incubation in the dark at room temperature for 18 h, the mixture was diluted ten-fold with distilled water, and trichloroacetic acid (TCA) added to 5% (w/v). Incubation at room temperature for a further 6 h was then performed to allow for precipitation of the peptides, before collection by centrifugation at 2,000 g for 30 min. The pellet was washed thrice by resuspension in 5.0% (w/v) TCA and centrifugation as before. The CNBr generated fragments were then dissolved in a minimum volume of 1.0 M NaOH (with subsequent pH correction to 7.5 with HCl) and then 10 mM potassium phosphate buffer pH 7.5. In some experiments acid-denatured but uncleaved α-crystallin was produced by the above protocol but with the omission of CNBr.

Oxygen free radical treatment of lens crystallins and non-lenticular proteins

α-, HMWα-, β_H-, β_L- and γ-crystallin, bovine serum albumin, ovalbumin, lysozyme, haemoglobin, carbonic anhydrase, alcohol dehydrogenase, β-amylase and thyroglobulin at 1.0 mg/ml in 10.0 mM potassium phosphate (pH 7.5) were filtered to sterility (through a 0.2 μm cellulose nitrate filter) and H_2O_2 (at 50 mM final concentration) was added to the protein solutions, followed by the addition of a sterile solution of $CuSO_4$ (at 100 μM final concentration). The samples were incubated at 37°C and 500 μl aliquots taken at various time intervals and added to tubes containing catalase (260 kU/ml). The samples were then left at room temperature for a further 30 min before the addition of TCA to 5% (w/v) and then cooled at 4°C for 30 min, prior to centrifugation at 12,000 g for 10 min. The supernatant of each sample was collected and BCA-protein estimation performed according to the manufacturer's instructions (Pierce (U.K.) Ltd., Cambridge, U.K.). The protein precipitates were retained for sodium dodecylsulphate, discontinuous buffer, polyacrylamide gel electrophoresis (SDS-PAGE) using 15% acrylamide resolving gels, according to the method of Laemmli[30] with staining in TCA according to the method of Neuhoff *et al.*[38]

RESULTS

Proteins were treated with H_2O_2 and copper and their fragmentation was determined by measurement of the acid-soluble material (fragments) using the BCA-method of protein analysis which detects peptide bonds. Using an oxygen free radical generating system of a fixed concentration of H_2O_2 (50 mM) and copper (100 μM), we first compared the fragmentation of BSA and α-crystallin (both at 1.0 mg/ml). Figure 1 demonstrates the very large difference in the susceptibility of BSA and α-crystallin to free radical induced fragmentation. Acid-soluble protein fragments were rapidly formed from BSA – up to approximately 90% of the total protein was found in this fraction within 4 h. Conversely, only minor fragmentation of α-crystallin to acid-soluble peptides was seen – at most 10% of the protein was cleaved in 24 h. The cleavage of the proteins was dependent on the presence of both copper and H_2O_2 together and, in addition, no autolysis of either protein occurred upon incubation in the absence of either agent (data not shown).

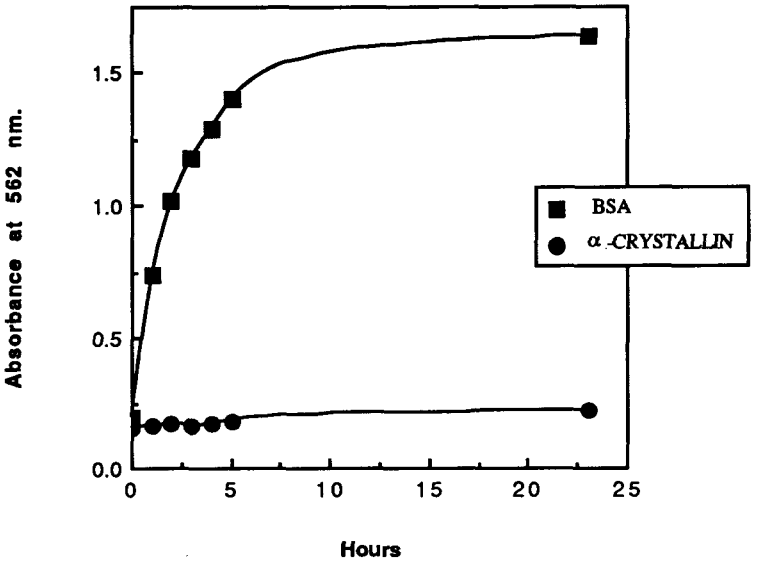

FIGURE 1 The comparative effect of hydroxyl radical attack of bovine serum albumin and α-crystallin. α-Crystallin and BSA (both at 1.0 mg/ml) were incubated at 37°C with copper (100 μM) and H_2O_2 (50 mM). Samples were taken at indicated time points and trichloroacetic acid-soluble peptide fragments were determined using the BCA reagent (detecting peptide bonds) by absorbance at 562 nm. Results are the mean of 3 experiments.

