Effect of a Single AGE Modification on the Structure and Chaperone Activity of Human αB-Crystallin†

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ABSTRACT: During aging, human lens proteins undergo several post-translational modifications, one of which is glycation. This process leads to the formation of advanced glycation end products (AGEs) which accumulate with time possibly leading to the formation of cataract. αB-Crystallin, a predominant protein in the lens, is a member of the small heat shock proteins (sHSPs) which are a ubiquitous class of molecular chaperones that interact with partially denatured proteins to prevent aggregation. This chaperone function is considered to be vital for the maintenance of lens transparency and in the prevention of cataract. In the present study, we introduced an analog of the advanced glycation end product, OP-lysine, at the 90th position of a mutated human αB-crystallin (K90C) by covalent modification of the cysteine residue with *N*-(2-bromoethyl)-3-oxidopyridinium hydrobromide. The AGE-modified K90C-αB-crystallin is termed as K90C-OP. We compared the structural and functional properties of K90C-OP with the original K90C mutant, with K90C chemically modified back to a lysine analog (K90C-AE), and with wild-type human αB-crystallin. Modified K90C-OP showed decreased intrinsic tryptophan fluorescence and bis-ANS binding without significant alterations in either the secondary, tertiary, or quaternary structure. K90C-OP, however, exhibited a reduced efficiency in the chaperoning ability with alcohol dehydrogenase, insulin, and citrate synthase as substrates compared to the other α-crystallin proteins. Therefore, introduction of a single AGE near the chaperone site of human αB-crystallin can alter the chaperoning ability of the protein with only minor changes in the local environment of the protein.

One of the post-translational modifications of proteins in living systems is glycation, which consist of modifications of the side chains of amino acids in proteins by reactive carbonyl components (*1*). First, reversible "early glycation products" are formed via the formation of Schiff bases, which then lead to the formation of advanced glycation end products (AGEs)[1] (*2*), which are relatively stable and tend to accumulate in biological systems with aging. These AGEs are important from a medical point of view since the concentration of AGEs increase in different medical complications such as diabetes (*2*), cataract formation (*3*), nephropathy (*4*), and retinopathy (*5*). A natural protective mechanism against glycation and other harmful post-translational modifications is protein turnover. Normally protein molecules have a limited life span and are periodically degraded and rebuilt. A remarkable exception to this is the proteins of the lens fiber cells, especially those in the lens nucleus, which are stable throughout the lifetime of the individual. Thus, the lens proteins are an appropriate subject to study the effect of AGEs on protein structure and function.

αB-Crystallin, a member of the small heat shock proteins, is present in substantial quantities in lens tissue where it is thought to have a role in maintaining lens transparency. The amount of αB-crystallin present in the soluble protein of young human lens is about 6% (*6*, *7*). Besides the lens, αB-crystallin has been shown to be expressed in a number of tissues such as skeletal and cardiac muscle and to a lesser extent in skin, brain, and kidney, suggesting that it has a general cellular function (*8*, *9*). The ubiquitous nature of αB-crystallin may be related to its chaperone-like property, which allows it to bind strongly to partially unfolded proteins, preventing the formation of large light-scattering aggregates (*10*). In the lens, this function is of paramount importance in preventing cataracts, as the lens proteins are subject to various protein modifications over the lifetime of the organism which makes them highly susceptible to denaturation and aggregation (*11*). Interestingly, αB-crystallin is also overexpressed in a number of neurological disorders such as Alzheimer's disease (*12*), diffuse Lewy body disease (*13*), Creutzfeldt−Jacob disease (*14*), and most prominently in Alexander's disease, in which it forms the major component of Rosenthal fibers (*15*). In many of these neurological diseases, αB-crystallin is found to be associated with intermediate filaments in cytoplasmic inclusion bodies (*15*), but the exact role that αB-crystallin may play in these various diseases is currently not known.

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[1] Abbreviations: AGE, advanced glycation end product; sHSPs, small heat shock proteins; ADH, alcohol dehydrogenase; CS, citrate synthase; bis-ANS, 1,1′-bi(4-anilino)naphthalene-5,5′-disulfonic acid; CD, circular dichroism; IPG, immobilized pH gradient; IEF, isoelectric focusing; HPLC, high-performance liquid chromatography; TCEP, tris-(2-carboxyethyl) phosphine hydrochloride; FRET, fluorescence resonance energy transfer.

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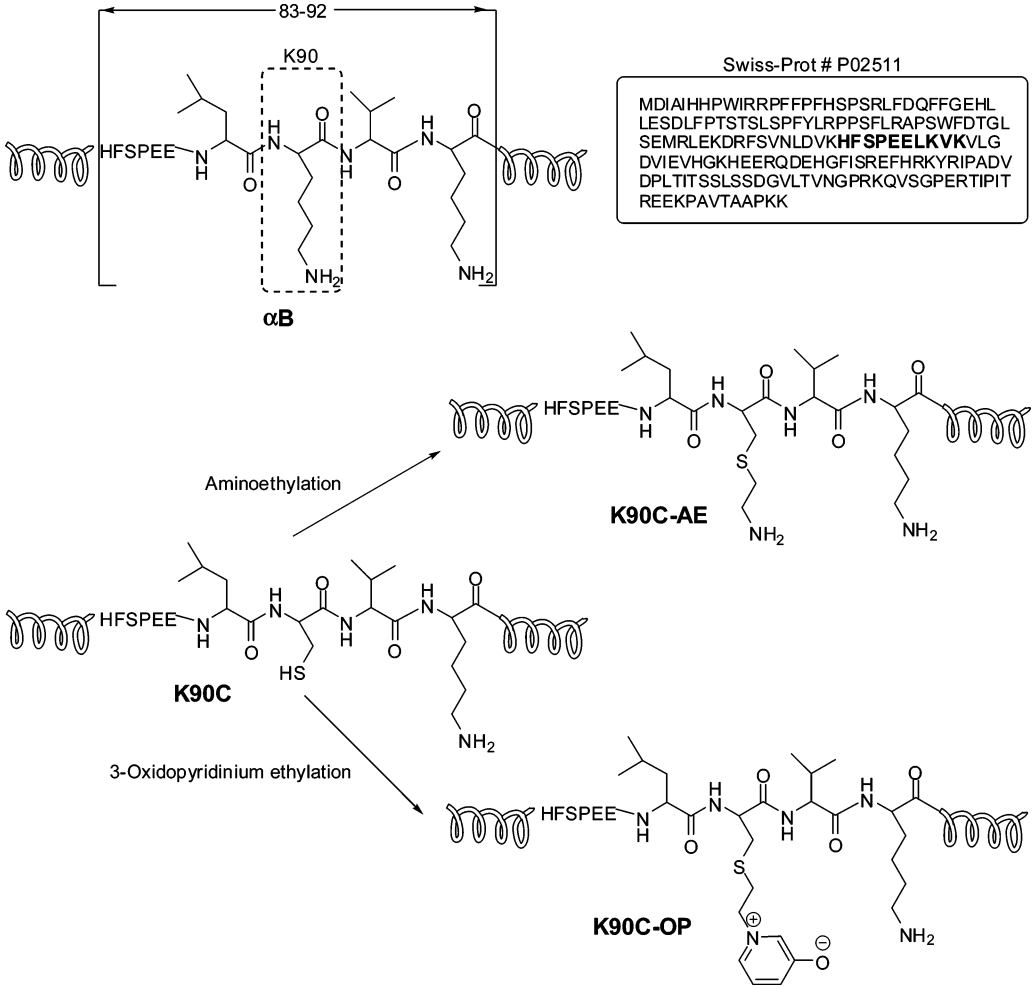


FIGURE 1: Schematic representation of the modification of αB-crystallin-K90C to K90C-AE (aminoethylation) and K90C-OP (3-oxidopyridinium ethylation). At the top is shown residue 90 of wild-type αB-crystallin and the amino acid sequence (inset).

