Peroxynitrite Reaction with Eye Lens Proteins: α-Crystallin Retains Its Activity Despite Modification

Geetha Thiagarajan, Jaganathan Lakshmanan, Madhavilatha Chalasani, and Dorairajan Balasubramanian

Purpose. Peroxynitrite is a highly potent reactive oxygen/nitrogen species present in the environment and also endogenously in the eye, that causes a variety of disorders. This study was undertaken to look at the oxidative damage that peroxynitrite causes to the proteins of the lens and the functional consequences thereof.

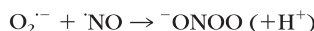
Methods. Peroxynitrite was allowed to react with α-, β-, and γ-crystallins. The formation of nitrotyrosine and nitrotryptophan, dityrosine, protein covalent cross-links, and chain degradation products were monitored by spectroscopy and SDS-PAGE. Conformational changes occurring in the protein were monitored with circular dichroism spectroscopy. The chaperoning ability of α-crystallin was assayed by monitoring its ability to inhibit the self-aggregation of two test proteins: β-crystallin and insulin.

Results. Peroxynitrite reaction produced nitrotyrosine, nitrotryptophan, and dityrosine, nondisulfide covalent cross-linked aggregates, and peptide chain degradation. The hydroxyl radicals produced by peroxynitrite caused more chain degradation than did the carbonate radicals. The oxidative reaction caused increased conformational disorder. The yield was highest in γ-crystallin and least in α-crystallin. The chaperoning ability of α-crystallin was not affected.

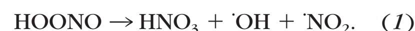
Conclusions. Peroxynitrite reacts with lens proteins, causing extensive covalent chemical changes. However, α-crystallin retains its chaperoning ability, despite the oxidative changes caused by the peroxynitrite reaction, indicating its functional robustness. (*Invest Ophthalmol Vis Sci.* 2004;45:2115–2121) DOI:10.1167/iovs.03-0929

Peroxynitrite ($ONOO^-$) is a highly potent reactive oxygen and nitrogen species, formed by the reaction of two reactive free radicals: superoxide anion ($O_2^{\cdot -}$) and nitric oxide ($^{\cdot}NO$). It is produced endogenously in various tissues of the eye, leading to pathologic conditions such as inflammation, uveitis, corneal damage, glaucoma, retinopathy, and cataracts,[1] particularly in situations in which ($^{\cdot}NO$) is overproduced.[2] The role of peroxynitrite in oxidative damage has been described in experimental autoimmune uveitis, in the orbit of diabetics with rhinocerebral mucormycosis, in inflammation, and in the stimulation of macrophages by retinal proteins.[3–6]

As is obvious from its structure, it is an isomer of the nitrate anion (NO_3^-), but approximately 36 kcal/mol higher in energy. It is an able one-electron oxidant, with an E_{redox} of 1.4 V at pH 7.0. It readily disproportionates into two potent oxidants: $^{\cdot}NO_2$ and $^{\cdot}OH$, or the nitrate anion as



$$O_2^{\cdot -} + {}^{\cdot}NO \rightarrow {}^{-}ONOO\ (+H^+)$$



$$HOONO \rightarrow HNO_3 + {}^{\cdot}OH + {}^{\cdot}NO_2. \quad (1)$$

The yield of the hydroxyl and NO_2 radicals is approximately 30% at pH 6.3 and declines at higher pH. Under physiological conditions, it reacts with carbon dioxide to produce a transient adduct that decomposes to yield 67% nitrate ($^{\cdot}NO_2$) and 33% carbonate ($CO_3^{\cdot -}$) radicals, as

$$ONOO^- + CO_2 \rightarrow (ONOOCOO^-) \rightarrow CO_3^{\cdot -} + {}^{\cdot}NO_2. \quad (2)$$

The reaction of peroxynitrite with proteins yields specific products that depend on the presence or absence of physiological concentrations of peroxynitrite. Thus, peroxynitrite is a source of potent free radicals that can oxidize lipids, thiols, amino acids, and biopolymers.[7] The hallmarks of the peroxynitrite-mediated attack on proteins is the formation of 6-nitrotryptophan, 3-nitrotyrosine, and 3,3′ dityrosine. Nitrotyrosines have been identified in various pathologic conditions and diseases, such as atherosclerosis,[8,9] Alzheimer's and related syndromes,[10,11] neurologic disorders including amyotrophic lateral sclerosis,[12] and others. Likewise, dityrosine has been identified as a marker of disease in a variety of instances, such as amyloid fibril formation,[13] Parkinson's disease,[14] atherosclerotic plaque formation,[15] cataract of the eye lens,[16,17] and oxidative stress such as occurs during exercise.[18]

One group of proteins that is significantly affected by oxidative stress are the crystallins, which are the structural constituents of the eye lens. Because their turnover in the tissue is extremely sluggish, damage to them accumulates over time, thereby affecting the transmission of light by the lens. We have looked at the reaction of peroxynitrite, in vitro, with the major constituent protein of the mammalian lens, α-crystallin. Apart from its structural role as the major constituent of the lens cortex, it is also believed to act in a chaperone-like fashion, inhibiting the aggregation and precipitation of the other lens proteins.[19] We have hence looked at how some of its properties are altered on reaction with peroxynitrite and found, to our surprise, that its structure and conformation were altered, but its chaperone-like functional activity was not.