The very different responses of BSA and α-crystallin to treatment with the hydroxyl radical generating system was confirmed by SDS-PAGE of the acid-precipitable fractions. The polyacrylamide gel of Figure 2 demonstrates the very rapid attack of the BSA by the hydroxyl radicals – the protein being extensively cleaved, as evidenced by streaking on the gel even at "zero time" (lane 6), following addition of copper and peroxide. In contrast, the two major bands of the α-crystallin subunits showed little change at either 0 or 2 h treatment. However, after 23 h treatment (lane 11) the α-crystallin bands showed minor differences in migration and, in addition, two new bands (fragments) below 20,000 M_r appeared. Controls of α-crystallin incubated without additions for 0 and 23 hours (lanes 12 and 13) demonstrate the lack of autolysis.

In order to determine whether our findings with α-crystallin and BSA were generally applicable to a range of lenticular and non-lenticular proteins, the responses of a number of crystallins (α, HMWα, β_H, β_L and γ) and proteins of non-lenticular origin (BSA, ovalbumin, lysozyme, haemoglobin, carbonic anhydrase, alcohol dehydrogenase, β-amylase and thyroglobulin) to treatment with the oxygen radical generating system (100 μM Cu and 50 mM H_2O_2) were therefore compared. Figure 3 shows the difference in BCA-detectable, acid soluble peptide fragments (Δ absorbance at 562 nm) produced following 5 h treatment with copper and peroxide, and clearly demonstrates the large differences in susceptibility between the lenticular and non-lenticular proteins. The least susceptible non-lenticular protein was haemoglobin and the most susceptible was ovalbumin. The different crystallin species were common in their resistance to cleavage; β_H-crystallin being totally uncleaved and β_L-crystallin the