One of the AGEs, present in lens proteins (OP-lysine) has been identified and characterized in our laboratory (*16*). This is one of the most abundant AGE modifications in both aged and cataractous lenses. Structurally, OP-lysine is a pyridinium heterocycle with specific absorbance at 320 nm at pH 7.0. It has a fluorescence maximum at 400 nm after excitation at 320 nm. Therefore, the incorporation of this moiety into a protein molecule can be easily characterized by spectroscopy.

In the work presented here we have studied the effect of a single OP-lysine analog on αB-crystallin structure and function. To the best of our knowledge, there are no reports in the literature that describe a similar approach. We used a human recombinant K90C-αB-crystallin mutant (*17*) for this study. Lys at position 90 in αB-crystallin was chosen as the most probable candidate for glycation-mediated modifications, since we have already demonstrated that this particular residue is heavily modified by ascorbic acid (*18*) in vitro. Moreover, the presence of another lysine (K92 in case of αB-crystallin) near the site of modification (K90C) would cause amplification of glycation at this site (*19*). The introduced Cys residue (Figure 1) can undergo a well-known alkylation reaction with alkyl bromides. A similar approach, named the "chemical modification rescue" method, was used to investigate the role of Lys residues in the active sites of enzymes (*20*).

In this work we compared the chaperone function of the modified K90C αB-crystallins shown in Figure 1 as well as their differences in protein structure.

EXPERIMENTAL PROCEDURES

Materials

Wild-type and mutant human K90C αB-crystallin were expressed in *E. coli* as described previously (*17*), purified by size exclusion and ion-exchange chromatography, and stored in 20 mM Tris + 500 mM NaCl, pH 8.0 at −70 °C until use. The expressed mutant was present in the water-soluble fraction from the bacterial lysate, and denaturation by chaotropic agents was not used at any point in the mutant preparations.

Aminoethyl-8, immobilized TCEP disulfide reducing gel (TCEP gel), Zeba desalt spin columns, and BCA protein assay kit were obtained from Pierce Biotechnology Inc. (Rockford, IL). 3-Hydroxypyridine, 2-bromoethanol, and 48% hydrobromic acid were purchased from Aldrich (Milwaukee, WI). Sequencing grade modified trypsin (EC 3.4.21.4) was purchased from Promega Corp (Madison, WI). Bis-ANS was obtained from Molecular Probes, Inc. (Junction City, OR). Bovine citrate synthase (CS) and yeast alcohol dehydrogenase (ADH) were purchased from Sigma and

Biozyme Laboratories, respectively. Other materials were of analytical grade.

Methods

Preparation of N-(2-Bromoethyl)-3-oxidopyridinium Hydrobromide. *N*-(2-Bromoethyl)-3-oxidopyridinium hydrobromide was prepared from 3-hydroxypyridine in two steps. A mixture of 5 g (52 mmol) of 3-hydroxypyridine and 10 mL (142 mmol) of 2-bromoethanol was heated at 110 °C in a 40 mL screw-top round-bottom glass flask with a Teflon cap. After 1 h the reaction mixture was intensively smeared with 50 mL of ethyl acetate to form a pale brown precipitate which was recrystallized from ethanol to yield 8 g (70% yield) of *N*-(2-hydroxyethyl)-3-oxidopyridinium hydrobromide. Electrospray ionization mass spectrometry (ESI-MS) for $C_7H_{10}NO_2^+$: *m*/*z* 139.90 (100%), 140.89 (8.2%).

The *N*-(2-hydroxyethyl)-3-oxidopyridinium hydrobromide (5 g, 23 mmol) was dissolved in 50 mL of 48% hydrobromic acid and stirred at 110 °C in a round-bottom flask, attached to a water condenser. After 48 h the reaction mixture was evaporated under reduced pressure at less than 30 °C to a final volume of 15 mL. The formed syrup was dissolved in ethanol and crystallized at +4 °C to give 2.2 g (33% yield) of *N*-(2-bromoethyl)-3-oxidopyridinium hydrobromide. ESI-MS for $C_7H_9BrNO^+$: *m*/*z* 201.80 (98% signal intensity), 203.80 (100% signal intensity), 204.81 (8% signal intensity), 202.81 (8% signal intensity). ESI-MS/MS yielded two fragments: $BrC_2H_4^+$ (*m*/*z* 108.7 or 106.7) and $C_5H_5NO^+$ (*m*/*z* 94.9).

Chemical Modifications of Mutant Protein (K90C). Both aminoethylation and 3-oxidopyridinium ethylation were performed with the same protein preparation, after reduction by TCEP gel. Typically, a 10 mg sample of K90C was diluted with 500 mM Tris pH 9.0 to a final volume of 1 mL and carefully bubbled with argon; 1 mL of TCEP gel was added, and the mixture was stirred at 37 °C. After 30 min, a gel was removed by centrifugation at 1000*g*. The supernatant was collected, and the gel was washed twice with 0.5 mL argon-treated 500 mM Tris, pH 9.0. The combined supernatants were divided into three 1 mL parts; one portion was left as a control, the second part was treated with 30 mg of *N*-(2-bromoethyl)-3-oxidopyridinium hydrobromide (final concentration 100 mM), and the third portion was treated with 40 μL of aminoethyl-8 reagent dissolved in methanol (25 mg in 0.5 mL; final concentration 7.5 mM). All reaction mixtures were blanketed by argon. Control and the 3-oxidopyridnium ethylation reaction mixtures were kept at room temperature overnight, and the aminoethylation reaction mixture was kept 5 h at 50 °C. Excess ethylation reagents were removed by extensive dialysis against 50 mM phosphate buffer, pH 7.0, in the presence of 150 mM NaCl. The purity of each protein and the yields after the modification were analyzed by matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) mass spectrometry and by two-dimensional (2D) gel electrophoresis after desalting samples over ZEBA columns in water and lyophilization. In all measurements 2−3 different preparations of each protein were analyzed.