Methods

Proteins and Peptides

α-, β-, and γ-crystallins were isolated and purified from bovine eye lens homogenate by gel-filtration chromatography[20] on a separation col-

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umn (Sephadex G-200; Roche Diagnostics, Mannheim, Germany) in a pH 7.4 buffer containing 50 mM Tris-HCl, 50 mM NaCl, 1 mM EDTA, and 3 mM sodium azide. The purified crystallins were then dialyzed, lyophilized, and stored at −20°C until used. RNase A, melittin, and insulin were obtained from Sigma-Aldrich (St. Louis, MO). Recombinant human αA-crystallin, purified from *Escherichia coli* was a kind gift of Mohan Rao, Centre for Cellular and Molecular Biology (Hyderabad, India). The chaperone-like functional element peptide—namely, the water-soluble derivative of the sequence 71-88 of αA-crystallin—with the sequence DFVIFLDVKHFSPEDLTVK, was obtained as a kind gift of Krishna K. Sharma of the University of Missouri–Columbia Medical School.

Synthesis of Peroxynitrite

Peroxynitrite was obtained by reacting ice-cold solutions of sodium nitrite (0.6 M) with H_2O_2 (0.7 M) in acidic medium (0.6 M HCl) and rapidly quenching the reaction in NaOH (1.5 M), as described previously.[21] The reaction mixture solution was frozen at −20°C, and the $ONOO^-$ concentrated in the upper layer was collected. Its concentration was measured at 302 nm using a molar extinction coefficient of 1670 $M^{-1} \cdot cm^{-1}$.

Peroxynitrite modification of test proteins was performed in the following manner. To the test proteins (1 mg/mL) in 50 mM sodium phosphate buffer (pH 7.4) chosen aliquots of $ONOO^-$ in 1.2 M NaOH were added, either in the presence or absence of 10 mM HCO_3^- with vigorous mixing. The mixture was incubated at room temperature for 15 minutes before any further analysis. For control samples, the same aliquots were added but without the peroxynitrite.

Peroxynitrite modification of the free amino acids tyrosine and tryptophan was achieved by the bolus addition of alkaline stock solution (1.2 M NaOH) of $ONOO^-$ (final concentration, 0.250–2.0 mM) to the reaction mixture (1 mL) containing 1 mg amino acid in 50 mM sodium phosphate buffer (pH 7.4), either in the presence or absence of 10 mM bicarbonate, immediately followed by vortex mixing and incubating for 15 minutes at room temperature.

Amino Acid Analysis

The amino acid analyses of the parent and the peroxynitrite-modified samples of α-crystallin were performed using an amino acid analyzer (LKB Alpha Plus; Pharmacia, Uppsala, Sweden). The samples were hydrolyzed under standard conditions using 6 N HCl at 110°C for 22 hours. Protection was performed for cys, met, and tyr, using proper protecting agents.

Photo-oxidation of α-Crystallin

Photo-oxidation was achieved by mixing α-crystallin (1 mg/mL) with 10 μM riboflavin and irradiating the mixture at 445 nm for 30 minutes, with the light source of a fluorescence spectrometer (F-2500; Hitachi, Tokyo, Japan), with the excitation monochromator slit wide open. Such a sensitizer-mediated reaction is known to oxidize target proteins efficiently.[22]

Spectral Measurements

The nitrated products of $ONOO^-$-modified crystallins and of monomeric tryptophan and tyrosine were estimated using absorption spectrometry. In the case of trp, the absorbance of 6-nitrotryptophan at 400 nm (ϵ_M = 5200 $M^{-1} \cdot cm^{-1}$) was measured.[23] With tyrosine, 0.5–1 N NaOH was added (4:1 vol/vol, final pH 11–11.5) and the 3-nitrotyrosine formed was estimated[24] by reading the absorbances at 428 nm (ϵ_M = 4200 $M^{-1} \cdot cm^{-1}$). Intrinsic fluorescence spectra due to tyrosine and tryptophan were recorded using the fluorescence spectrophotometer (F-2500; Hitachi) with excitation wavelengths of 275 and 290 nm, respectively. The excitation and emission band-passes were set at 2.5 nm. Dityrosine emission was monitored with an excitation wavelength of 320 nm and emission near 400 nm, and its quenching monitored using 0.5 M borate/boric acid pH 8.7 buffer, which is a specific quencher of this fluorophore.[25] The fluorescence of the extrinsic probe 8-anilinonaphthalene 1-sulfonic acid (ANS) was monitored using an excitation wavelength of 375 nm and emission in the 500 nm region. Circular dichroism (CD) measurements were performed with a spectropolarimeter (J715; Jasco, Tokyo, Japan). Sample concentrations were 1.0 mg/mL in 50 mM sodium phosphate buffer (pH 7.4). The near and far UV-CD spectra were recorded using 1-cm and 0.02-cm path length cells, respectively.