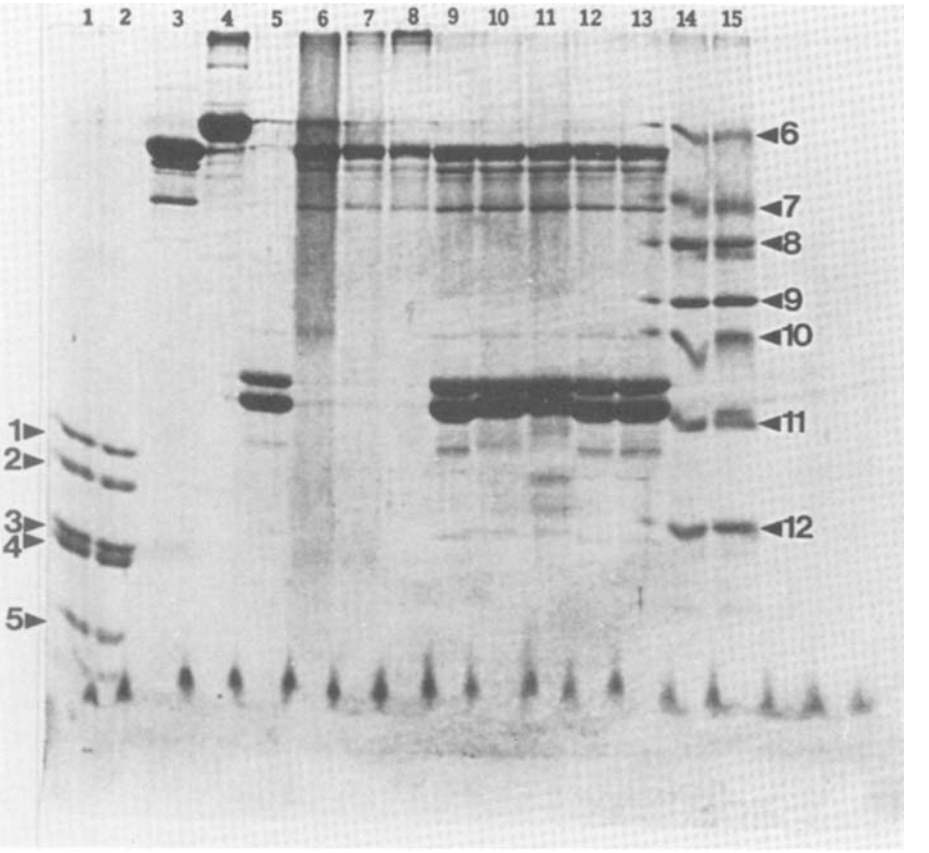

FIGURE 2 The comparative effect of hydroxyl radical attack of bovine serum albumin and α-crystallin as shown by SDS-PAGE. Lanes 1 and 2, molecular weight markers (myoglobin CNBr-peptides M_r 16950, 14400, 8160, 6210 and 2510; markers 1–5 respectively); lanes 14 and 15, molecular weight markers (M_r 66000, 45000, 36000, 29000, 24000, 20100, 14200; markers 6-12 respectively). Lane 3, catalase (untreated). Lane 4, BSA (untreated). Lane 5, α-crystallin (untreated). Lanes 6, 7 and 8, BSA treated with copper (100 μM) and H_2O_2 (50 mM) for 0, 2 and 22 h respectively. Lanes 9, 10 and 11, α-crystallin treated similarly with copper/peroxide for 0, 2 and 22 h respectively. Lanes 12 and 13, α-crystallin incubated for 0 and 22 h respectively in the absence of copper/peroxide.

least resistant. SDS-PAGE again confirmed the fragmentation of the non-lenticular proteins; Figure 4 shows the complete loss of the non-lenticular proteins following 5 h incubation with the hydroxyl radical generating system, yet the resistance of α-crystallin.

In an attempt to partially characterize the apparent resistance of the crystallins to oxygen radical insult, the denaturation and prior fragmentation of α-crystallin was investigated and found to markedly affect the stability of the protein. When α-crystallin (1.0 mg/ml) was acid-denatured or chemically cleaved using CNBr (to large non-acid soluble polypeptides) the protein lost its resistance to the free radical induced fragmentation (Figure 5). Hence the resistance of α-crystallin resides in a correct protein conformation and is not solely a result of the presence of randomly arranged appropriate amino acid residues scavenging the hydroxyl radicals or binding copper

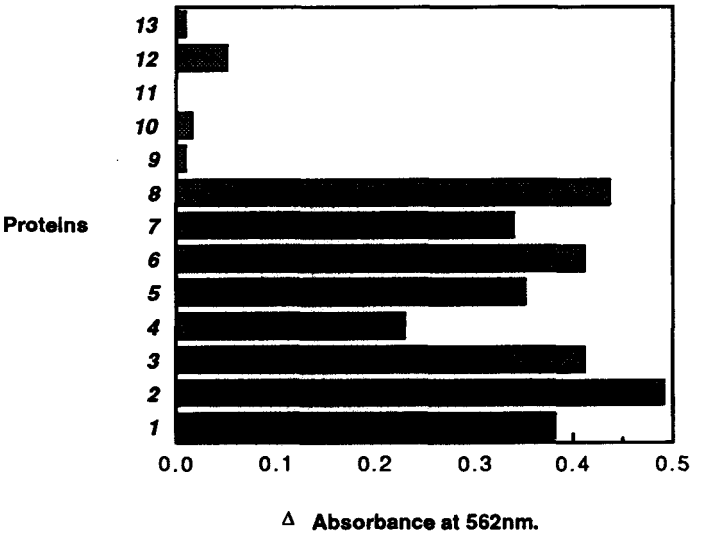

FIGURE 3 The comparative susceptibility of crystallins and non-lenticular proteins to copper and hydrogen peroxide-mediated cleavage. Proteins: 1 — BSA, 2 — ovalbumin, 3 — lysozyme, 4 — haemoglobin, 5 — carbonic anhydrase, 6 — alcohol dehydrogenase, 7 — β-amylase, 8 — thyroglobulin, 9 — α-crystallin, 10 — HMWα-crystallin, 11 — β_H-crystallin, 12 — β_L-crystallin, 13 — γ-crystallin were treated with copper and peroxide and acid-soluble, BCA-reactive fragments were detected as described in Figure 1. Results, the mean of 5 experiments, are the difference in absorbance at 562 nm for 0 and 5 h treatment. Standard error of the mean for BSA and α-crystallin were 0·010 and 0·007, respectively.

ions. Heat treatment (100°C for 5 min), however, prior to incubation with the copper and hydrogen peroxide did not change the protein's resistance to free radical mediated cleavage; the heated crystallin remained as stable as the native untreated protein.