Mass Spectrometry Studies. MALDI-TOF mass spectra were acquired on an Applied Biosystems Voyager DEPro instrument operated in the positive ion linear delayed extraction mode at 25 000 V accelerating potential. The sample was desorbed/ionized with a 20 Hz 337 nm nitrogen laser set to 1074 units. Spectra were the summed/averaged result of three 512-shot acquisitions (0.5 ns bin size, 50 mV full scale, 25 MHz input bandwidth) and were processed with "Data Explorer" (version 4.0.0.0) software. Horse skeletal muscle apomyoglobin $[M + H]^+$ (average 16 952.56 Da, 2.5 pmol on target) and bovine pancreatic α-chymotrypsinogen A (25 657.1 Da if all cysteines are cross-linked, 7.5 pmol on target) were used for external mass calibration of the instrument. All samples for analysis were stored at −20 °C between the time of receipt and the time of analysis. Each sample was dissolved in water. Two microliters of the reconstituted sample was mixed with 18 μL of SA matrix solution, and a 1 μL aliquot of the mixture was applied to a polished stainless steel target. The sample/matrix mixture was stirred on the target with the pipettor tip until crystals appeared. The crystals were allowed to dry under ambient conditions before they were washed twice for 3−5 s with 2−2.5 μL of 0.1% TFA. The crystals were allowed to dry between washings.

Two-Dimensional PAGE Electrophoresis. Two-dimensional PAGE was performed with equipment and materials purchased from Bio-Rad Laboratories (Hercules, CA). Lyophilized protein samples (20−30 μg per strip) were reconstituted in RedyPrep sample buffer provided by the manufacturer. A volume of 185 μL of protein solution was loaded per strip, and each strip underwent passive rehydration at room temperature for 11−16 h. Immobilized pH gradient (IPG) strips pH 5−8 and pH 3−10, 11 cm in length, were used for isoelectric focusing in a Protean IEF (isoelectric focusing) cell. The IEF run was performed using a three-step protocol: (1) 250 V, 20 min, linear; (2) 8000 V, 2.5 h, linear; (3) 8000 V, 20000 V·h, rapid; total 5.3 h and 30 000 V·h. Strips were loaded onto second dimension gels after a two-step equilibration in equilibration buffer I (containing DTT) and equilibration buffer II (containing iodoacetamide) as provided by the manufacturer. Criterion precast gels (10.5−14% Tris−HCl) with IPG comb were used for the second dimension which was run in a Criterion cell. Gels were stained by Bio-Safe coomassie, destained with several changes of water, and imaged by a Nikon Coolpix 2500 digital camera. Images were manually calibrated for MW and pI.

Trypsin Digestion of Proteins. Digestion of proteins with trypsin was performed according to the procedure described on the Promega Web site (http://www.promega.com/tbs/9piv5113/9piv5113.pdf). Briefly, lyophilized proteins were solubilized with 50 mM ammonium bicarbonate (pH 7.8) at a concentration of 100 μg each. They were digested with 4 μg of modified trypsin at 37 °C for 12 h. The addition of 10% TFA to adjust the reaction mixture to pH 2 terminated the digestion reaction.

MALDI-TOF/TOF MS and MS/MS Analyses of Tryptic-Digested Proteins. Portions of the digested samples were diluted in 700/290/10 (v/v/v) acetonitrile/water/88% formic acid to produce solutions with peptide concentrations of about 2 pmol/μL. The diluted samples were mixed 1/1 with α-cyano-4-hydroxycinnamic acid and spotted on a MALDI target for analysis in the ABI 4700 MALDI-TOF/TOF mass spectrometer. The mass scale was calibrated using external peptide standards since very little signal was observed for

trypsin autolysis peptides in the samples. Mass spectra and targeted MS/MS were acquired.

Structure−Function Studies of Modified and Unmodified Proteins. The structural variations between unmodified and modified K90C αB-crystallins were determined by spectroscopic analysis. Phosphate buffer (0.05 M) containing 0.15 M NaCl (pH 7.0) was used in all measurements unless otherwise specified. All UV spectra were performed on a Cary UV−vis model spectrophotometer.

Fluorescence Spectra. The intrinsic tryptophan fluorescence spectra of the unmodified and modified K90C-crystallins (0.2 mg/mL) were recorded using a Jasco FP-750 spectrofluorometer at room temperature. The excitation wavelength was set to 295 nm, and the emission was recorded between 300 and 400 nm. Emission and excitation slits were set at 5 nm.

For further verification of the modification of K90C by the OP derivative, we recorded the fluorescence spectra of the proteins by exciting the proteins at 320 nm, the characteristic absorption maxima of OP-lysine (*16*). Protein samples of 1 mg/mL in phosphate buffer were used for this purpose.

Bis-ANS Fluorescence Studies. The fluorescence spectrum of bis-ANS bound to modified and unmodified proteins was measured using a Jasco FP750 spectrofluorometer. The bis-ANS solution, 20 μM final concentration, was added to 0.2 mg/mL modified and unmodified protein solutions in phosphate buffer. The mixture was thoroughly mixed and then incubated for 10 min. Fluorescence emission spectra were then recorded at 400−600 nm using an excitation wavelength of 390 nm. The excitation and emission slits were set at 5 nm.

Circular Dichroism Studies. The far-UV CD spectra of purified proteins were recorded at 25 °C over a range of 200−250 nm on a Jasco 815 spectropolarimeter using 0.25 mg/mL concentration of the protein solutions in 10 mM phosphate buffer, pH 7.2. A quartz cell of 0.1 cm path length was used, and five spectra for each sample were taken and averaged. The percentage of various secondary structures was determined from the secondary structure prediction software provided with the Jasco 815 spectropolarimeter.

Near-UV CD spectra of the proteins in phosphate buffer were recorded in the same spectropolarimeter at 25 °C over a range of 240−310 nm using a 0.5 cm path length cell. Three spectra of each sample were taken and averaged.

Light-Scattering Studies. The quaternary structural parameters of the modified and unmodified proteins were determined by static light-scattering measurements. Purified proteins in phosphate buffer were injected (100 μg) into a TSK G5000PWXL (Tosoh Bioscience) size exclusion column fitted to an HPLC with refractive index detector (RID) (Shimadzu Scientific Instruments, Inc.) and equilibrated with phosphate buffer. The HPLC was attached to a multiangle light-scattering detector (DAWN). The molar masses of the samples were determined using ASTRA (5.3.0.18) software developed by Wyatt Technology.

Assays of Chaperone-Like Activity. The aggregation of proteins upon heating was monitored by measuring the increase in apparent absorption caused by light scattering (*10*). Assays for chaperone-like function of modified and unmodified αB-crystallin K90C were performed at 37, 25, and 43 °C using alcohol dehydrogenase, insulin, and citrate synthase, respectively, as substrate proteins (*17*). This methodology has been described in detail previously (*17*).

