Molecular Vision 2006; 12:205-14 <http://www.molvis.org/molvis/v12/a23/>
Received 21 September 2005 | Accepted 20 March 2006 | Published 27 March 2006

Carnosine inhibits modifications and decreased molecular chaperone activity of lens α-crystallin induced by ribose and fructose 6-phosphate

Hong Yan,[1,2] John J. Harding[1]

[1]Nuffield Laboratory of Ophthalmology, University of Oxford, Oxford, UK; [2]Department of Ophthalmology, Tangdu Hospital, Fourth Military Medical University, Xi'an, China

Purpose: α-Crystallin, a major structural protein in the lens, prevents heat- and oxidative stress-induced aggregation of proteins and inactivation of enzymes by acting as a molecular chaperone. Modification of α-crystallin by some posttranslational modifications results in conformational changes and decreases in chaperone activity, which may contribute to cataractogenesis in vivo. Carnosine (β-alanyl-L-histidine), an endogenous histidine dipeptide, prevents protein modifications including glycation and oxidation. The purpose of this study was to further explore whether carnosine can protect α-crystallin against glycation by a sugar and a sugar phosphate, and in particular to find whether it can protect against its decreased chaperone activity. Additionally, we investigated whether carnosine could directly react with a sugar and a sugar phosphate.

Methods: Bovine lens αL-crystallin was separated by size-exclusion chromatography on a Sephacryl S-300 HR column. αL-crystallin was incubated with different concentrations of fructose 6-phosphate (F6P) and ribose with or without carnosine for different times. The chaperone activity of αL-crystallin was monitored using the prevention of thermal aggregation of βL-crystallin. The modified αL-crystallin was examined by SDS-PAGE and fluorescence measurements. The absorbance spectra of solutions of carnosine and sugars were investigated.

Results: Carnosine inhibited the crosslinking of αL-crystallin induced by F6P and ribose in a dose- and time-dependent manner. It protected αL-crystallin against its decreased chaperone activity induced by 100 mM F6P during four days incubation, but not against ribose-induced change. Control αL-crystallin gave 96% protection against aggregation of βL-crystallin after four days incubation, but only 85% protection was achieved in the presence of F6P, rising to 96% (p=0.0004) in the presence of carnosine. After more extensive modification by sugar and a sugar phosphate, there was no significant protective effect of carnosine on αL-crystallin cross-linking or chaperone activity. The tryptophan fluorescence of modified αL-crystallin was remarkably decreased in the presence of F6P and ribose. However, the decrease was less when 50 mM carnosine was present during eight days incubation with F6P. Carnosine did not maintain the fluorescence when ribose was used. The nontryptophan fluorescence was increased with a shift to longer wavelengths in a time-dependent manner. Carnosine readily reacted with F6P and ribose thereby inhibiting glycation-mediated protein modification as revealed electrophoretically. The increased absorbance was time-dependent, suggesting adducts may be formed between F6P, ribose, and carnosine.

Conclusions: This is the first report showing that carnosine can protect the chaperone activity of α-crystallin. This chaperone may protect against cataractous changes. In addition to demonstrating the effects of carnosine on prevention crosslinking, our studies also bring out important evidence that carnosine reacts with F6P and ribose, which suggests carnosine's potential as a possible nontoxic modulator of diabetic complications.

α-Crystallin, a major structural protein in the lens, has a chaperone-like function, binding unfolding lens proteins and protecting various proteins against aggregation induced by heating, chaotropic agents, reduction, and chemical modification [1-3]. Even at low concentrations it also provides full protection against modification-induced inactivation of several enzymes [2,4]. The role of α-crystallin as a molecular chaperone may explain how the lens stays transparent for so long. Furthermore, α-crystallin is not exclusively a lens protein but it is found outside the lens, having an extensive tissue distribution, which may have a wider functional significance [1]. Increased expression of αB-crystallin has been seen in various neurodegenerative diseases, which are considered as conformational diseases as is the cataract [2]. The loss of chaperone activity of α-crystallin may contribute to the pathogenesis of these conformational diseases.

