Enhancement of Chaperone Function of α-Crystallin by Methylglyoxal Modification †

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Enhancement of Chaperone Function of α-Crystallin by Methylglyoxal Modification†

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ABSTRACT: The molecular chaperone function of α-crystallin in the lens prevents the aggregation and insolubilization of lens proteins that occur during the process of aging. We found that chemical modification of α-crystallin by a physiological α-dicarbonyl compound, methylglyoxal (MG), enhances its chaperone function. Protein-modifying sugars and ascorbate have no such effect and actually reduce chaperone function. Chaperone assay after immunoprecipitation or with immunoaffinity-purified argpyrimidine-α-crystallin indicates that 50−60% of the increased chaperone function is due to argpyrimidine-modified protein. Incubation of α-crystallin with DL-glyceraldehyde and arginine-modifying agents also enhances chaperone function, and we believe that the increased chaperone activity depends on the extent of arginine modification. Far- and near-UV circular dichroism spectra indicate modest changes in secondary and tertiary structure of MG-modified α-crystallin. LC MS/MS analysis of MG-modified α-crystallin following chymotryptic digestion revealed that R21, R49, and R103 in αA-crystallin were converted to argpyrimidine. 1,1′-Bis(4-anilino)naphthalene-5,5′-disulfonic acid binding, an indicator of hydrophobicity of proteins, increased in α-crystallin modified by low concentrations of MG (2−100 μM). MG similarly enhances chaperone function of another small heat shock protein, Hsp27. Our results show that posttranslational modification by a metabolic product can enhance the chaperone function of α-crystallin and Hsp27 and suggest that such modification may be a protective mechanism against environmental and metabolic stresses. Augmentation of the chaperone function of α-crystallin might have evolved to protect the lens from deleterious protein modifications associated with aging.

The small heat shock protein, α-crystallin, is a major protein component of the lens. α-Crystallin forms polydisperse aggregates of molecular masses 500−1000 kDa composed of 20 kDa subunits. Two related proteins, αA- and αB-crystallins, constitute α-crystallin, and they are generally in a ratio of 3:1 αA:αB. αA-Crystallin occurs primarily in the lens but is also present in the retina (*1*); αB-crystallin is also found in the retina as well as in the heart and kidney (*2*). In these tissues, as well as in the lens, αB-crystallin is thought to protect cells from a variety of stresses. Notably, the amount of both αA- and αB-crystallins appears to increase in rat retina following intense light exposure (*1*). In retinal pigment epithelial cells, αB-crystallin increases following oxidative stress, and RPE cells stably transfected with αB-crystallin are more resistant to oxidative damage (*3*). αB-Crystallin is reported to prevent TNF-α-induced apoptosis (*4*) and to inhibit autocatalytic maturation of caspase-3 (*5*). Numerous additional studies have demonstrated a molecular chaperone function for α-crystallin (*6*−*8*), where it prevents thermally and chemically induced aggregation of a variety of proteins. The chaperone function of α-crystallin is thought to be one way to prevent aggregation of lens proteins during aging. A number of studies suggest that posttranslational modification of α-crystallin induced by oxidation (*9*), UV photolysis (*10*), and Maillard reactions (*11*−*13*) make it a weaker chaperone. Phosphorylation has been reported to reduce α-crystallin's chaperone function (*14*), but this finding remains somewhat controversial (*15*). Crystallins also appear to undergo tyrosine nitration in response to photooxidation (*16*); however, the impact of this modification on chaperone function is not known.

The Maillard reaction is the nonenzymatic reaction of aldehydes and ketones with amino groups on proteins. Several sugars, lipid peroxidation products, and sugar metabolites can initiate this reaction, which then produces a variety of adducts on proteins. These modifications, known collectively as advanced glycation end products (AGEs),[1] include chromophoric, fluorophoric, and amino acid cross-

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linking adducts on proteins (*17*). Several AGEs have been detected in the human lens, and their concentrations increase with aging and cataract formation (*18*−*23*). Recent studies suggest that α-dicarbonyl compounds, such as methylglyoxal (MG), glyoxal, and 3-deoxyglucosone are the major intermediates in AGE formation. MG originates mostly from the glycolytic intermediates glyceraldehyde 3-phosphate and dihydroxyacetone phosphate (*24*). Concentrations of MG are higher in the lens than in plasma, and MG concentrations are elevated in diabetes (*24*).

Our studies and those of others showed that MG reacts rapidly with proteins to produce stable end products on proteins in tissues and plasma. Several of these end products have been isolated, including methylglyoxal lysine dimer (MOLD), a lysine−lysine cross-linking structure (*25*, *26*), N^{ϵ}-(carboxyethyl)lysine (*23*), a modification of lysine, and argpyrimidine, a fluorescent arginine modification (*27*). Some of these protein modifications were detected in the human lens (*23*), and it was noted that brunescent cataractous lenses had much greater concentrations of AGEs than other types of cataractous or noncataractous lenses (*27*, *28*).

We found relatively large quantities of argpyrimidine in the human lens α-crystallin fraction, which then prompted us to examine the effect of MG modification on the chaperone function of this protein. From work done by others, we predicted that MG-induced modification of α-crystallin would diminish its chaperone activity. Unexpectedly, we found that the MG-induced modification of human lens α-crystallin actually enhanced its chaperone function. We then focused upon the mechanism through which MG alters α-crystallin to increase its chaperone function.

EXPERIMENTAL PROCEDURES

Materials. 1,1′-Bis(4-anilino)naphthalene-5,5′-disulfonic acid (bis-ANS), dithiothreitol (DTT), bovine insulin, alcohol dehydrogenase (ADH), sugars, ascorbate, glyoxal, 2,3-butanedione, phenylglyoxal, and trinitrobenzenesulfonic acid (TNBS) were obtained from Sigma Chemical Co., St. Louis, MO. 1,2-Cyclohexanedione was from Aldrich, Milwaukee, WI. Methylglyoxal (MG) from Sigma was purified by low pressure distillation. Citrate synthase from Roche Diagnostics was dialyzed against PBS before use. Hsp27 was from Stressgen, Canada. Acetic anhydride was from Fisher Scientific, Pittsburgh, PA. Human lenses were obtained from the National Disease Research Interchange, Philadelphia, PA, and bovine lenses were from Pel-Freeze Biologicals, AR. The argpyrimidine monoclonal antibody was a gift from Dr. Koji Uchida, Nagoya University, Japan.