DISCUSSION

Studies by Garland *et al.*,[27] McDermott *et al.*[31] and Ortwerth *et al.*[32] have suggested that cleavage of lens crystallins and the production of aggregates can be achieved using transition metal ions and/or ascorbate. Ascorbate and ions such as Fe^{3+} and Cu^{2+} may combine to produce the reduced ion and H_2O_2, and these species may subsequently react to form hydroxyl radicals via a Fenton type reaction. Thus the combination of H_2O_2 and copper (both species being elevated in the cataractous lens[13,20,22,23]) can be employed directly to produce protein fragments of BSA[14] and crystallin.[28] However, comparison of the rates of fragmentation of α-crystallin and BSA revealed a vast difference in susceptibilities. This observation may partially explain the exceptionally long incubation times used by other workers to achieve modifications of crystallin in oxidative systems, e.g. 80 h treatment of crystallins with ascorbate and copper/iron,[27] 96 h treatment of α-crystallin with iron alone[31] and 8 weeks treatment of α-crystallin with ascorbate alone.[32,33] Similarly, other crystallins (HMWα, β_H, β_L and γ) were also resistant to cleavage mediated by copper and hydrogen peroxide, whereas all the non-lenticular proteins investigated (BSA, ovalbumin, lysozyme, haemoglobin, carbonic anhydrase, alcohol dehydrogenase, β-amylase and thyroglobulin) were very susceptible.

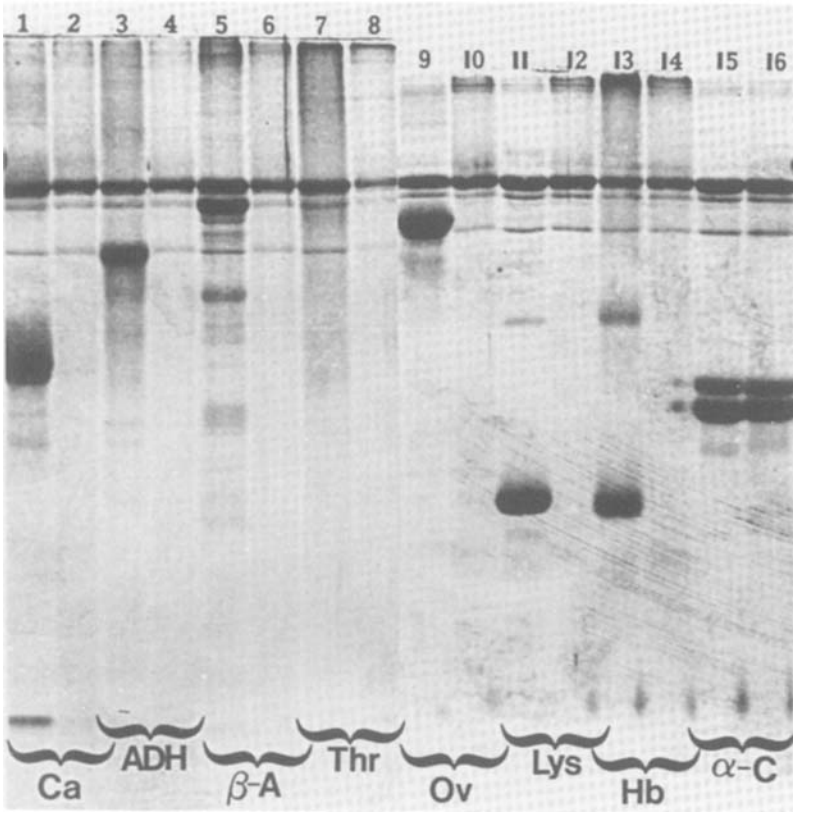

FIGURE 4 The comparative susceptibility of crystallins and non-lenticular proteins to copper and hydrogen peroxide-mediated cleavage as shown by SDS-PAGE. Lanes 1 and 2, carbonic anhydrase. Lanes 3 and 4, alcohol dehydrogenase. Lanes 5 and 6, β-amylase. Lanes 7 and 8, thyroglobulin. Lanes 9 and 10, ovalbumin. Lanes 11 and 12, lysozyme. Lanes 13 and 14, haemoglobin. Lanes 15 and 16, α-crystallin. Proteins at 1.0 mg/ml were treated with copper (100 μM) and H_2O_2 (50 mM) for 0 (lanes 1, 3, 5, 7, 9, 11, 13 and 15) and 5 h (lanes 2, 4, 6, 8, 10, 12, 14 and 16).