α-Crystallin acts as a molecular chaperone to prevent the aggregation and inactivation of other proteins. Specific areas important for chaperone binding and function have been identified throughout the NH_2-terminal-region and COOH-terminal extension [5,6,7]. α-Crystallins are aggregates assembled from polypeptides of 10-25 kDa that share a common central domain of about 90 residues (the "alpha-crystallin domain") with variable NH_2- and COOH-terminal extensions [7]. The α-crystallin domain, sharing homology with other heat shock

Correspondence to: Hong Yan, Department of Ophthalmology, Tangdu Hospital, Fourth Military Medical University, Xi'an 710038, China; Phone: 0086 29 84777445; FAX: 0086 29 84777445; email: yhongb@fmmu.edu.cn

proteins, can affect chaperone-activity when it is truncated. However, the quaternary structure of α-crystallin is necessary for its chaperone-like activity [8].

Posttranslational modifications (PTM) of lens crystallin, consequent to aging or diseases such as diabetes, may result in conformational changes and aggregation of these proteins and lead to lens opacification and cataract formation. All PTMs known to cause intra- or intermolecular crosslinking of α-crystallin decrease chaperone function [3,9,10]. Glycation, the nonenzymic reaction of sugars with protein, occurs normally but to an increased extent in aging and diabetic complications [2]. Glycation, a nonenzymic modification, is a process by which sugar reacts with protein. It can lead to protein crosslinking and further aggregates, which contribute to pathogenesis of conformational diseases, including cataract and complications of diabetes [2]. Glycation and later (crosslinking) glycation products cause a decrease in chaperone function of α-crystallin [9,11].

Carnosine, a naturally occurring dipeptide (β-alanyl-L-histidine), is found predominantly in long-lived tissues including the brain, innervated muscle, and the lens in surprisingly high amounts (up to 20 mM in human muscle) [12]. Carnosine can delay senescence and extend the life-span of cultured human fibroblasts, kill transformed cells, and protect cells against aldehydes and an amyloid peptide fragment. It appears to possess antiglycating, antioxidant, and free-radical scavenging activity [12,13]. The imidazolium group of histidine or carnosine stabilizes adducts formed at the primary amino group and may play an important role for an anticrosslinking agent [15]. β-Alanine, a component of carnosine with neurotransmitter activity, has chaperone-like activity to suppress thermally-induced inactivation of lactate dehydrogenase, suggesting that it may play a cellular role in the preservation of enzyme function [14].

Carnosine can nonenzymatically react with deleterious hexoses, pentoses, and trioses and protect α-crystallin against glycation and crosslinking induced by sugars [15], diminish the modification of α-crystallin by methylglyoxal-treated ovalbumin [16], and disaggregate glycated α-crystallin [17]. It also inhibits inactivation and crosslinking of enzymes, including superoxide dismutase, by glycation [15,18] and oxidation [19,20]. It protects esterase and catalase but not glyceraldehyde 3-phosphate dehydrogenase against sugars and a steroid [21,22], and aspartate aminotransferase against glyceraldehyde 3-phosphate [23]. The potential biological and therapeutic significance of carnosine against human age-related cataract has been reported [24]. N-acetylcarnosine (NAC), a prodrug of L-carnosine, accumulates in the aqueous humor of rabbit eyes more easily than L-carnosine [25], suggesting its better penetration and potential functions.

Carnosine is identified as an antioxidant and antiglycation agent. It inhibits crosslinking of proteins [15,17], protects against inactivation of enzymes induced by glycation, oxidation, and a steroid [15,18-22], and protects neural cells from malondialdehyde-induced toxicity [26]. In addition, carnosine can modulate the reactivity of the glycated protein toward α-crystallin [16] and directly reacts with sugars [15] and free radicals [27]. Protein crosslinking is a consequence of glycation that induces formation of carbonyl (CO) groups. Carnosine can react with protein CO groups and thereby modulate their deleterious interaction with other polypeptides [16].