Assay for Chaperone Function of α*-Crystallin.* Chaperone activity of α-crystallins was assayed in 96-microwell plates using a microplate reader (Molecular Devices, Model 190, Sunnyvale, CA). The total reaction volume was 250 μL. Additional assays, using a volume of l mL, were done in a Beckman DU spectrophotometer. Five hundred micrograms of insulin was incubated with 10 mM DTT in 0.1 M potassium phosphate buffer (pH 7.0) containing 2 mM EDTA at room temperature, and light scattering was monitored at 650 or 360 nm (*29*). ADH [200 μg in 0.05 M potassium phosphate buffer (pH 7.0) containing 0.1 M NaCl] was incubated at 48 °C, and the light scattering was monitored at 360 nm (*30*). Citrate synthase (320 μg of protein in 0.1 M HEPES buffer, pH 7.4) was incubated at 42 °C, and light scattering was monitored at 360 nm (*31*). Unless stated otherwise all components were reduced by 75% for microwell plate assays.

Modification of Lysine Residues on α*-Crystallins by Acetic Anhydride and Incubation with MG.* Purified α-crystallins from human lenses were treated with acetic anhydride (40 μmol/mL) as described by Ortwerth et al. (*32*) and dialyzed overnight in two changes (2 L each) of 0.1 M potassium phosphate buffer. The lysine amino groups were estimated by reaction with TNBS (*19*). Samples of acetic anhydride treated α-crystallin and native α-crystallin (10 mg of protein each) were treated with 25 mM MG in 0.1 M potassium phosphate buffer (pH 7.4) at 37 °C for 7 days. Control experiments were run simultaneously without MG. The samples were then centrifuged and concentrated by centrifugation using 10K MWCO Centricon concentrators (Amicon, Inc., Beverly, MA).

Incubation of α*-Crystallin and* α*A- and* α*B-Crystallins with* α*-Dicarbonyls, Ascorbate, and Sugars.* α-Crystallin (5 mg/mL) was incubated in 0.1 M sodium phosphate buffer (pH 7.4) with carbohydrates for 3 and 7 days at 37 °C. The mixtures were passed through 0.2 μM filters before incubation. Several carbohydrates were used for these experiments: glucose, fructose (40 mM each), or ascorbate (20 mM), DL-glyceraldehyde (20 mM), and MG (5 mM). α-Crystallin was also incubated with varying concentrations of MG (2−4000 μM). Similarly, αA- and αB-crystallins were incubated with 5 mM MG for 1 week. All samples were dialyzed against PBS before being assayed for chaperone function. Hsp27 (0.1 mg/mL) was similarly incubated with 50 and 100 μM MG for 1 week.

Incubation of α*-Crystallin with Arginine-Modifying Agents.* Bovine lens α-crystallin (10 mg/mL) in 0.1 M sodium phosphate buffer (pH 7.4) was sterile filtered and then incubated with agents known to modify arginine in proteins. The protein was incubated with 2.5 or 10 mM each of MG, glyoxal, phenylglyoxal, 1,2-cyclohexanedione, or 2,3-butanedione for 1 week at 37 °C. Following incubation, all samples were dialyzed against PBS for 48 h.

Circular Dichroism Spectroscopy. Far-UV (with 1.0 mm cell) and near-UV (with 5.0 mm cell) CD measurements were recorded at 25 °C using an Aviv 52DS spectropolarimeter. Protein concentrations were maintained at 1.0 mg/mL. The reported CD spectra are an average of at least five scans, smoothed by a curve-fitting program. The CD spectra are expressed as molar ellipticity.

Identification of Argpyrimidine in MG-Modified α*-Crystallin by LC MS/MS.* Bovine lens α-crystallin at a concentration of 13.6 mg/mL in 0.1 M sodium phosphate buffer, pH 7.4, was incubated with 25 mM MG for 6 days at 37 °C, dialyzed extensively against PBS for 48 h, and lyophilized. Sequence-grade chymotrypsin (Sigma, 2% w/w) was added to MG-modified α-crystallin in 50 μM sodium carbonate buffer, pH 8.0, and the mixture was incubated at 37 °C for 6 h. Digestion was continued for another 3 h with a second

[1] Abbreviations: AGEs, advanced glycation end products; MG, methylglyoxal; ADH, alcohol dehydrogenase; CS, citrate synthase; bis-ANS, 1,1′-bis(4-anilino)naphthalene-5,5′-disulfonic acid; DTT, dithiothreitol; TNBS, trinitrobenzenesulfonic acid; Hsp27, heat shock protein 27.

addition of the same enzyme (1% w/w). The chymotryptic peptides were separated in two batches (800 μg each) by RP-HPLC using a 5 μm Vydac C_{18} column (4.6 × 250 mm;, The Separations Group, Hesperia, CA). A linear gradient of 0−50% acetonitrile in water with 0.1% trifluoroacetic acid over 45 min at a flow rate of 1.0 mL min^{-1} was used for separation of peptides. The column effluent was collected in 1.0 mL fractions, dried in a Speed Vac concentrator, and reconstituted in 100 μL of water. Four microliters from each fraction was analyzed for argpyrimidine by a competitive ELISA as described by Padayatti et al. (*27*). Ten microliters from fractions that exhibited argpyrimidine immunoreactivity was subjected to liquid chromatography electrospray tandem mass spectrometry (LC MS/MS) as described in detail elsewhere (*16*, *33*) using a Cap LC system (Micromass) and a quadrupole time-of-flight mass spectrometer (QTOF2; Micromass, Beverly, MA). Protein identifications from MS/MS data utilized Micromass software ProteinLynx Global Server, MassLynx, version 3.5, and the Swiss-Protein and NCBI protein sequence databases. Identification of argpyrimidine-containing peptides was achieved by incorporating into database searches a mass addition of 80 Da to the monoisotopic residue mass of arginine.

Immunoaffinity Purification of Argpyrimidine-Containing α-Crystallin. α-Crystallin (5 mg/mL in 0.1 M phosphate buffer, pH 7.4) was incubated with 5 mM MG for 1 week at 37 °C and then dialyzed against PBS as described above. A monoclonal antibody to argpyrimidine was chemically coupled to CNBr-activated Sepharose (Amersham Biosciences, Piscataway, NJ) according to the manufacturer's instructions. MG-modified α-crystallin was then incubated in PBS with the antibody-coupled gel overnight at 4 °C. The gel was thoroughly washed, and the bound protein was eluted with 0.1 M glycine hydrochloride buffer (pH. 2.7). The pH of the eluate was adjusted to 7.0 with 1 M Tris-HCl buffer (pH 9.0). The eluted protein was then dialyzed against PBS and assayed for chaperone activity.