Assay for Protein Aggregation

Chaperone-like activity of the unmodified and ONOO-modified α-crystallin was studied with a target protein aggregation assay.[26] The isothermal aggregation of the proteins insulin and lysozyme (0.2 mg/mL in 10 mM phosphate buffer [pH 7.4], containing 100 mM NaCl) was initiated by the addition of 25 μL of 1 M dithiothreitol (DTT) to 1.2 mL of the protein at 37°C. The extent of aggregation was measured as a function of time by monitoring the scattering at 465 nm in a fluorescence spectrophotometer (F-4000 Hitachi). The thermal aggregation of β_L-crystallin was monitored in 10 mM phosphate buffer (pH 7.4), containing 100 mM NaCl at 60°C. The buffer, containing unmodified or peroxynitrite-treated α-crystallin/peptide, was preincubated at 60°C for 10 minutes before the addition of 60 μL β_L-crystallin to make a final concentration of 0.2 mg/mL. Aggregation was monitored by measuring the light-scattering as just described.

Gel Electrophoresis

Polyacrylamide gel electrophoresis (PAGE) was performed with a commercial system (Mighty Small; Hoefer, San Francisco, CA), using 12% acrylamide gels with added sodium dodecyl sulfate (SDS) and β-mercaptoethanol. Agarose (1.4%) gel electrophoresis was used to estimate the size of the modified crystallin in the "native" state, and the protein bands were visualized with Coomassie blue staining.

Trypsin Digestion of αA-Crystallin

Human αA-crystallin was incubated with trypsin in 50 mM Tris-Cl buffer (pH 7.4) at 37°C for 25 minutes, in the ratio of αA-crystallin: trypsin of 100:1. The action of trypsin was inhibited with BPTI (10 μg/mL) at 37°C for 30 minutes. Individual peptide fractions were separated by reverse-phase HPLC (Zorbax 300SB-C18 column; 9.4 × 250 mm, 5-μm; Agilent, Palo Alto, CA), using 0.1% trifluoroacetic acid (TFA) for 55 minutes followed by a linear gradient of 0.4% per minute acetonitrile in water. Detection was performed at 214 nm. Each fraction was then lyophilized and modified with peroxynitrite in a manner similar to that used for α-crystallin. Chaperone activity of each peptide fraction before and after modification was studied using both insulin and β_L-crystallin as target proteins.

Results

Modification of Tyr and Trp Residues

Earlier studies have indicated that the peroxynitrite reaction with tyr and trp side chains leads to nitrotyrosine and nitrotryptophan and also dityrosine, but the relative yields of the nitration reaction and dityrosine formation depend on the presence of dissolved CO_2 in the medium and the concentration of $ONOO^-$ itself.[27,28] Figure 1A bears this out in the case of α-crystallin, where the amounts of nitrotyr and nitrotrp increase with concentration of the oxidant itself and the presence of added bicarbonate at all concentrations of peroxynitrite. In all further experiments, we mixed 1 mg/mL protein solution with 1 mM peroxynitrite (±10 mM bicarbonate) and let the reaction run for 15 minutes, as the standard or uniform reaction condition.

Among the bovine lens proteins, the βγ-crystallins have the highest number of tyr and trp residues.[20] Thus, they would be expected to produce more nitrated residues as well as dity-