The reasons for the unique stability of the crystallins can only be speculated upon at present. One possibility is that the primary sequences of the different crystallin proteins are common in their abundance of one or more amino acids which may actively scavenge the radical species. This hypothesis was explored by acid denaturation and CNBr cleavage of α-crystallin. If the stability to oxygen radical attack was a function solely of the presence of certain amino acids, then the acid denatured protein and the CNBr peptides should have been as stable as the native protein. This was clearly not the case; the treatments resulted in cleavage to levels similar to that observed with BSA. Hence the correct (native) crystallin conformation was required for its resistance to the copper/peroxide-mediated cleavage. Heat-treatment of α-crystallin, however, did not result in a loss of resistance to oxygen radical cleavage; the treated protein being as stable as the native species. This result may possibly be a function of the extreme thermostability reported for α-crystallin.[34]

One hypothetical explanation of the resistance of the crystallins to radical induced cleavage could be that the crystallins may actively scavenge reactive oxygen species

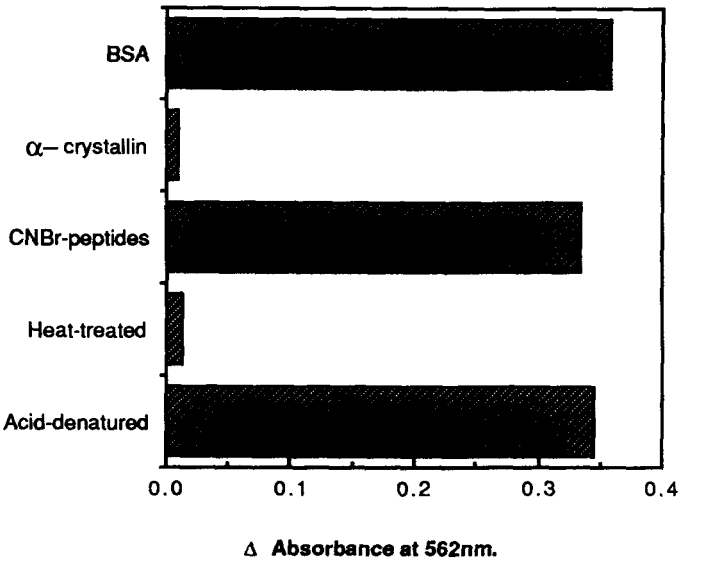

FIGURE 5 The effect of crystallin conformation on its susceptibility to copper/H_2O_2-mediated fragmentation. BSA and α-crystallin in its native form or as CNBr-peptides, heat-treated α-crystallin or acid denatured α-crystallin, were exposed to copper (100 μM) and H_2O_2 (50 mM) for 5 h. The difference in acid-soluble, BCA-reactive fragments at 0 and 5 h treatment, were detected as described in Figure 1.

via an inherent enzymic activity, e.g. a superoxide dismutase (SOD) or catalase-like activity. It is thus interesting to note that certain crystallins have been demonstrated to possess highly similar amino acid sequences to a number of enzymes and indeed, to possess an inherent level of those enzyme activities.[39] For example, certain structural crystallin proteins have been reported to possess lactate dehydrogenase, argino-succinate lystate and α-enolase, activities and very high sequence homologies with a number of other enzymes such as prostaglandin-F synthetase and glutathione S-transferase.[39] None of these enzyme activities, it should be noted however, have been reported to be SOD, catalase, glutathione peroxidase or any known anti-radical activity. Hence the suggestion of such a stabilizing and inherent enzyme activity is purely speculative. In addition no catalase activity was found associated with the α-crystallin preparations used in the present experiments (data not shown).

Hence, regardless of the means by which this resistance against oxygen free radical attack occurs, it is clear that the crystallins may be unique proteins in this respect and, in conjunction with their thermoresistance,[34] are thus structural proteins of remarkable stability – able to withstand much of the insult to which they may be exposed to during their endurance as the longest lived proteins of the mammalian body. The demonstrated resistance of crystallins to radical-induced cleavage, even under the high and non-physiological copper and peroxide levels used in this study, does not however detract from the fact that crystallin fragments are a feature of aged and cataractous lenses. Although other processes such as inadvertent proteolysis and spontaneous peptide bond cleavage by succinimide formation[35–37] may contribute to fragment formation, it is likely that oxidative processes still play a major role. It is therefore possible that, although relatively stable to attack, the crystallins in the aged lens (and especially in the lens core where the radical scavenging systems become

significantly impaired) could ultimately be fragmented over a period of many years. The persistence of such fragments and their contribution to high molecular weight aggregate formation would be dependent on a concurrent impairment of the proteolytic machinery responsible for their removal.[28]

Acknowledgements

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