RESULTS

Chemical Modification of Proteins and Confirmation of Modification. Recombinant proteins were purified by a combination of gel filtration and ion-exchange chromatography in 20 mM Tris buffer, pH 8.0−8.5. The preparations allowed direct alkylation of the SH group and allowed us to avoid additional manipulations of the starting protein. Control proteins were treated under the same buffer conditions as the reaction mixtures and incubated in the absence of modifying agent. We tried to keep conditions as mild as possible to prevent any protein structural changes except for those introduced by the modifications. According to the literature, a Cys residue at position 90 does not form a disulfide bond during preparation (*17*). However, Ellman's analysis showed only 70% of free thiol groups in several preparations. To increase the yield of modification we reduced the possible residual 30% of disulfides by TCEP gel.

Schematic representation of modification of the proteins is shown in Figure 1. For aminoethylation we chose *N*-(iodoethyl)-trifluoroacetamide (aminoethyl-8, Pierce), which reacts exclusively with sulfhydryl groups without polymerization. The trifluoroacetyl moiety was hydrolyzed during the alkylation reaction. The incorporation of 3-oxidopyridinium vinyl into the K90C molecule was made by reaction with especially synthesized 3-oxidopyridinium ethyl bromide as described in Methods.

The completeness of the modifications and the purity of the products were confirmed by MALDI-TOF spectrometry (Figure 2). Masses (*m*/*z*) obtained from mass spectrometric analyses were similar to the theoretically calculated values for all proteins. The addition of 43 Da for K90C-AE and 121 Da for K90C-OP show that only one residue was added per protein molecule. In all samples an ion intensity for protein dimers was detected but represented only about 5−10% that of the intensity of the protein monomers.

Tryptic digestion and MS and MS/MS analyses of peptide mixtures were performed to confirm the selectivity of modifications. From the collection of peptides resulting from this cleavage we paid attention to the fragment (83−90) of αB-crystallin (Table 1, row 1) and the fragment (83−92) of K90C (Table 1, row 2) with absolute masses 986.5 and 1188.6 Da, respectively. Although there are no Cys−Cys dimers in the proteins, peptides could very likely form such dimers either during the tryptic digestion or the sample desorption/ionization in MALDI-TOF MS. So the fragment with mass 2374.1 (Table 1, row 2) was also taken into account. The expected modified peptide was confirmed for K90C-OP (1309.7 Da; the net addition of 121.053 Da to (83−92); Table 1, row 5) and for K90C-AE (1004.5 Da; the net addition of 43.042 Da to (83−90); Table 1, row 3). The fragment 1231.6 Da (Table 1, row 4) was not found in the K90C-AE digested peptides mixture. It confirms the similarity of enzymatic susceptibility of wild-type αB-crystallin and its thiolysine analog (the aminoethylated K90C mutant). Trypsin hydrolysis of the AE derivative showed a cleavage at residue 90, which is consistent with

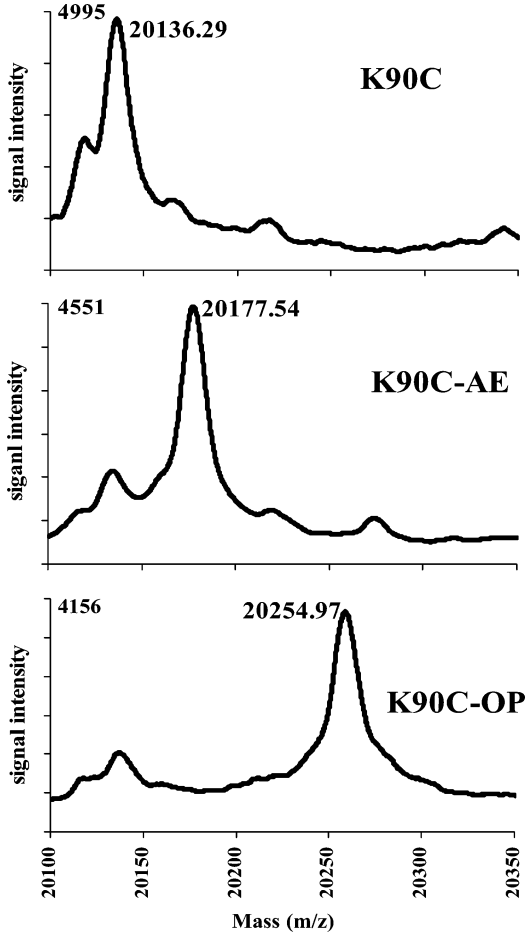


FIGURE 2: MALDI-TOF mass spectra of K90C-αB-crystallin, K90C-AE, and K90C-OP with molecular masses of 20 136.29, 20 177.54, and 20 254.97, respectively.

the conversion of the Cys residue to an amino acid recognized by trypsin as a lysine. The comparison of intensities of modified (1309.6 or 1004.5 Da) and unmodified (1188.6 plus 2374.1 Da) fragments could be used for an indirect estimation of yields of modifications as 86% for K90C-OP and 74% for K90C-AE.

To confirm the presence of the OP modification in K90C, we also recorded the UV absorption and fluorescence spectra of the modified and unmodified K90C-αB-crystallins. The UV absorbance and fluorescence spectra (Figure 3, parts A and B) of OP-modified K90C showed the appearance of the specific 3-oxidopyridinium chromophore with absorbance at 330 nm and emission at 400 nm when excited at 330 nm. The spectra were collected at pH 7.0 because the chromophore's absorbance maximum changes to 290 nm at low pH (*16*).

The 2D electrophoretic protein analysis (Figure 4) with isoelectric focusing in the first dimension and SDS−PAGE in the second dimension was performed using two different pH gradients (pH 3−10 and pH 5−8). All analyzed proteins showed a high level of purity and were about 20 kDa in size. The isoelectric point was found to be 7.76 for both wild-type αB-crystallin and K90C-AE. Changing Lys at position 90 to Cys leads to a decrease in the pI to 7.59. Incorporation of the zwitterionic OP fragment (Figure 1) caused a decrease in the negative charge of protein molecule, and so the pI of K90C-OP was 7.45.

All measurements in the following experiments are done in duplicate from two to three different preparations of each protein.