Immunoprecipitation. α-Crystallin (10 mg/mL) was incubated with 2.5 mM MG in 0.1 M sodium phosphate buffer for 1 week at 37 °C under sterile conditions followed by dialysis against PBS for 48 h. Argpyrimidine antibody (10.8 μg of protein in 1.8 μL) was added to 10 μL of the sample (100 μg of protein), and the mixture was incubated at 4 °C for 24 h. Following this incubation, 30 μL of protein A−Sepharose (Amersham Biosciences) suspension was added to the mixture, and incubation was continued at room temperature for 2 h longer. The mixture was centrifuged, and the supernatant (5 μL) was withdrawn for the chaperone assay with citrate synthase. A control experiment was run simultaneously with samples that lacked antibody but contained all other components.

Bis-ANS Binding Experiments. α-Crystallin (40 g/mL) was incubated with bis-ANS (7.5 μM) as described by Reddy et al. (*34*). Fluorescence was measured at 490 nm (excitation at 390 nm) in a microplate fluorescence reader (SpectraMax Gemini XS; Molecular Devices).

Amino Acid Analysis. Proteins were hydrolyzed for 24 h at 110 °C in 6 N HCl containing 0.05% β-mercaptoethanol. After hydrolysis, samples were dried thoroughly and dissolved in citrate buffer. Amino acid analysis was performed on a Beckman 6300 system by postcolumn ninhydrin derivatization.

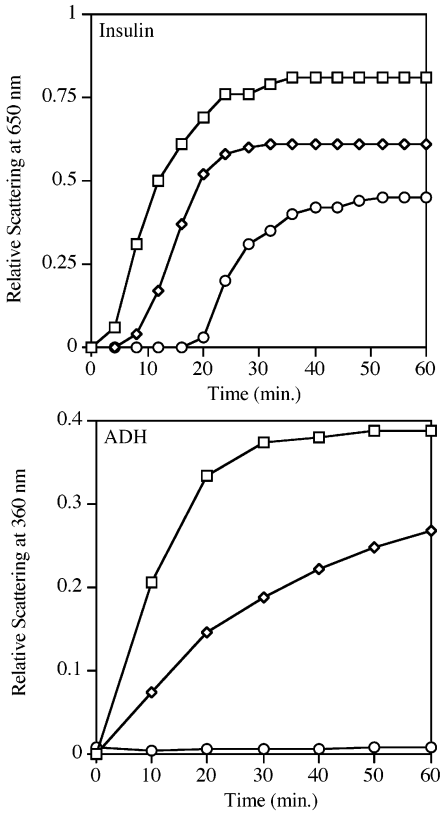

FIGURE 1: Methylglyoxal (MG) enhancement of human lens α-crystallin chaperone function. α-Crystallin was isolated from human lenses (donor age 45−60 years) by gel filtration on a Sephadex G-200 column. α-Crystallin (10 mg/mL) was incubated under sterile conditions in 0.1 M phosphate buffer (pH 7.4) with or without 5 mM MG for 1 week at 37 °C and then dialyzed against PBS. Chaperone activity was measured by DTT-induced insulin B-chain aggregation (500 μg of insulin) and thermal aggregation of alcohol dehydrogenase (ADH, 200 μg). The assay volume was 1.0 mL and used 30 μg of α-crystallin. Data points represent an average from two assays. Key: (□) protein alone; (◇) protein + α; (○) protein + α-MG.

Other Methods. Protein was measured by the Bradford method using a Bio-Rad protein assay kit with bovine serum albumin as the standard. Argpyrimidine was measured by a competitive ELISA (*27*). Statistical significance was calculated by the paired Student's *t*-test; a p value of <0.05 was considered statistically significant.

RESULTS

Initially, we compared the capability of human lens α-crystallin with and without MG modification to prevent the chemically (DTT) or thermally induced protein aggregation of insulin or alcohol dehydrogenase (ADH). Figure 1 shows that MG modification improved chaperone function of α-crystallin by nearly 2-fold when insulin was used as a target protein. The enhanced chaperone function of α-crystallin was even more dramatic when assayed using thermal aggregation of ADH; MG-modified α-crystallin completely prevented protein aggregation while unmodified α-crystallin afforded only about 40% protection over the 60 min incubation.

To determine if this phenomenon applies to other Maillard reaction initiators, we incubated calf lens α-crystallin with selected sugars and ascorbate. The chaperone function was assessed by DTT-induced aggregation of the β-chain of

Table 1: Effect of Sugars, Ascorbate, and MG on the Chaperone Function of α-Crystallin[a]

carbohydrate	% increase (+) or decrease (−) in chaperone function	
	3 days	7 days
control	0	0
glucose (40 mM)	0	−12.3
fructose (40 mM)	−12.5	−35.8
ascorbate (20 mM)	−17.5	−27.1
glyceraldehyde (20 mM)	+41.5	+72.2
methylglyoxal (5 mM)	+46.3	+90.0

[a] Each value represents an average of two independent assays of DTT-induced aggregation of insulin β-chain (125 μg) with 80 μg of α-crystallin in a volume of 250 μL. OD measurements were made after 60 min of incubation. All incubations were compared with control (α-crystallin alone).

insulin. Of the carbohydrates tested, only MG and glyceraldehyde increased the chaperone function of α-crystallin. Fructose and ascorbate actually decreased chaperone function after only 3 days of incubation (Table 1), and more marked effects were noted after 7 days of incubation. In contrast, both MG and glyceraldehyde enhanced the chaperone function. The chaperone function in the MG-incubated samples increased by 46% when compared to unmodified α-crystallin; DL-glyceraldehyde generated an effect comparable to that of MG when present at four times higher concentration. We noted similar increases in chaperone activity when the target protein was citrate synthase (data not shown). Bovine serum albumin similarly modified with MG failed to inhibit aggregation of citrate synthase or insulin in chaperone assays (data not shown).