We know that carnosine works by reacting with carbohydrates, oxidized lipids, and oxygen radicals. However, the effectiveness of carnosine against decreased chaperone activity of α-crystallin by those insults in addition to its antiglycation effects remain to be elucidated. Based on our previous studies of decreased chaperone activity of α-crystallin by glycation [9] and a steroid [11], the aim of the present study was to further investigate the possibility that carnosine can prevent glycation-induced modifications and whether carnosine can protect α-crystallin against decreased chaperone activity induced by glycation, all of which are pertinent to cataract formation. Here we report evidence that carnosine protects the chaperone activity of α-crystallin against glycation and reacts with sugars by forming adducts in addition to inhibiting crosslinking by glycation.

METHODS

Materials: Bovine lenses were obtained from a local abattoir. Sephacryl S-300 HR was purchased from Pharmacia (Milton Keynes, UK). Carnosine, fructose 6-phosphate (F6P), ribose, and all other chemicals were obtained from Sigma Chemicals (Poole, Dorset, UK).

Isolation of crystallins: αL-Crystallin and βL-crystallin were isolated from bovine lenses by Sephacryl S-300 HR gel permeation chromatography (100x2.7 cm) using the method previously described in the literature [28,29]. Frozen lenses were thawed, decapsulated, weighed, and added to seven times mass volume of buffer (50 mM sodium phosphate, 0.2 M KCl, pH 6.7). The suspension was homogenized gently then centrifuged at 22,440x g for 40 min at 4 °C. The supernatant was then loaded onto the column and eluted at a rate of 30 ml/h. The fractions corresponding to αL-crystallin and βL-crystallin were pooled and dialyzed against three changes of distilled water over 24 h at 4 °C, then freeze-dried. The purity was demonstrated by SDS-PAGE, and the protein stored at -20 °C until required.

Incubation of αL-crystallin with F6P and ribose: All incubations were carried out in 50 mM sodium phosphate buffer (containing 0.05% NaN_3), pH 7.0 at 37 °C unless otherwise stated. In the preliminary experiment, αL-crystallin samples (30 mg) were dissolved in 15 ml of 100 mM F6P or 100 mM ribose with or without 50 mM and 100 mM carnosine. The solutions were then filtered through sterilized Millipore filters (0.2 μm) into sterilized 30 ml glass vials and incubated at 37 °C in a shaking water bath. At time 0, and after 4 and 8 days, samples (3 ml) were removed from each of the solutions and dialyzed against five changes of distilled water over 24 h using microdialysis tubing (molecular weight cut-off 12-14 kDa) to remove excess glycating agents. The sample was then freeze-dried and analyzed by SDS-PAGE. The βL-crystallin heat-induced aggregation assay was used to determine the chaperone ability of the modified and unmodified α-crystallin.

In order to study possible protective effects of carnosine on the extensive modifications of α-crystallin, a second set of incubations was conducted. αL-Crystallin was incubated with 100 mM F6P and 100 mM ribose with or without 50 mM and 100 mM carnosine for 0, 15, and 25 days at 37 °C.

In an attempt to investigate whether the reaction between carnosine and sugars was responsible for part of the yellowing in the earlier experiments, carnosine was incubated with 100 mM F6P and 100 mM ribose over 25 days without any proteins.

SDS-PAGE: SDS-PAGE was carried out using Laemmli's discontinuous buffer system by using a Bio-Rad Mini-Protean II dual slab mini gel apparatus (Bio-Rad Laboratories Ltd., Bath, UK) according to the manufacturer's instructions [30]. Samples were boiled in loading buffer (0.5 M Tris buffer, pH 6.8 containing 10% (w/v) SDS, 5% (v/v) 2-β-mercaptoethanol, 0.05% (w/v) bromophenol blue, and 10% (v/v) glycerol) for 5 min prior to electrophoresis. Samples (up to 10 µl) were added to each well, and electrophoresis was carried out using 12% separating gels and 4% stacking gels at a constant voltage of 200 volts. Protein bands were visualized by 0.1% Coomassie Blue R-250 stain in fixing solution (containing 40% [v/v] methanol and 10% [v/v] acetic acid) for 20 min, then destained with 40% (v/v) methanol and 10% (v/v) acetic acid for 1-2 h. Gels were scanned using an Epson Perfection 1200U Scanner (800 dpi resolution supported by Epson Twain 4 software) to quantify the levels of modification, and samples were compared with controls.