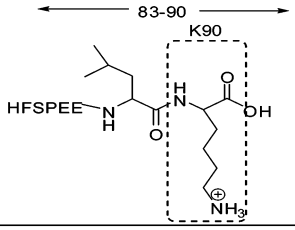
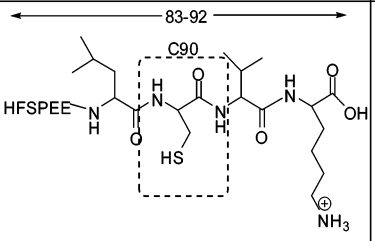
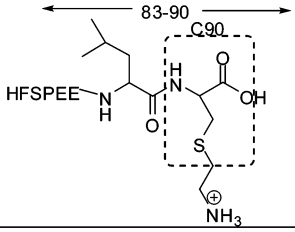
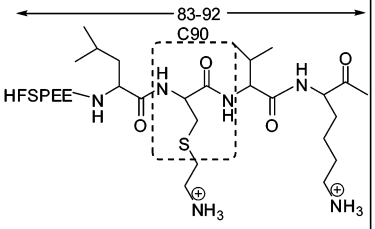
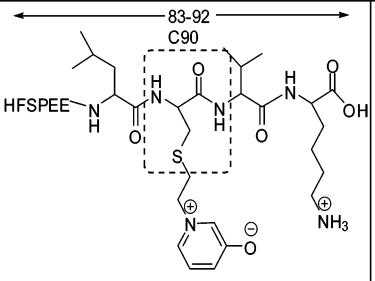
Tryptophan Fluorescence Spectra. The fluorescence characteristics of tryptophan residues depend strongly on the microenvironment and thus provide a sensitive probe of the conformational state of proteins. The tryptophan fluorescence spectra of the modified (K90C-OP and K90C-AE) and unmodified proteins (wild-type recombinant αB-crystallin and αB-K90C) were recorded from 300 to 400 nm after excitation at 295 nm. All the proteins showed tryptophan emission maxima at 340 nm indicating an approximate native conformation of the protein. The fluorescence intensity of the K90C-OP is comparably less than that of unmodified K90C (Figure 5A). This might be due to the energy transfer that occurs between the tryptophan residues and the OP molecule in OP-K90C (Figure 3B) suggesting that the OP aromatic ring of K90C-OP is in close proximity to a tryptophan moiety in the protein molecule.

Bis-ANS Fluorescence Spectra. We also investigated the structural differences among the modified and unmodified proteins by analyzing bis-ANS binding. This dye is known to bind to surface-exposed hydrophobic patches and is helpful in analyzing relative hydrophobicity and conformational changes of the proteins. The bis-ANS fluorescence spectrum of the modified and unmodified proteins has been recorded at 400−600 nm after excitation at 390 nm. The modified as well as the unmodified protein showed emission maxima at 498 nm, but the bis-ANS fluorescence intensity of the OP-modified protein at the emission maximum is considerably less (50%) than that of the unmodified proteins (Figure 5B). This indicates that although the proteins exhibit surface-exposed hydrophobic surfaces, the total accessible hydrophobic surfaces provided by the modified protein (K90C-OP) are significantly less than the control proteins.

Dynamic Light-Scattering Studies. The quaternary structure of the modified as well as the unmodified proteins has been studied by light scattering. The oligomeric masses of the proteins were determined using HPLC fitted with a gel filtration column and refractive index detector, attached to a static light-scattering instrument. Figure 6 shows the bar graph for the average of the molar mass of the modified and unmodified proteins from three different protein preparations. Wild-type αB-crystallin has an average MW of 600 kDa, which is consistent with other reports found in literature (*21*). K90C and both derivatives exhibited a slightly smaller MW (∼540 kDa) as shown in Figure 5. This slight change in molecular size is within the standard error range and therefore is an insignificant change in the molar mass. Taken together, there appears to be no drastic change in the oligomeric size of the modified and unmodified proteins.

Near-UV Circular Dichroism Studies. The near-UV−CD spectra of the modified and unmodified proteins were taken at 25 °C between 240 and 310 nm. A significant uplift of the CD signal is observed in the aromatic region (250−290 nm) without any appearance or disappearance of the signature peaks for K90C-OP compared to the other proteins (wild-type αB-crystallin, K90C, and K90C-AE), which might be due to the introduction of the extra aromatic OP-ring in the modified K90C-OP (Figure 7A). This observed CD signal

Table 1: Major Peptides Found in the Tryptic Digestion Mixtures of Modified and Unmodified Proteins

	Peptide	[M+H], Da	Found in… (intensity)	Comments
1		986.5	αB-crystallin (4413)	To confirm presence of expected peptide [83-90] of αB sequence
2		1188.6 and 2374.1 (dimer)	K90C (1504+2262) K90C-AE (771+672) K90C-OP (577+387)	To confirm presence of unmodified peptide [83-92] and its Cys-Cys bridged dimer in K90C sample and treated K90C samples
3		1004.5	K90C-AE (4039)	To confirm presence of AE modification at Cys90 of [83-90] region of K90C
4		1231.6	Not found	To confirm presence of AE modification at Cys90 of [83-92] region of K90C
5		1309.6	K90C-OP (5967)	To confirm presence of OP modification at Cys90 of [83-92] region of K90C

change within the aromatic region for K90C-OP does not necessarily indicate a possible loss or gain in tertiary structure although one can argue there was a local conformational change within the protein molecule which does not necessarily affect quaternary structure of the entire protein.

Far-UV Circular Dichroism Studies. The far-UV CD spectrum of both the modified and unmodified proteins has been recorded at 200−250 nm (Figure 7B). K90C and K90C-OP exhibits exactly similar far-UV secondary spectral features although we see an apparent decrease and slight increase in secondary structural conformation in case of wild-type αB-crystallin and K90C-AE, respectively. These subtle changes noticed in the case of wild-type αB-crystallin and K90C-AE are within the standard error limits. Therefore, no significant change in the far-UV CD structure is seen for the modified as well as the unmodified protein (Figure 7B).

The percentage of secondary structural elements has been predicted by the software (Jasco secondary structure estimation) provided in the Jasco 815 spectropolarimeter (Table 2). A set of several soluble proteins included in the software package was used as a base of reference to estimate the secondary structures of the experimental proteins. Calculations of the secondary structural elements were essentially the same and within the standard error limits for all four proteins. No drastic change was observed in the percentages of α-helix, β-sheet, or random coiled structures for the modified and unmodified proteins.

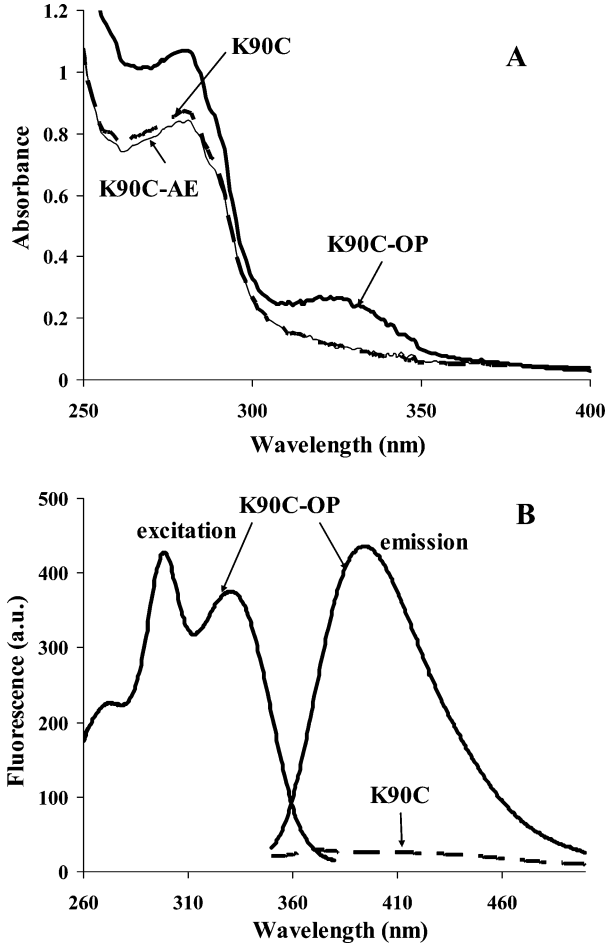


FIGURE 3: (A) UV absorption spectra of K90C, K90C-AE, and K90C-OP. (B) Fluorescence excitation spectrum (emission at 400 nm) and emission spectrum (excitation at 330 nm) of K90C-OP. Shown in the dashed line is the emission spectrum of K90C when excited at 330 nm. Protein samples of 1 mg/mL were taken in 50 mM phosphate buffer, 0.15 M NaCl, pH 7.0.