We initially modified α-crystallin by incubation with 5 mM MG. Since this concentration is much higher than the micromolar levels of MG found in the human lens, we also exposed α-crystallin samples to concentrations of MG that more closely approximate those within the lens. Incubation of α-crystallin with 2−100 μM MG for 7 days also resulted in enhanced chaperone function. Figure 2 shows that the lowest concentration of MG tested (2 μM) enhanced the chaperone function by ∼9% and that this phenomenon increased with increasing concentrations of MG. A similar but less pronounced effect was seen when insulin was used as the target protein (Figure 2). These data suggest that MG at physiological concentrations can effectively enhance the chaperone function of α-crystallin.

MG modifies arginine and lysine residues in proteins. The reaction with arginine is favored, because the guanidino group of arginine readily interacts with the dicarbonyl of MG. We wanted to determine which of these two amino acid modifications is responsible for increased chaperone function. We first treated α-crystallin with acetic anhydride, which reduced the reaction of the protein with TNBS by approximately 60%, suggesting significant modification of lysine residues (Figure 3A). Acetylation of the ϵ-amino group of lysine residues did not adversely affect the chaperone function of α-crystallin, which is consistent with the report by Ortwerth et al. (*32*). In fact, acetylation of the α-crystallin lysine slightly improved the chaperone-like function (∼12%). Modification with 25 mM MG produced unexpected results, enhancing the chaperone activity almost equally (75−80%) in acetylated and native α-crystallin (Figure 3B). These data

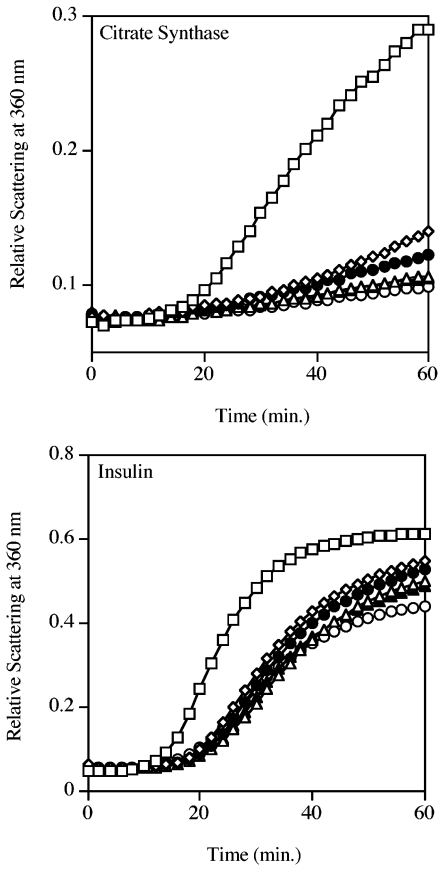

FIGURE 2: Low concentrations of MG affect chaperone function of α-crystallin. α-Crystallin was incubated with 2−100 μM MG as in Figure 1 and assayed for chaperone function by citrate synthase (CS) and insulin aggregation. Citrate synthase (40 μg) in 0.1 M HEPES buffer (pH 7.4) was incubated at 43 °C in the presence or absence of 5 μg of α-crystallin. Insulin (125 μg) was incubated in the presence or absence of 110 μg of α-crystallin. Chaperone assays were done in triplicate in a microwell plate, and representative data are shown. The total volume in each assay was 250 μL. Key: (□) protein alone; (◇) protein + α; (●) protein + α-MG (2 μM); (△) protein + α-MG (8 μM); (▲) protein + α-MG (25 μM); (○) protein + α-MG (100 μM).

indicate that modification of lysine residues by MG cannot account for the increase in chaperone activity.

We used several dicarbonyl compounds that preferentially modify the guanidino group of arginine to see if this residue was involved in the increased chaperone function. α-Crystallin from calf lenses was incubated for 1 week under sterile conditions (pH 7.4 and 37 °C) with either 2.5 or 10 mM arginine-modifying reagents. We found the highest chaperone activity in samples that were incubated with MG, followed by glyoxal, phenylglyoxal, 1,2-cyclohexanedione, and 2,3-butanedione reagents (10 mM) (Figure 4). This pattern was observed for both the citrate synthase and insulin aggregation assays. The enhancement of chaperone function was more marked in samples treated with 10 mM MG than in those with 2.5 mM MG, and effects were more prominent in the citrate synthase assay compared to the insulin assay. Amino acid analysis of proteins treated with 10 mM reagents showed a decreased arginine content. For MG-modified proteins arginine was 1.28 mol/100 mol of amino acids compared to 7.98 for controls; it was also reduced in glyoxal (1.30), phenylglyoxal (5.22), 1,2-cyclohexanedione (5.87), and 2,3-butanedione (5.58) modified proteins. These results indicate

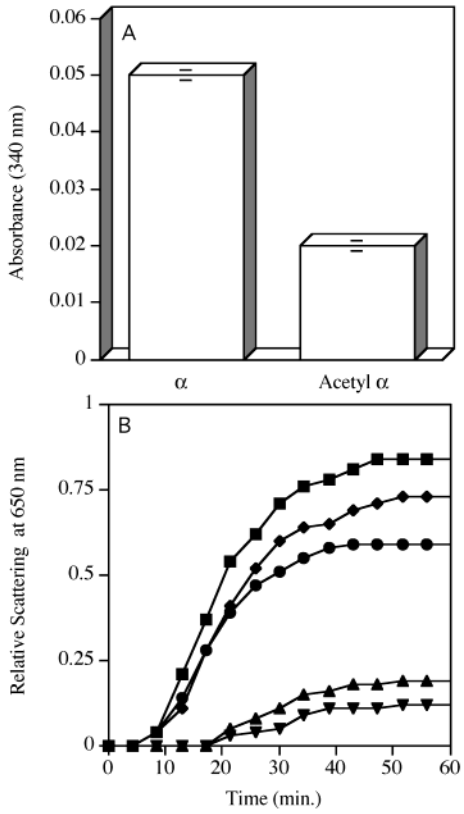

FIGURE 3: Lysine and chaperone function of MG-modified α-crystallin. (A) Lysine residues in α-crystallin were modified with acetic anhydride. (B) The modified protein was incubated with 25 mM MG for 1 week at 37 °C. The chaperone activity of acetylated and unmodified α-crystallin was assessed by insulin β-chain aggregation assay using 500 μg of insulin and 30 μg of α-crystallin in a volume of 1.0 mL. Data points are an average from three assays. Key: (■) insulin alone; (◆) insulin + α; (●) insulin + acetyl α; (▲) insulin + α-MG; (▼) insulin + acetyl α-MG.

a direct link between the enhancement of chaperone activity and the extent of arginine modification.