βL-Crystallin heat-induced aggregation assays: The chaperone activity of α-crystallin was assessed by its ability to prevent the increase in turbidity upon heating solutions of βL-crystallin as described previously [3,31]. This is the most widely used method for chaperone function in the α-crystallin field. Bovine βL-crystallin (1.87 mg) was dissolved in 6 ml of degassed 0.05 M sodium phosphate buffer, pH 6.7, and the solution was then passed through a Gelman 0.2 µm pore-sized sterilized Millipore filter into a sterilized glass container. Final concentrations of 250 µg/ml βL-crystallin and 100 µg/ml αL-crystallin gave a final mass/mass ratio of 1:2.5 αL-crystallin to βL-crystallin in 1 ml plastic cuvettes (Whatman, Maidstone, UK). Light scattering resulting from heat-induced aggregation was monitored continuously at 360 nm and 55 °C for 60 min. Chaperone function was represented as the percentage of protection afforded by αL-crystallin against the scattering produced by the βL-crystallin control aggregation after 60 min. All assays were repeated in triplicate.

Tryptophan and nontryptophan fluorescence measurements: The loss of protein tryptophan fluorescence, an indicator of conformational change or tryptophan modification, is a marker of crystallin integrity. The tryptophan fluorescence in αL-crystallin solution after incubation with or without carnosine was measured by using a Perkin-Elmer LB 50 B luminescence spectrophotometer as described previously in the literature [3]. The excitation wavelength was set to 280 nm, slit width 5 nm, and the fluorescence emission was recorded between 280-550 nm. The nontryptophan fluorescence spectra were obtained with an excitation wavelength at 340 nm. αL-Crystallin solutions (0.04 mg/ml) in 0.05 M sodium phosphate buffer (pH 6.7) were used unless otherwise stated.

Absorbance readings of the αL-crystallin samples were measured at various wavelengths (220-400 nm) on a UVIKON 930 Kontron spectrophotometer. αL-Crystallin solutions (0.4 mg/ml) in 0.05 M sodium phosphate buffer (pH 6.7) were used unless otherwise stated. Absorbance readings of the preparations of carnosine with sugars after incubations were also measured at 220-400 nm.

Statistical analysis: Results are reported as the mean±SD, unless otherwise specified, of at least three separate experi-

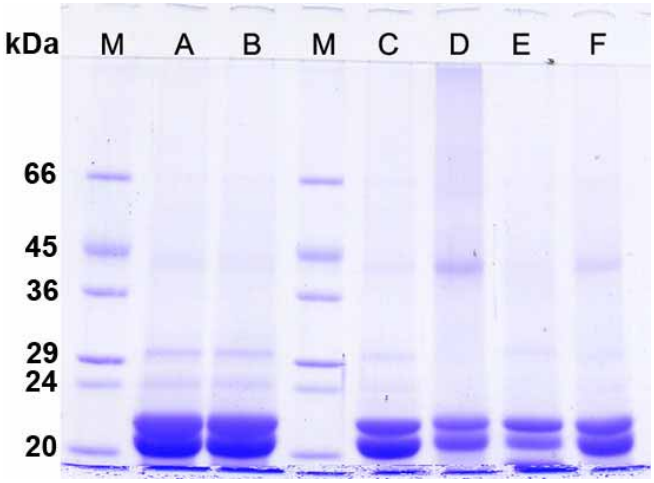


Figure 1. SDS-PAGE analysis of the effect of carnosine on fructose 6-phosphate-induced modifications of αL-crystallin. Fructose 6-phosphate (F6P) crosslinked αL-crystallin to form a dimer during a 4 d incubation but this crosslinking was decreased in the presence of carnosine. Lane M: Molecular weight marker. Lane A: control αL-crystallin at start. Lane B: αL-crystallin with 100 mM F6P at start. Lane C: control αL-crystallin at four days. Lane D: αL-crystallin with 100 mM F6P at four days. Lane E: αL-crystallin with 100 mM F6P and 100 mM carnosine at four days. Lane F: αL-crystallin with 100 mM F6P and 50 mM carnosine at four days.