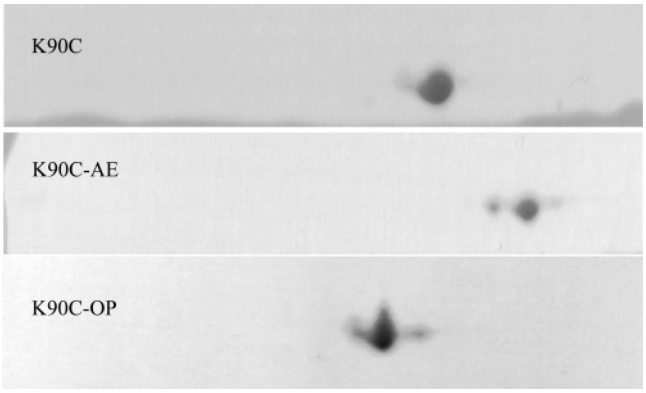


FIGURE 4: Two-dimensional PAGE of K90C, K90C-AE, and K90C-OP.

Chaperone Activity of the Modified and Unmodified Proteins. Chaperone-like activities of modified and unmodified proteins were performed using alcohol dehydrogenase (ADH), citrate synthase (CS), and insulin as the substrates (Figure 8A−C). The OP-modified protein showed reduced efficiency in protection against EDTA-induced aggregation of ADH at 37 °C, thermal aggregation of CS at 43 °C, and DTT-induced insulin aggregation at 25 °C compared to the other αB-crystallins at the same concentration. At a weight/weight ratio of 1:10 for chaperone to substrate, K90C-OP

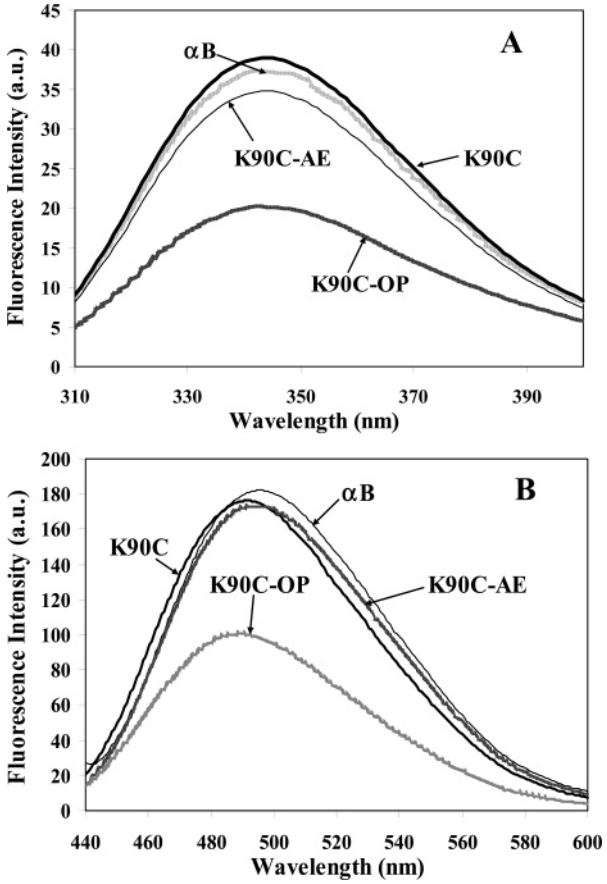


FIGURE 5: (A) Intrinsic tryptophan fluorescence emission spectra when excited at 295 nm for wild-type αB-crystallin, K90C, K90C-AE, and K90C-OP. Protein samples of 0.2 mg/mL were taken in 50 mM phosphate buffer, 0.15 M NaCl, pH 7.0. in a total volume of 1 mL; (B) bis-ANS fluorescence spectra of wild-type αB-crystallin, K90C, K90C-AE, and K90C-OP when excited at 390 nm. The 20 μM bis-ANS was incubated with 0.2 mg/mL of each of the proteins for 15 min in 50 mM phosphate buffer, 0.15 M NaCl, pH 7.0. The spectra are the average of several scans.

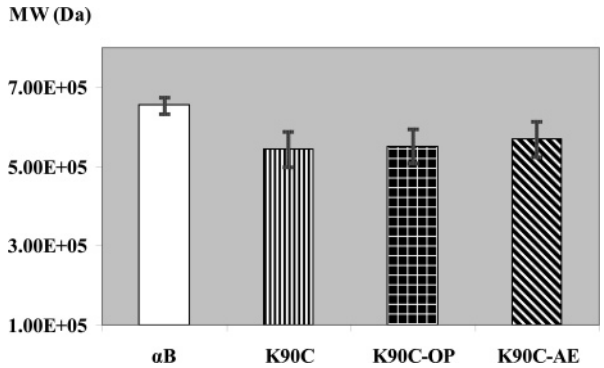


FIGURE 6: Oligomeric masses of wild-type αB-crystallin, K90C, K90C-AE, and K90C-OP. Molecular weights (MW) were obtained from static light-scattering measurements, processed by ASTRA software. Average molar masses and standard deviation were calculated from MW of three different protein preparations.

showed only 28% protection compared to 92% with the other proteins when ADH was used as the substrate. With citrate synthase the percentage protection with K90C-OP was 72% as compared to 88% protection with the other proteins. With insulin the percentage protection was only 5% with K90C-OP as compared to 50% protection with the other proteins. These differences might be due to slightly different substrate binding sites for ADH, insulin, and CS as substrates. In a