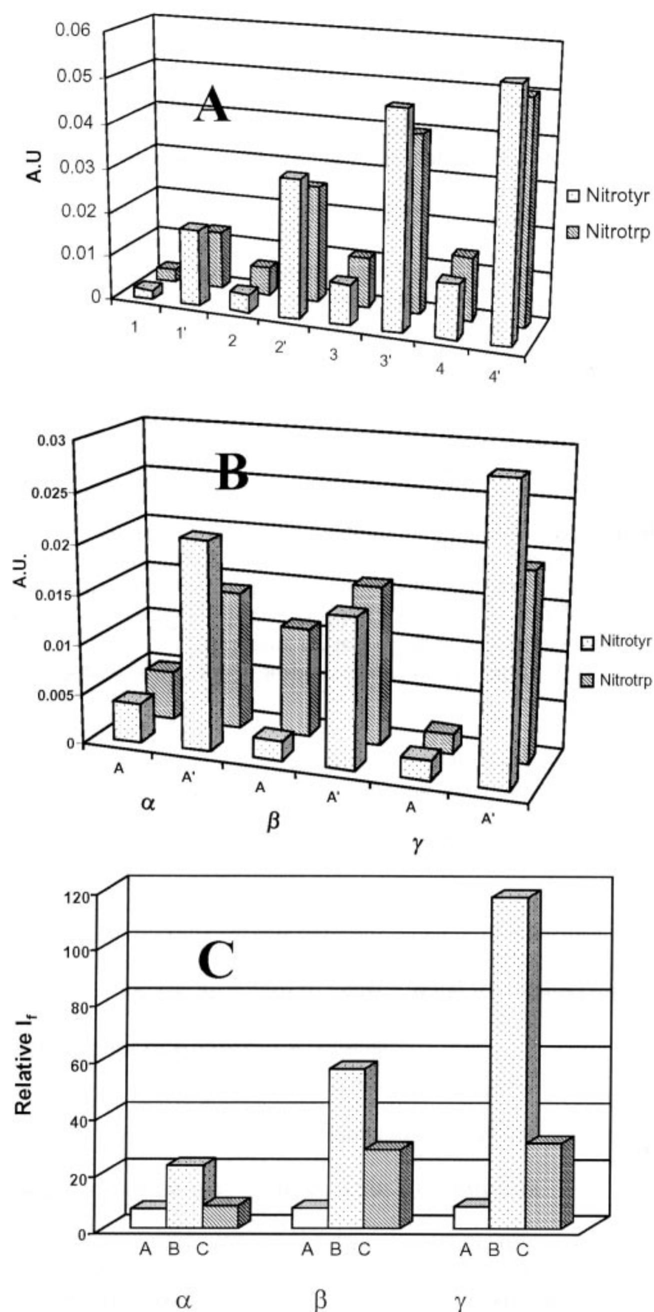


FIGURE 1. (**A**) Nitration of the tyr and trp residues in α-crystallin increases with increasing concentration of peroxynitrite, both in the absence and presence of 10 mM bicarbonate. *Lanes 1*, *2*, *3*, and *4* correspond to the reaction with 0.25 mM, 0.5 mM, 1.0 mM, and 2 mM $ONOO^-$, respectively, whereas *lanes 1′*, *2′*, *3′*, and *4′* represent the addition of 10 mM HCO_3^- to each of these, respectively. In the *y*-axis, AU, absorption units of nitrotyr and nitrotrp. (**B**) Relative yields of nitrotyr and nitrotrp in α-, β-, and γ-crystallins. In each case, lanes marked *A* and *A′* represent reactions with 1 mM peroxynitrite in the absence and presence of 10 mM bicarbonate. (**C**) Relative yields of dityrosine in α-, β-, and γ-crystallins in the control (A) and after reaction with 1 mM peroxynitrite in the absence (B) and presence of 10 mM sodium bicarbonate (C). The *y*-axis denotes the relative emission intensity of dityrosine at 400 nm. In all cases, the protein concentration was 1 mg/mL in 50 mM phosphate buffer (pH 7.4).

rosine links. This expectation were borne out as illustrated in Figures 1B and 1C, where we compare the results obtained with γ-and α-crystallins. We had earlier shown that among the crystallins, the γ-family form dityrosine linkages most readily, whereas α-crystallin yields the least.[29,30]

Chain Cross-linking and Degradation

Figure 2 shows the SDS-PAGE profile of the reaction products of the crystallins with peroxynitrite. Reaction with peroxynitrite led to high-molecular-weight aggregates and also degradation products, in some instances (β- and γ-crystallins). The proportion of the low-molecular-weight degradation products was reduced when the reaction was conducted in the presence of bicarbonate. (Densitometric analysis of the protein bands in the gel revealed a maximum reduction in the degradation products' intensity of from 28% to 7% in the case of γ-crystallins, when the reaction was performed in the presence of bicarbonate. With β-crystallin, the intensity was reduced from 41% to 34%, and with α-crystallin the degradation was minor.) As seen in equations (1) and (2), peroxynitrite by itself disproportionates to produce $^{\cdot}OH$ but generates $CO_3^{\cdot-}$ when CO_2 is added ($^{\cdot}NO_2$ is produced in both instances).

It seems that $^{\cdot}OH$ causes more peptide chain degradation than $CO_3^{\cdot-}$. We found similar results when the reactions were conducted with another protein, bovine pancreatic RNase A (RNase A). Analysis of the reaction products with a few other proteins would be worthwhile to arrive at any generalization. It has been noted[1] that $^{\cdot}OH$ is a stronger one-electron oxidizing radical than $^{\cdot}NO_2$ or $CO_3^{\cdot-}$.

Figure 3A shows the effect of peroxynitrite reaction on the conformation of α-crystallin. The secondary structure (largely β-sheet conformation) of the native protein is reduced (band around 218 nm) and a greater degree of chain disorder (band around 205 nm) is noticed. The effect is more pronounced when the protein is reacted with $ONOO^-$ in the presence of bicarbonate. The tertiary structure is altered too, as Figure 3B reveals, The band intensities in the 250- to 260-nm region, ascribable to the phe chromophore, are seen to increase, whereas the weak band around 290 nm, ascribable to the trp chromophore, is significantly altered, indicating some tertiary structural rearrangements around the aromatic residues after the reaction.