Several arginine modifications of MG have been reported. Argpyrimidine, which is found in lens crystallins, is a prominent modification (*27*). To determine if argpyrimidine was responsible for the enhanced chaperone function, we isolated argpyrimidine-modified α-crystallin by immunoaffinity chromatography using a monoclonal antibody for argpyrimidine. Figure 5A shows that the immunoaffinity-purified protein retained its chaperone function, accounting for nearly 50% of the increase in activity of the MG-modified α-crystallin.

Immunoprecipitation experiments further supported these findings. When we used the monoclonal antibody to immunoprecipitate argpyrimidine-bearing proteins, we found a significant ($p < 0.05$) reduction in chaperone function; the antibody removed 50−60% of the activity of MG-modified α-crystallin (Figure 5B). Together, these results would indicate that the argpyrimidine modification in α-crystallin is largely responsible for increased chaperone function.

The far-UV circular dichroism (CD) spectrum of unmodified α-crystallin recorded in this study exhibited a peak minimum at 214 nm and was consistent with a high percentage of β-sheet/β-turn structure (Figure 6A) and with UV CD results published elsewhere (*29*, *35*). When tested under identical conditions, MG-modified α-crystallin showed higher ellipticity than the unmodified α-crystallin, and this effect was dependent on MG concentration; with increasing MG concentration, there was an increase in the ellipticity. These data suggest a change in protein conformation after MG modification. The near-UV CD spectra, which reflect the tertiary structure of proteins, showed decreased intensity between 270 and 290 nm in 1000 μM MG-modified protein (Figure 6B). This is indicative of a change in tryptophan and tyrosine microenvironment and suggests a partial unfolding of the protein. It was noted that at MG concentrations higher than 25 mM, both the secondary and tertiary structures were altered, as assessed by UV CD spectroscopy (data not shown).

To probe for the presence of argpyrimidine, MG-modified bovine α-crystallin was digested with chymotrypsin, the digest was fractionated by RP-HPLC, and the resulting chromatography fractions were analyzed by an ELISA for argpyrimidine immunoreactivity. Two distinct peaks of argpyrimidine immunoreactivity were detected, corresponding to fractions 28−29 and 37−38. On the basis of a mass addition of 80 Da to the residue weight of arginine, LC MS/MS analysis of fraction 28−29 identified argpyrimidine-containing α-crystallin peptide V^{94}EIHGKHNE**R***QDD-HGY^{109}, and analysis of fraction 37−38 revealed argpyrimidine-containing peptides Y^{18}PS**R***LF^{23} and Y^{48}**R***$QSLF^{53}$ (Figure 7). Thus, in this preparation of MG-modified α-crystallin, amino acid residues R21, R49, and R103 have been converted to argpyrimidine. Analysis of the complete α-crystallin structure was not performed, and additional argpyrimidine as well as other modifications, such as MOLD, MODIC (*36*), and N^{ϵ}-(carboxyethyl)lsyine (*23*), could have been present in the modified protein.

The chaperone function of α-crystallin is attributed to hydrophobic regions of the protein, and we wondered if MG modification increased its hydrophobicity. We used bis-ANS, a fluorescent dye that binds to hydrophobic regions on proteins (*12*), to determine changes in hydrophobicity of the altered α-crystallin. α-Crystallin modified by low MG concentrations (up to 100 μM) showed an increase in binding of bis-ANS; the binding with 100 μM MG-modified protein was 11% more than the unmodified protein, but it was considerably lower with 500−4000 μM MG-modified protein (Figure 8). Bis-ANS binding did not correlate with the chaperone function, as the chaperone function improved linearly with increasing MG concentration. These results suggest that the bis-ANS binding and the improvement in chaperone function are unrelated processes.

We also tested bovine αA- and αB-crystallins to determine if MG modified one of these polypeptides or both. Changes in chaperone activity were followed by assays using citrate synthase (Figure 9) and insulin (data not shown). The αB-crystallin showed no enhancement of chaperone function in either assay, but the modified αA-crystallin clearly showed enhanced activity, indicating that modification of αA-crystallin is most likely responsible for the enhanced chaperone function in MG-modified α-crystallin.

We next wanted to see if the increased chaperone function is unique to α-crystallin or if it occurs in other small heat shock proteins as well. Hsp27 is a ubiquitous heat shock protein with a significant sequence homology to α-crystallin. We incubated Hsp27 with 50 and 100 μM MG for 1 week at 37 °C. Like the modified α-crystallin, MG-modified Hsp27 showed increased chaperone function when assayed by citrate