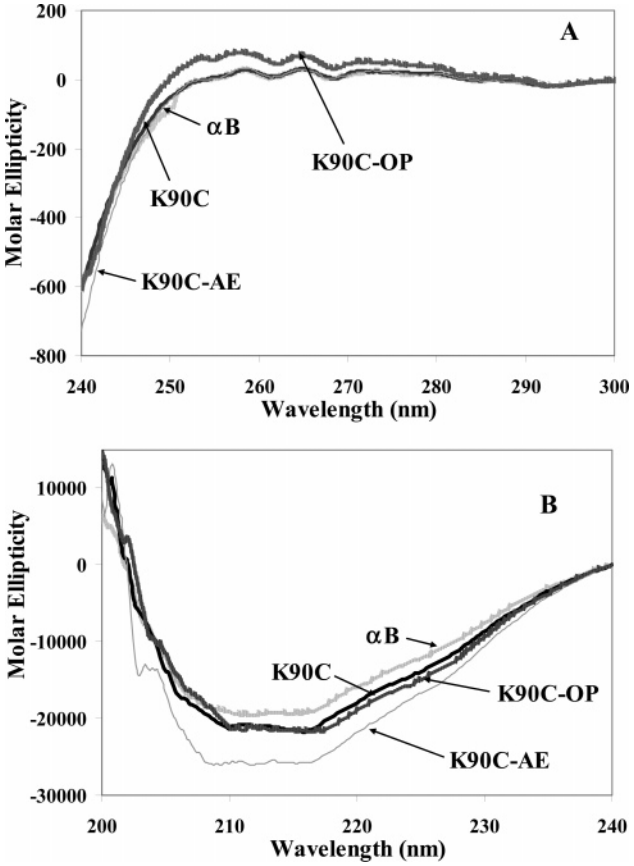


FIGURE 7: Near-UV (A) and far-UV (B) CD spectra of wild-type αB-crystallin, K90C, K90C-AE, and K90C-OP. For near-UV CD experiments, protein concentrations of 1.5 mg/mL were used and the cell path length was 0.5 cm. In the case of far-UV CD spectra, protein concentrations of 0.2 mg/mL were used with a cell path length of 0.1 cm. For both cases, the reported CD spectra are the average of several scans.

Table 2: Secondary Structural Elements Calculated from Far-UV CD Data[a]

	percentage of secondary structural elements			
protein	α-helix	β-sheet	β-turns	random coil
Wt αBC	16	31	31.4	21.6
K90C	16.5	36.7	26.9	20.0
K90C-AE	14.6	30.8	29.9	24.6
K90C-OP	22.3	29.3	28.5	20.0

[a] The secondary structural elements from the ellipticity values were calculated using the Jasco secondary structure prediction software included with the spectropolarimeter. The references used for the estimation were a set of soluble proteins included with the software.

previous study, we have shown that truncated mini-αB-crystallin at the same ratios exhibit a different percent of protection using different substrates (ADH and insulin) (*22*). Similar results were obtained with several other preparations of the proteins. However, when a higher amount (100−400 μg) of K90C-OP was used, we could obtain almost complete protection against aggregation of the substrate proteins. This suggests a lower binding affinity for K90C-OP and partially unfolded substrate proteins, possibly due to OP modification at position 90, which has been reported to be part of the chaperone site (*22*). The perturbed hydrophobic environment provided by the modified protein might also play a role in the chaperoning ability of the protein.

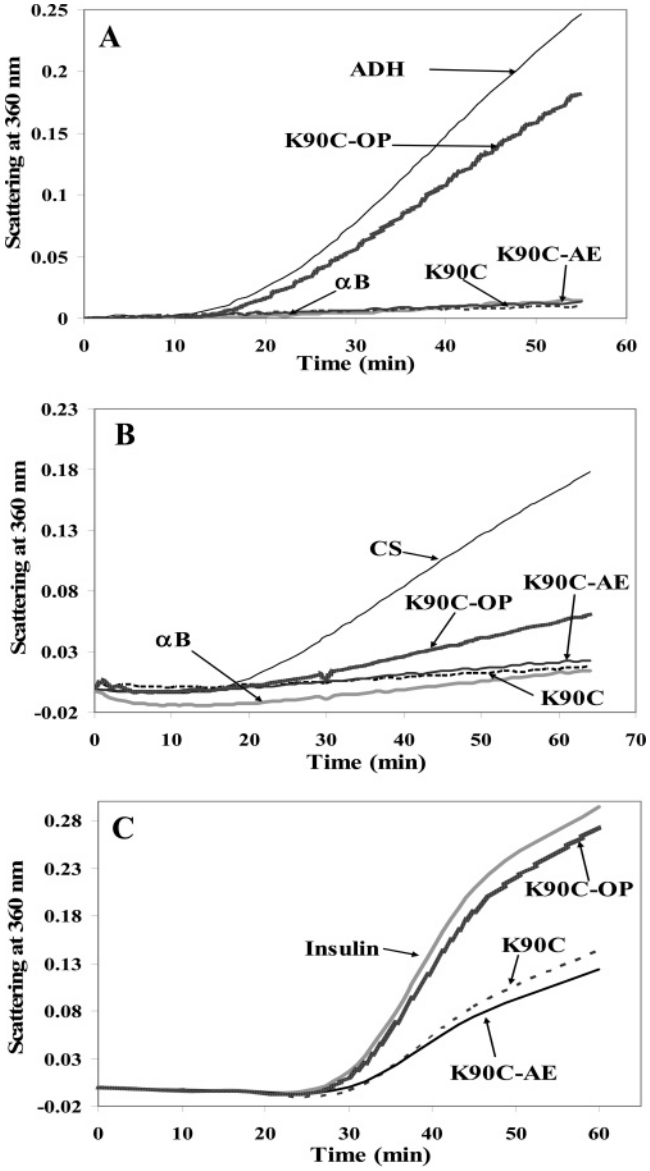


FIGURE 8: Chaperone assays of wild-type αB-crystallin, K90C, K90C-AE, and K90C-OP using ADH at 37 °C (A), CS at 43 °C (B), and insulin at 25 °C (C) as the substrates. Chaperone/substrate were taken in a w/w ratio of 1:10 in (A) and (B) and 1:2 in (C).

DISCUSSION

α-Crystallin, a small heat shock protein in the eye lens, has been shown to function as a molecular chaperone (*10*). The molecular chaperone function of α-crystallin may be instrumental in the maintenance of lens transparency vis-à-vis the prevention of cataract. Despite the large amount of information available on the pathological significance of AGE-modified proteins in cataractogenesis, relatively little is know about the effect of AGE formation on the changes in structure and chaperone-like function of α-crystallin. Recently, Nagaraj et al. (*23*) have demonstrated that methylglyoxal-modified α-crystallin shows increased chaperone-like activity in aggregation assays although another group reported that methylglyoxal modification of alpha-crystallin decreased chaperone activity (*24*). In contrast with aggregation assays, methylglyoxal/glyoxal-modified α-crystallin showed a decreased chaperone-like activity in a functional assay (enzyme inactivation) (*25*). The oxidation products of

ascorbate readily form AGEs on proteins. Analyses of lens chromophores, fluorophores and mass spectral analysis (*26*) of ascorbate modified lens proteins and proteins isolated from human cataracts show considerable homology (*26*). Although there are numerous studies with regard to the pathological significance of AGE-modified proteins in cataractogenesis, relatively little is known about the effect of a single AGE residue on α-crystallin structure and function.