Chaperone Function of α-Crystallin

The protein α-crystallin is known to act in a chaperone-like fashion and enable the solubility (inhibit the aggregation and precipitation) of test proteins such as β- or γ-crystallins, lysozyme, or insulin.[26,31] We tested the ability of peroxynitrite-modified α-crystallin in this connection and found surprising results. Figure 4A shows the isothermal aggregation of insulin induced by the addition of DTT and inhibition of this process by the addition of α-crystallin. Despite the oxidative modification and changes in the chain conformation, the peroxynitrite-treated protein was just as good as the parent molecule, indeed somewhat better in protecting insulin from aggregation. Figure 4B shows the results of a similar experiment, conducted using the thermal aggregation of β-crystallin as the target system. Here again, we see that the peroxynitrite-modified α-crystallin displayed a chaperone-like ability comparable to that of the unmodified parent molecule. (In another set of experiments, with lysozyme as the target protein, we again found peroxynitrite-modified α-crystallin to display chaperone-like activity just as efficiently as the parent).

α-Crystallin is thought to exert its chaperone-like action due to its ability to present a hydrophobic receptor surface to the aggregation-prone target protein.[26,31] This receptor surface can be blocked by the addition of the peptide melittin, which thus leads to inhibition of the chaperoning ability of α-crystallin, as shown by Sharma et al.[32] Figure 4B shows that this was the case in our system as well, in which the chaperone-like

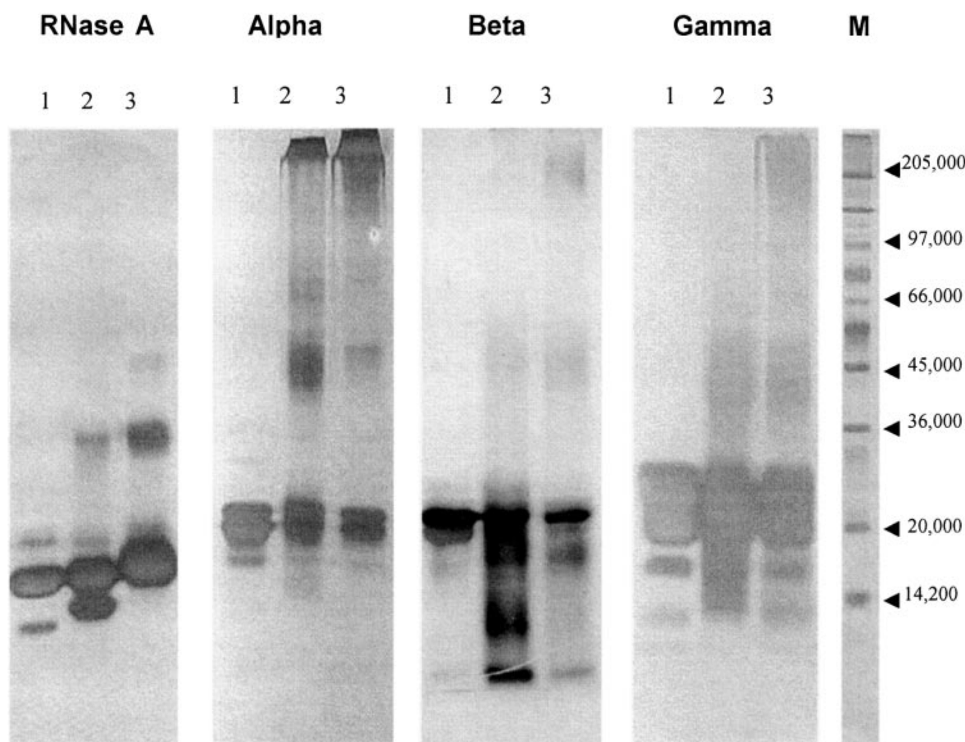


Figure 2. SDS-PAGE profiles of RNase A (RNase), α-, β-, and γ-crystallins (concentrations 1 mg/mL in each case). *Lane 1*: the unmodified protein; *lane 2*: profile after 15 minutes reaction of the protein with 1 mM $ONOO^-$; *lane 3*: profile after reaction with 1 mM $ONOO^-+10$ mM HCO_3^-. The last column displays the molecular weight markers.

activities of both parent α-crystallin and its peroxynitrite-modified product were lost with the addition of melittin to the solution. (The increased scatter is due to enhanced turbidity, which the three-protein mixture tends to exhibit.) Both the modified and native α-crystallins presented a similar type of surface to the target protein.

This ability of the peroxynitrite-modified molecule to retain its chaperone function was unanticipated. We considered the possibility of a change in the size or quaternary structure on modification, which could offer more binding sites for the target protein. Agarose gel electrophoresis of the control and modified proteins in their native states was run, but the two samples moved essentially to the same extent in the gel, suggesting no noticeable change in the quaternary structures. We also considered the possibility of the modified protein exposing a relatively greater hydrophobic surface to which the target protein can bind and thus stay in solution, and checked this possibility by using the method of ANS binding to the parent and modified α-crystallins. The ability of this fluorescence probe to increase its emission intensity when placed in an apolar environment[33] is used to assess the relative hydrophobicities of the protein before and after peroxynitrite reaction. However, we found that ANS bound less well to the modified protein than with the parent α-crystallin (as estimated by the intensity of the fluorescence of protein-bound ANS). It thus appears that the modified protein does not offer as much of a hydrophobic surface as the parent. Furthermore, to make sure that the activity was not due to the presence of any residual unmodified α-crystallin present in the samples, we tried different ratios of target to α-crystallin; as Table 1 shows, we found that the modified protein prevented aggregation even at ratios of 1:4.