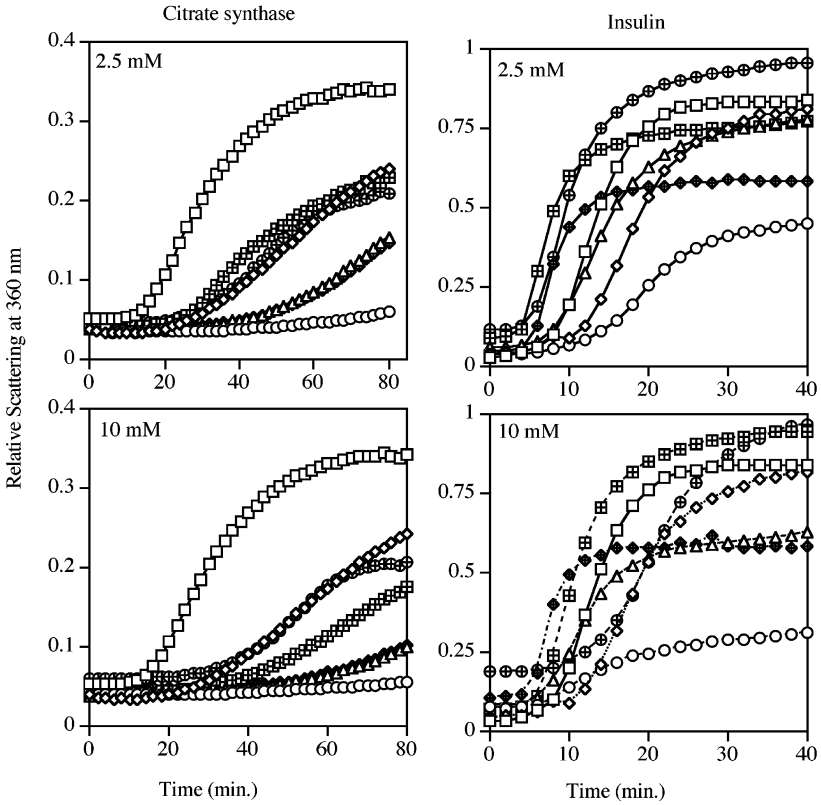

FIGURE 4: Arginine modification and chaperone function of α-crystallin. Calf lens α-crystallin (10 mg/mL) was sterile filtered and incubated in 0.1 M sodium phosphate buffer (pH 7.4) with 2.5 or 10 mM each of methylglyoxal, glyoxal, phenylglyoxal, 1,2-cyclohexanedione, and 2,3-butanedione for 1 week at 37 °C. The incubated proteins were dialyzed against PBS for 48 h. The left panels show chaperone assay with citrate synthase (50 μg), and the right panels show assay with insulin (125 μg). α-Crystallin was 30 μg in the citrate synthase aggregation assay and 110 μg in the insulin aggregation assay. All assays were done in triplicate in 96-well plates, and representative data are shown. The total volume in each assay was 250 μL. Key: (□) protein alone; (◇) protein + α; (○) protein + α-MG; (△) protein + α-glyoxal; (⊞) protein + α-1,2-cyclohexanedione; (◆) protein + α-phenylglyoxal; (⊕) protein + α-2,3-butanedione.

synthase aggregation (Figure 10), and the efficacy was related to the extent of modification by MG. The sample modified by 100 μM MG was nearly 50% more effective than the one modified by 50 μM MG. The fact that MG-induced modification of another small heat shock protein similarly enhances chaperone function suggests broader implications for pathological processes in tissues other than the lens.

DISCUSSION

Previously, we found significant quantities of argpyrimidine in the human lens associated with α-crystallin (*27*). This prompted the present study to examine how argpyrimidine affects the chaperone function of α-crystallin. Other studies have shown that the Maillard reaction of α-crystallin with sugars depresses chaperone function (*37*, *38*). Our own studies with sugars supported these observations, but the finding that MG enhanced chaperone function was unexpected. One previous study reported that N^{ϵ}-(carboxymethyl)-lysine (CML), a glycoxidation product formed from the reaction of sugars with proteins, enhanced the chaperone function of α-crystallin (*39*). However, because these investigators used CML chemically coupled to protein, it was not obvious that CML formed through the Maillard reaction would produce similar results. In contrast to our own observations, Derham and Harding reported that chemical modification of α-crystallin by MG reduces its chaperone function (*40*). Our study was done with highly purified MG while Derham and Harding used a commercial preparation of MG to modify α-crystallin, raising the question of whether contaminants might have affected their results. These investigators also reported that low concentrations of 1,2-cyclohexanedione had no effect on the chaperone function of α-crystallin, an observation confirmed by our own findings.

The human lens contains relatively large amounts of MG; its levels are 20 times higher than in plasma. Arginine is the major target for MG modification, although lysine is also modified. Since we found that lysine modification did not account for the MG-induced enhancement of chaperone function, we assume that modification of arginine is a likely cause of the observed increase.

A few arginine modifications of MG have been described, and among these, two that are found in proteins are 5-hydroimidazolone (*41*) and argpyrimidine (*27*, *42*). Argpyrimidine has been detected in several tissues, and because we found it in the human lens, we reasoned that argpyrimidine modifications could affect the chaperone function. Our present results from immunoprecipitation and with the immunoaffinity-purified, argpyrimidine-enriched α-crystallin (Figure 5) strongly support 50−60% of the increased chaperone function to be associated with the argpyrimidine-modified protein.

The data on bis-ANS binding with MG-modified protein (Figure 8) suggest that α-crystallin undergoes partial unfolding during its reaction with MG and such unfolding may expose hydrophobic sites that otherwise are not available for the chaperone function. We detected higher bis-ANS

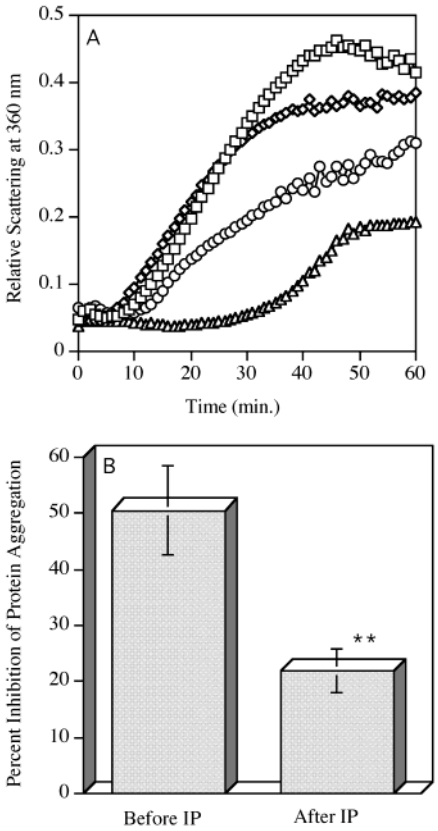

FIGURE 5: Contribution of argpyrimidine modification to increased chaperone function of α-crystallin. (A) α-Crystallin was incubated with 5 mM MG for 1 week and subjected to immunoaffinity chromatography on anti-argpyrimidine antibody-coupled CNBr-activated Sepharose. The immunoaffinity-purified material (IA-purified α-MG), MG-modified α-crystallin (α-MG), and unmodified α-crystallin (α) (24 μg each) were assayed by citrate synthase (CS, 40 μg) aggregation. Key: (□) CS alone; (◇) CS + α; (○) CS + IA-purified α-MG; (△) CS + α-MG. (B) To confirm the contribution of argpyrimidine to enhanced chaperone function, both MG-modified and unmodified α-crystallin were incubated first with a monoclonal antibody against argpyrimidine and then with protein A−Sepharose. The samples were centrifuged, and the chaperone function was assessed in the supernatant using the CS assay. The reduction in chaperone function by immunoprecipitation (IP) was statistically significant ($p < 0.05$).