To date there has been no report showing how a specific AGE modification at a single site in a protein molecule can affect protein structure and/or function. In the present study, we used this approach to assess the effect of OP-lysine modification (AGE formation) at a single residue on the structure and function of a mutant αB-crystallin (K90C). We selected K90C-αB-crsytallin for modification because residue 90 is within the chaperone site in αB-crystallin (*22*) and is also the most accessible glycation site (*18*). Also, the presence of lysine (K92) near the site of modification (K90) may amplify the glycation process (*19*). To show that during modification we are not destroying the structural properties of the protein due to modification, we also modified K90C-αB-crystallin to an aminoethyl derivative (K90C-AE). This modification converts the Cys residue at position 90 to an amino acid similar to lysine and theoretically should possess structural and functional properties similar to wild-type αB-crystallin. In this way a specific modification can be introduced into a folded lens protein much as it does in vivo, as opposed to amino acid substitutions, deamidations, or truncations which have the potential to affect protein folding during expression in *E. coli*. So our modifications were carried out as they are in vivo, namely, as post-translational modifications on fully folded proteins. Amino acid substitution can easily alter protein folding. Even a change in the rate of translation is sufficient to alter protein folding in vivo (*27*). Therefore, in all our experiments we used wild-type αB-crystallin, K90C-αB-crystallin, and K90C-AE as controls and compared the structural and functional differences with an AGE-modified protein (K90C-OP).

First of all, our tryptic digestion and mass spectrometric results indicate that we were able to successfully introduce a single modification at a single site in the protein molecule. The appearance of specific 3-oxidopyridinium chromophore in case of K90C-OP with absorbance at 330 nm (Figure 3A) and emission at 400 nm when excited at 330 nm (Figure 3B) and its absence in the control proteins also confirms that we have successfully modified K90C with the OP derivative.

We also see that introduction of an AGE (OP-lysine) at the 90th position within the chaperone site of αB-crystallin causes some changes in the isoelectric point (pI) of the protein, but the change in the pI did not affect the structure of the modified protein (K90C-OP). K90C as well as the other two derivatives (K90C-AE and K90C-OP) showed an oligomeric size of ∼540 kDa similar to wild-type αB-crystallin which had a molecular size of 600 kDa (Figure 6). Previously, Santhoshkumar and Sharma (*17*) also reported no change in the quaternary, tertiary, or secondary structure between wild-type αB-crystallin and mutant K90C-αB-crystallin. The tertiary structures of all the proteins except K90C-OP are similar (Figure 7A). A small increase in the CD signal was observed in case of K90C-OP in the aromatic region which does not indicate a gain or loss of tertiary structure but can be attributed to some local conformational changes in or around the tryptophan moiety/moieties of the protein molecule due to the presence of an extra aromatic OP-ring at position 90. Had there been a significant gain or loss of tertiary structure in the modified protein, it would likely have been reflected in the quaternary structure, which did not show any significant change. Local conformational changes around the tryptophan moieties of a protein molecule can occur without altering the quaternary, tertiary, or secondary structure of a protein molecule. This was shown by drug−protein interaction (*28*).

We also notice that although introduction of an OP-lysine analog at the 90th position (the C-terminal residue in the chaperone site of αB-crystallin) does not significantly cause any global change in the structure of the protein, it significantly reduces (50% decrease) the fluorescence intensity of the tryptophan emission spectra at the same concentrations (Figure 5A). Such a change can be speculated to be due to the close proximity of possibly Trp 60 to residue 90 in the structure of protein. Wild-type αB-crystallin has two tryptophan residues—one near the amino terminus (residue 9) and the other near the chaperone site (residue 60). According to homology modeling of human αB-crystallin (*29*) it is evident that W9 is far away from the chaperone site, whereas W60 is close to the chaperone site. So we have speculated that the reduced tryptophan fluorescence intensity in the case of K90C-OP may be due to the fluorescence resonance energy transfer (FRET) that occurs between the OP-ring at position 90 and the nearest tryptophan, i.e., W60.

Concomitant with the decrease in the fluorescence intensity of the tryptophan emission spectrum of K90C-OP, we notice a 50% decrease in the bis-ANS fluorescence spectrum as well. The bis-ANS fluorescence spectrum of the modified and unmodified proteins showed emission maxima at 498 nm, but the bis-ANS fluorescence intensity of the OP-modified protein at the emission maximum was considerably less (50%) than that of the unmodified proteins (Figure 5B). This suggests a possible shielding of some of the accessible hydrophobic surfaces of K90C-OP due to the close proximity of the OP-ring near a tryptophan residue. It is well-known that surface-exposed accessible hydrophobic patches play a significant role in chaperone activity of αB-crystallin (*30*). This agrees with our assay of chaperone activity of K90C-OP with alcohol dehydrogenase, insulin, and to a lesser extent citrate synthase. We notice reduced efficiency in the chaperoning ability of K90C-OP compared to the other modified αB-crystallins, with all substrates tested. Therefore we see that the reduction in accessible hydrophobic surfaces in the modified protein (K90C-OP) may be due to the close proximity of the OP-ring around the tryptophan moiety/moieties of K90C-OP as evident from the energy transfer from tryptophan to the OP-ring which in turn affects the chaperone function of the protein. It is also another indication that tryptophan moieties of αB-crystallin offer significant hydrophobic surfaces for bis-ANS and substrate binding. In one study, it has been reported that local conformational changes around the tryptophan moieties of the protein due to drug binding alter the function of the protein without alterations in the quaternary, tertiary, or secondary structure of the protein (*28*). We also notice that changes in the tryptophan emission spectra and bis-ANS binding spectra

correlate with a change in the chaperoning ability of K90C-OP compared to the control proteins without a major change in the total global conformation in the protein.

In aged human lenses, OP-lysine is a significant AGE modification, yet it accounts for only 600 pmol/mg protein (*16*) in cataract lenses of Indian origin. If evenly distributed between the various crystallins, this would be equivalent to approximately one subunit modified for every 80 subunits of α-crystallin. Mass spectral analysis, however, argues that there are over 200 modifications, of which 100 can be accounted for by ascorbate glycation (*31*). The aggregate number of glycation modifications could therefore be one per α-crystallin subunit, which could represent a significant loss of chaperone activity in vivo, possibly leading to increased crystallin aggregation and ultimately to cataract formation. The accumulation of even low levels of this modification along with other known modifications may be detrimental to lens function. In this study, the AGE modification has been carried out at an amino acid residue (residue 90) which is part of the chaperone site (*22*), and so we observe a change in the chaperone ability of the protein without any gross conformational change in the structure of the protein. The question arises whether a single AGE modification at a single site outside the chaperone site (at the N- or C-terminus motif of αB-crystallin) can alter the chaperoning ability of the protein. Studies are underway in our laboratory to further investigate whether single AGE modification outside the chaperone site could affect structure or function of the αB-crystallin.

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