Search for Functional Sites in the Molecule

We then checked another possibility suggested by the work of Sharma et al.,[32] who have reported that the functional element for the chaperone-like activity of bovine lens α-crystallin lies in the 19-residue sequence KFVIFLDVKHFSPEDLTVK (residues 71-88 in the αA-crystallin) and the eight-residue sequence FSVNLDVK (residues 75-82 of αB-crystallin). We therefore decided next to study the effect of peroxynitrite

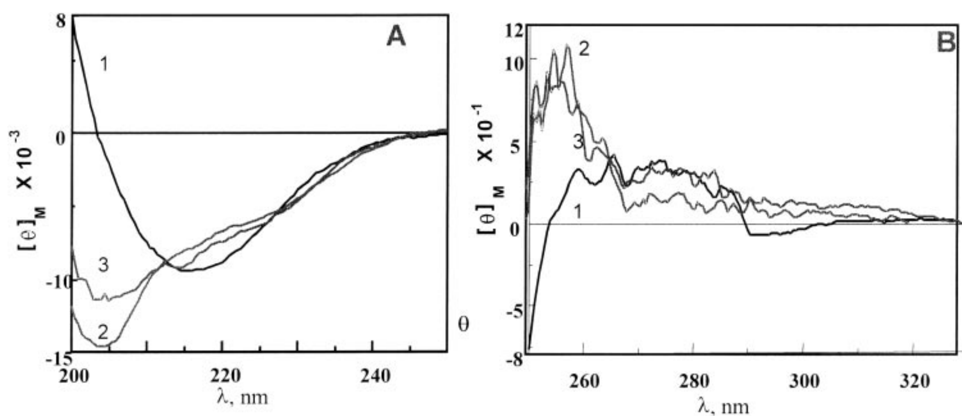
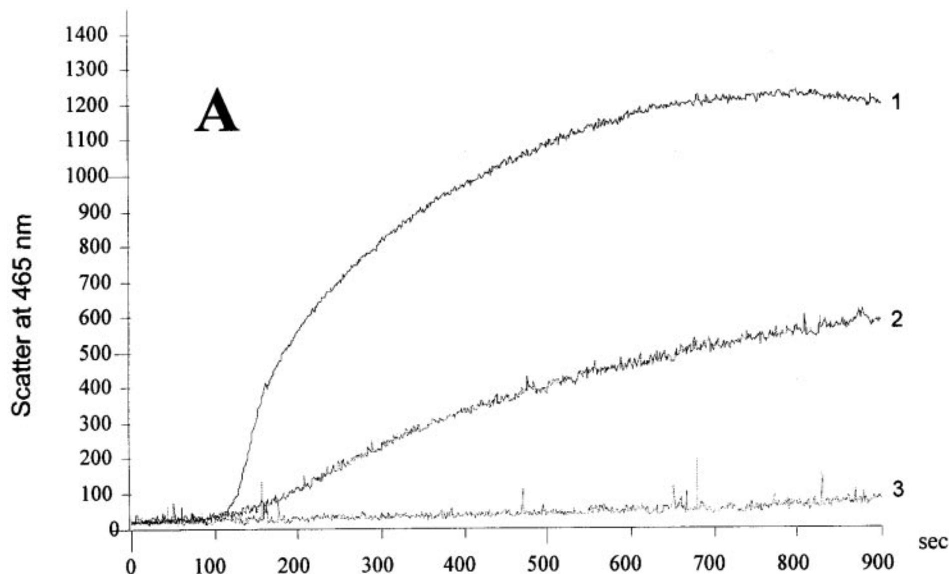


Figure 3. (**A**) Far-UV CD spectra of solutions α-crystallin (1) before and (2) and (3) after reaction for 15 minutes with 1 mM $ONOO^-$ in the absence and presence of 10 mM HCO_3^-, respectively. (**B**) Near-UV CD spectra of the samples. Ellipticity units are in degree square centimeters per decimole.