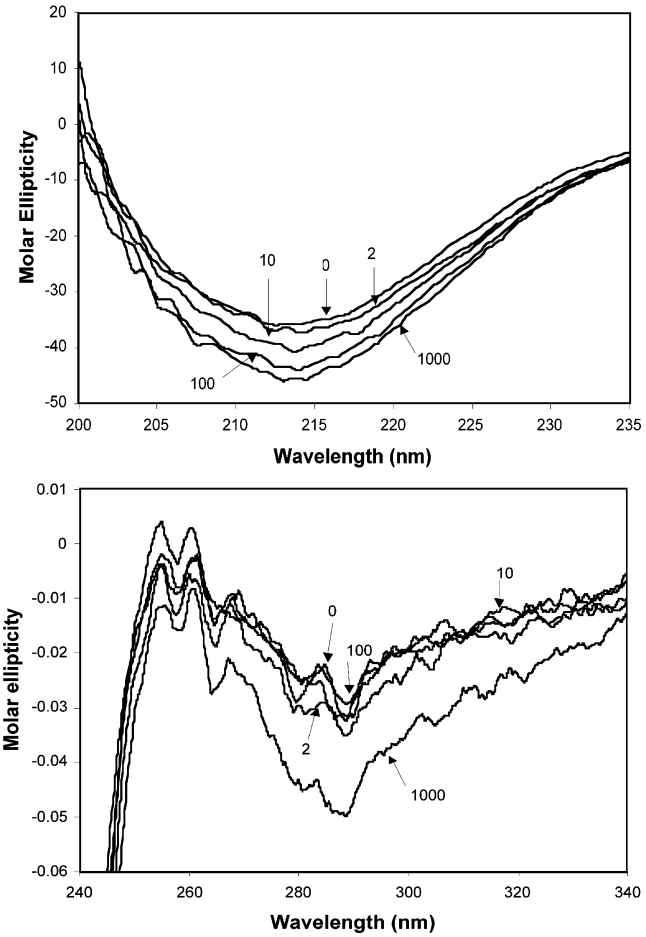

FIGURE 6: Far-UV (top) and near-UV (bottom) CD spectra of unmodified and MG-modified α-crystallin. The data represent the average from at least five scans at 25 °C from a sample containing 1 mg/mL protein. The numbers indicate μM MG used for α-crystallin modification.

binding with α-crystallin modified with low concentrations of MG (2−100 μM); however, the binding was reduced at higher MG concentrations (500−4000 μM). Analysis of proteins by SDS−PAGE showed significant cross-linking above 500 μM MG (data not shown). The observation of enhanced chaperone function in cross-linked α-crystallin in the present study is in contrast to the observation of Sharma and Ortwerth of a reduction in the chaperone function in chemically cross-linked α-crystallin (*43*). Difference in the chemical nature of cross-links and selective modification of arginine residues by methylglyoxal may be the reasons for the dissimilarity. Whether cross-linking of α-crystallin led to decreased binding of bis-ANS needs to be investigated. Bis-ANS binding and the improvement in chaperone function seem to be unrelated, as the chaperone function increased linearly with increasing MG concentrations (from 2 to 4000 μM). Even though the chaperone function was increased at low MG concentrations, no significant changes in secondary and tertiary structures of α-crystallin were detectable by UV CD spectroscopy (Figure 6), suggesting the possibility of mild structural perturbation, and such mild structural changes may be sufficient for enhancing the chaperone function of α-crystallin as reported by others (*34*, *44*, *45*).

Our mass spectrometric analyses of MG-modified bovine αA-crystallin support argpyrimidine modifications at residues R21, R49, and R103, and these modifications may contribute to increased chaperone function of α-crystallin. In contrast to our findings, several studies indicate that site-directed mutations, such as R116C in αA-crystallin and R120G in αB-crystallin, can reduce the chaperone function (*46*−*48*). Arginine at these sites may be essential for chaperone function; however, we suspect that structural alterations resulting from the above mutations cause the loss of chaperone function. In contrast to mutations, arginine residues in MG-modified α-crystallin are not replaced but are chemically modified to argpyrimidine, resulting in enhanced chaperone function. Our results indicate that chaperone function is enhanced in MG-modified αA-crystallin but not in comparably modified αB-crystallin. This finding suggests that the phenomenon may be more prevalent in the lens, since, unlike αB, αA is found mainly in the lens.

α-Crystallin's chaperone activity decreases during aging (nuclear alpha but not cortical), during cataract formation (*49*), and in diabetes (*50*). A number of studies report accumulation of advanced glycation end products (AGEs) in lens crystallins during aging and cataract formation (*21*−*23*, *51*, *52*). We recently found that argpyrimidine ac-