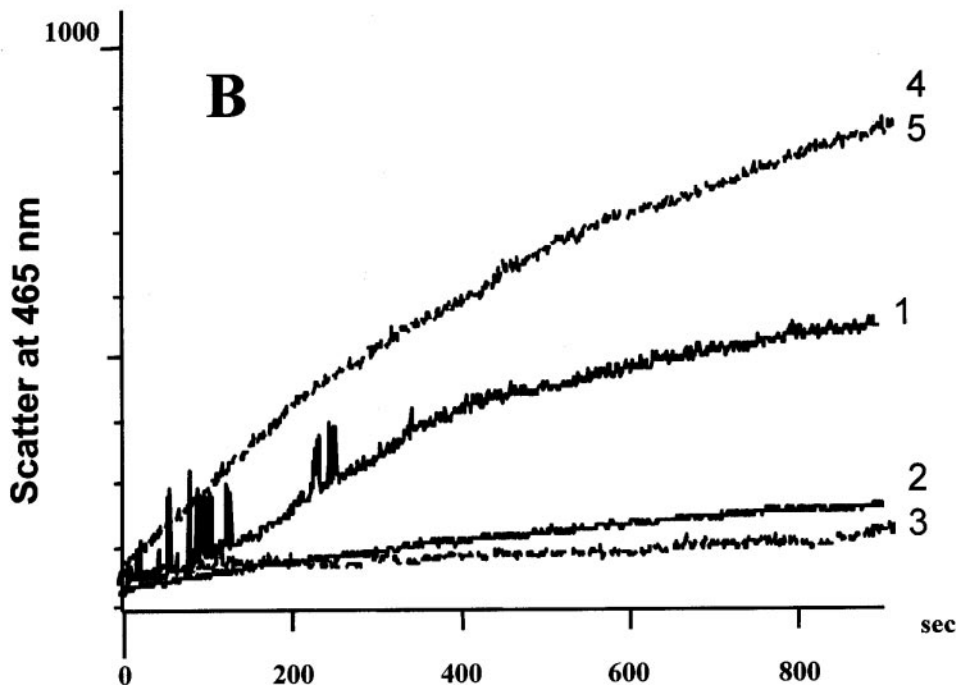


FIGURE 4. **(A)** The aggregation and precipitation of insulin, brought about by the addition of DTT at room temperature, inhibited by unmodified and peroxynitrite-modified α-crystallin. *Curve 1*: light-scattering by insulin alone; *curves 2* and *3*: light-scattering in the presence of unmodified and modified α-crystallin, respectively. The insulin: crystallin ratio used was 2:1. **(B)** Inhibition of the thermal aggregation of β-crystallin by unmodified and modified α-crystallin and abolition of this protective action on the addition of the peptide melittin. *Curve 1*: light-scattering by β-crystallin alone; *curves 2* and *3*: light-scattering in the presence of unmodified and modified α-crystallin, respectively; *curves 4* and *5*: light-scattering after the addition of melittin to the protein mixtures used to obtain *curves 2* and *3*. The target-to-chaperone protein ratio was 1:1, and the melittin content was 100 μg/mL.

treatment on the structure and conformation of the functional element sequence 71-88 of αA-crystallin. Based on considerations of solubility, we chose to work with the sequence DFVIFLDVKHFSPEDLTVK, which has been shown by these authors to be just as active. Although we found this peptide to protect the target protein from aggregating and precipitating out of solution (Fig. 5B, with lysozyme as the target protein), it failed to do so after it was reacted with peroxynitrite. This loss

TABLE 1. Light-Scattering at 465 nm, 450 Seconds after Initiation of the Reaction with Peroxynitrite (PON)

	α-Crystallin:Insulin				α-Crystallin:β_L-Crystallin				
α-Crystallin	**0:1**	**1:2**	**1:3**	**1:4**	**0:1**	**1:1**	**1:2**	**1:3**	**1:4**
Unmodified	2400	900	1450	7280	1580	680	880	2080	2230
PON modified	2400	670	880	2430	1580	530	675	1100	1325

Light-scattering is expressed in arbitrary units, as in Figure 4.

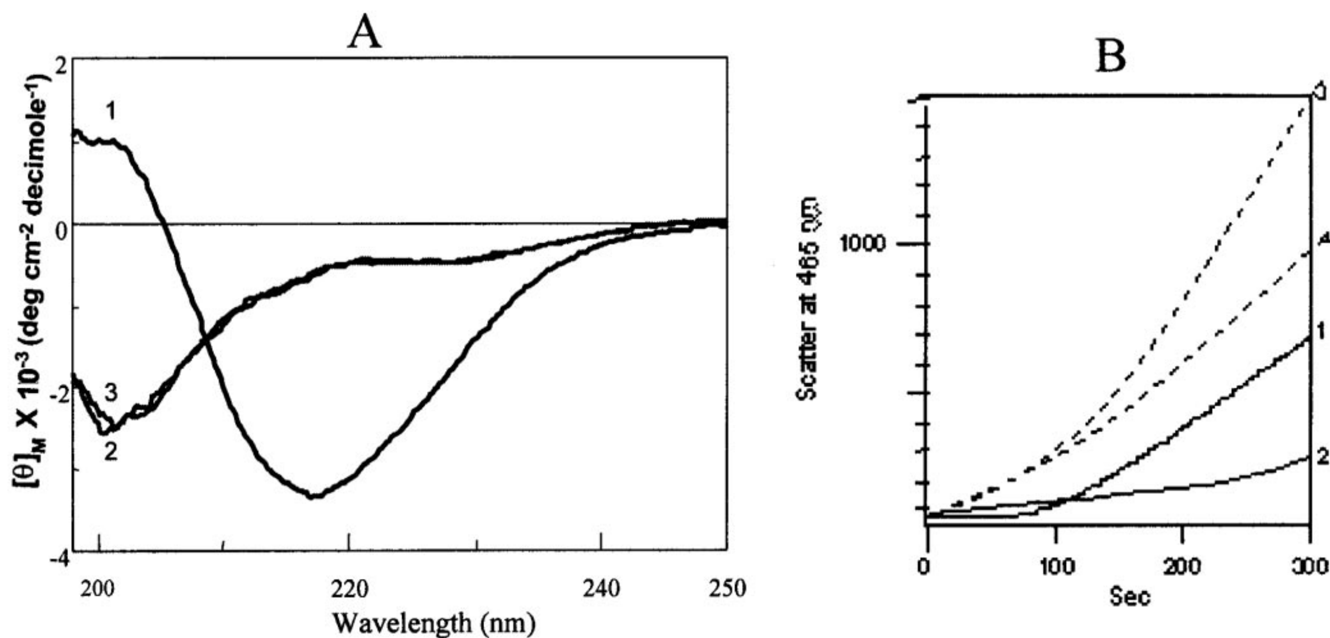


FIGURE 5. **(A)** Far-UV CD spectra of the chaperone-element-peptide before (*curve 1*) and after reaction for 15 minutes with 1 mM peroxynitrite in the absence (*curve 2*) and presence of 10 mM bicarbonate (*curve 3*). CD units are in millidegrees per square centimeter per decimole. **(B)** Assay of the chaperone-like action of the peptide toward the DTT-induced self-aggregation of lysozyme at room temperature. *Curve 1*: lysozyme alone; *curve 2*: in the presence of unmodified peptide; *curves 3* and *4*: in the presence of the peptide treated with 1 mM peroxynitrite in the absence and presence of 10 mM bicarbonate, respectively.

of activity is intriguing because the sequence does not contain tyr or trp residues. However, HPLC analysis of the reaction product of the peptide with peroxynitrite showed what appear to be covalent oligomers. We suspect that the residues his and phe may be affected on oxidation. That his is oxidized to active asx residues and cross-links with lys is known.[34,35]

This result suggests that there may be other regions in the αA-crystallin molecule that contribute to its chaperone-like ability. We thus performed limited digestion of human αA-crystallin, isolated the individual peptide fragments and checked the chaperone-like ability of each of these before and after oxidation. Of interest, we found at least two fractions displaying chaperone-like activity both against insulin and β_L-crystallin even when oxidized by peroxynitrite. We have initiated a project to determine the sequence and conformational characteristics of these fractions and will report on them shortly.

DISCUSSION

Peroxynitrite reaction produced nitrotyrosine, nitrotryptophan, and dityrosine residues in the proteins, and nondisulfide covalent cross-linked aggregates, as well as to peptide chain degradation products. The hydroxyl radicals produced by peroxynitrite caused more chain degradation than the carbonate radicals. The oxidative reaction caused a loss in the secondary structure of the protein and increased conformational disorder.

However, the chaperoning ability of α-crystallin was not affected. Direct information regarding the structure of α-crystallin is lacking because neither the oligomer nor its composite subunits have been crystallized. Therefore, it is difficult to predict which stretches of amino acids will participate in the chaperone-like activity of the protein. Earlier studies on limited tryptic digestion of α-crystallin demonstrated that the C-terminal region of αA-crystallin is important for its activity, which is consistent with the hypothesis that posttranslational changes that are known to occur at the C-terminal region may have significant effects on the ability of αA-crystallin to protect against protein denaturation in vivo.[36]

The overall results, however, indicate that α-crystallin can act as a chaperone under conditions of oxidative stress. Though most earlier reports[37–39] had also indicated this possibility and our present results bear this out, Cherian and Abraham[40] reported that oxidation, mixed disulfide formation and glycation of α-crystallin significantly diminished its chaperone-like activity. As a comparison, we photo-oxidized α-crystallin in solution, using riboflavin as the sensitizer, and monitored the chaperoning ability of the oxidized protein. Here again, though spectral changes indicated modifications in the structure and conformation of the molecule, the photo-oxidized molecule displayed approximately 90% chaperoning ability of the native unmodified α-crystallin, when tried against the target protein, β-crystallin. These results are somewhat similar to those obtained by Dhir et al.,[39] who found that α-crystallin photo-oxidized at 300 nm, and the W9F mutant of the protein, both retain chaperoning ability. Likewise, Weinreb et al.[41] found that the protein from young calf lenses does not lose its chaperoning ability after 24 hour of UVA light exposure, though the same protein from old lenses shows decreased activity after similar treatment.

The function of the eye lens is to receive and focus light, and it is thus under continual photic stress, which is largely oxidative in character. Because oxidative stress is known to be present under usual physiological conditions, it appears probable that the protein α-crystallin has evolved to be able to contribute to the mechanisms that maintain the animal lens in a transparent state. It is also worth noting that whereas the other lens proteins, such as the δ-, ε-, τ-, ζ-, and ρ-crystallins, show taxon specificity,[42,43] α-crystallin is found ubiquitously, and in high homology, across animal species. Also, it has high homology in its sequence with the small heat shock proteins (sHSPs)[44] and, just like the latter, displays chaperone-like properties, which are of functional use in the lens. Its structural and functional robustness thus

appears to have been selected through evolution, particularly for its role in the eye lens, where molecular turnover is extremely sluggish.

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