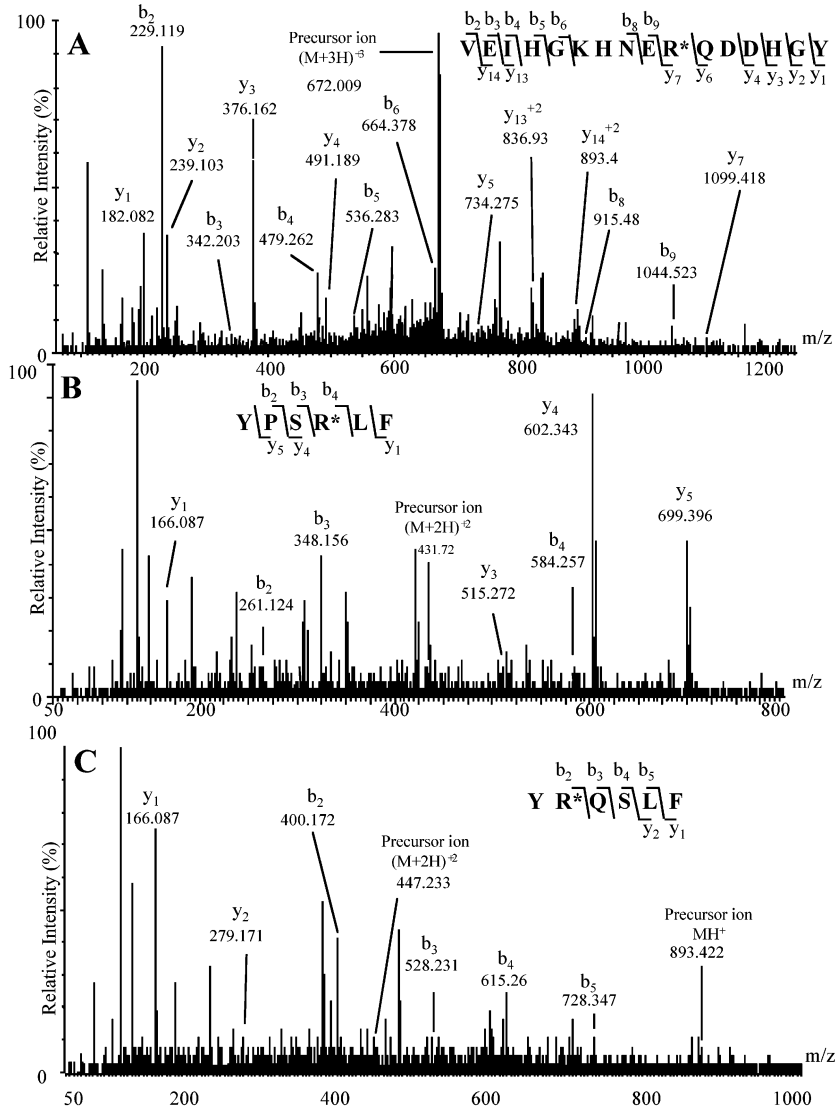

FIGURE 7: Sites of argpyrimidine in MG-modified α-crystallin. MS/MS spectra are shown identifying Arg21, Arg49, and Arg103 as argpyrimidine residues (R*) in MG-modified bovine α-crystallin. Panels: (A) α-crystallin residues 94−109, (B) α-crystallin residues 18−23, and (C) α-crystallin residues 48−53.

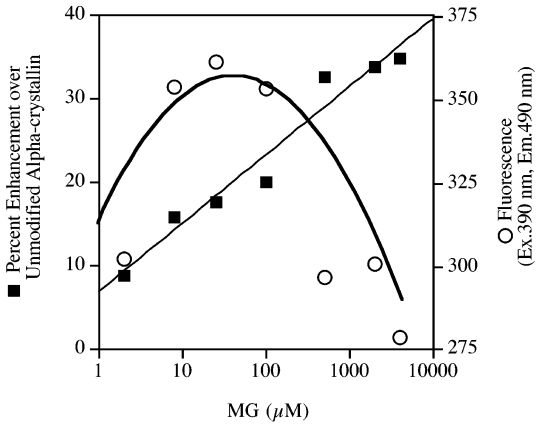

FIGURE 8: Effect of MG modification on the hydrophobicity of α-crystallin. MG-modified and unmodified proteins were incubated with bis-ANS and dialyzed against PBS. Fluorescence of these samples was measured at 490 nm (excitation at 390 nm). Chaperone assays were done using citrate synthase as the target protein in a microwell plate. The total volume in each assay was 250 μL.

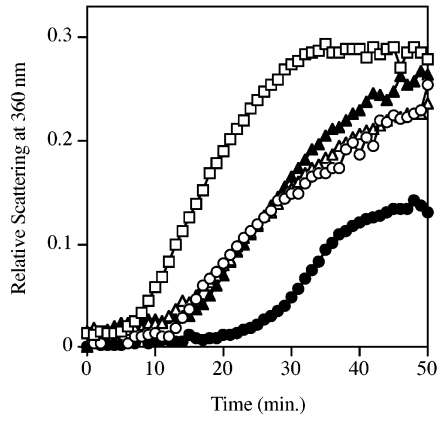

FIGURE 9: Effect of MG modification on chaperone function of calf lens αA- and αB-crystallin. The proteins were incubated with 5 mM MG for 1 week and dialyzed against PBS, and 2 μg of protein of each was tested for chaperone function against thermal aggregation of citrate synthase (CS, 40 μg) in an assay volume of 250 μL. Chaperone assays were done in triplicate in a microwell plate, and representative data are shown. The total volume in each assay was 250 μL. Key: (□) CS alone; (○) CS + αA; (●) CS + αA-MG; (△) CS + αB; (▲) CS + αB-MG.

cumulates at relatively high concentrations in brunescent cataractous lenses when compared to noncataractous aging

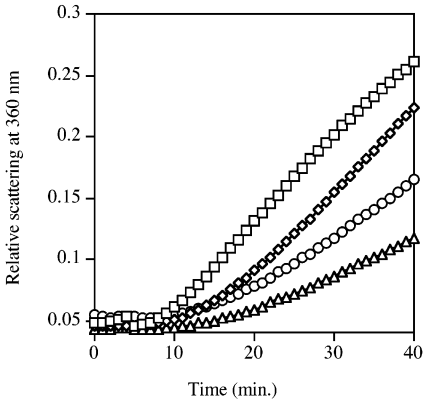

FIGURE 10: Effect of MG modification on the chaperone function of Hsp27. Hsp27 was modified with 50 and 100 μM MG as described, and the chaperone function was assessed using citrate synthase (CS, 40 μg; Hsp27, 10 μg). The sample modified by the higher concentration of MG was nearly twice as effective than the one modified by the lower concentration. Chaperone assays were done in triplicate in a microwell plate, and representative data are shown. The total volume in each assay was 250 μL. Key: (□) CS alone; (◇) CS + Hsp27; (○) CS + Hsp27-MG (50 μM); (△) CS + Hsp27-MG (100 μM).

lenses (*27*). A number of in vitro studies also report decreased chaperone function when α-crystallin is incubated with sugars. These observations suggest a causal link between formation of AGEs in crystallins and loss of chaperone function; however, our finding that dicarbonyl modification enhances α-crystallin chaperone function argues otherwise. Lens crystallins undergo numerous modifications during aging and cataract formation, including oxidation, deamidation, and glycation by sugars and ascorbate, and such modifications are likely to decrease the chaperone function of α-crystallin. Thus, the enhancement of the α-crystallin's chaperone function by dicarbonyls may be an evolutionary adaptation to offset the loss of chaperone function in aging and cataractogenesis. Cataract formation could result when chemical modifications of the lens by oxidation, deamidation, and glycation override the favorable effects of MG. Alternatively, it might be argued that MG modification keeps the lenses clear, despite potentially damaging modifications that occur with aging.

MG modification also enhanced the chaperone activity of Hsp27, a small heat shock protein. Unlike α-crystallin, Hsp27 is ubiquitously expressed throughout the body and plays a role in various cellular functions, including apoptosis and actin polymerization (*53*−*55*). We previously noted that Hsp27 is a major site for argpyrimidine formation in rat glomerular mesangial cells (*56*), and other investigators reported argpyrimidine modification of Hsp27 in cultured human cancer cells (*57*). However, it is not yet clear how such modifications affect the lens.

In summary, we find that a physiological dicarbonyl compound enhances the chaperone function of α-crystallin. This increased chaperone function could protect against such physiological stresses as hyperglycemia in diabetes and oxidative stress during aging and cataract formation.

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