Table 1. The effect of carnosine on the fructose 6-phosphate-induced decrease in chaperone activity of αL-crystallin

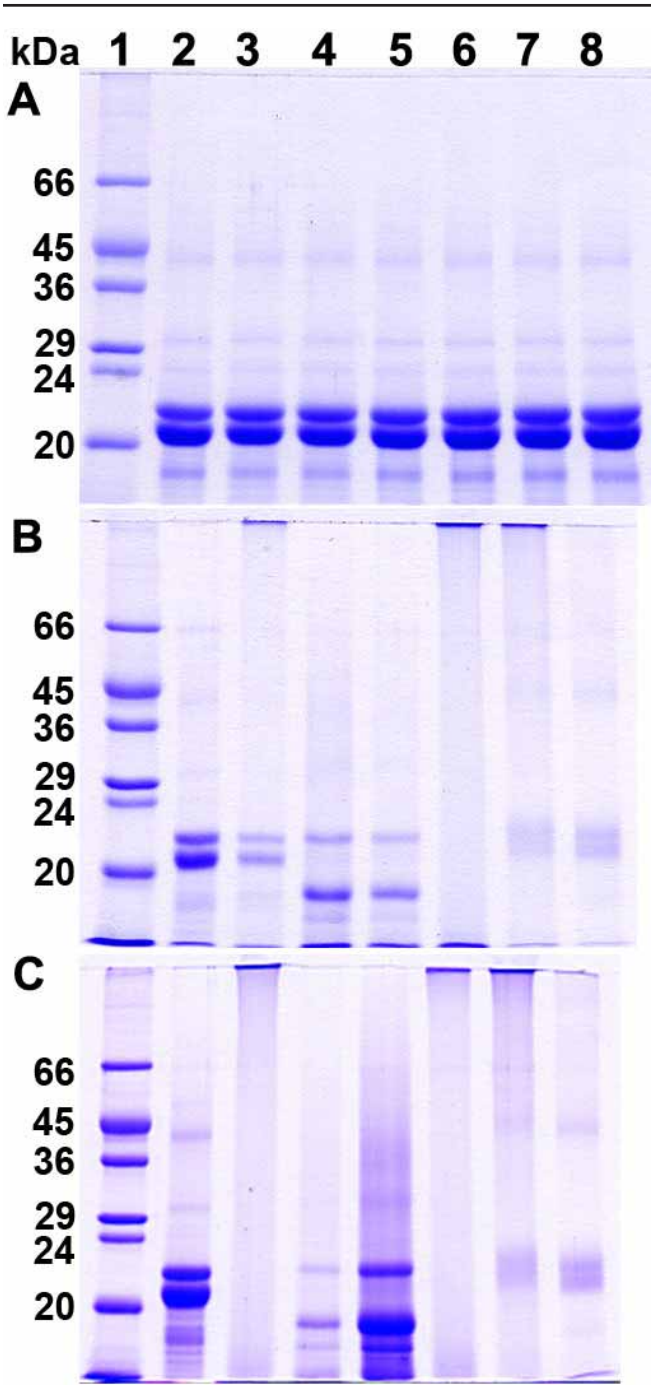
αL-crystallin preparation	0 day	4 day
α	95.1±1.1	95.5±0.5
α+F6P	94.1±1.1	84.7±1.7*
α+F6P+50 mM C	94.7±1.2	95.8±0.2**
α+F6P+100 mM C	94.7±1.7	95.6±1.3***

Percent protection against aggregation at start and four days for combinations of αL-crystallin (α), 100 mM fructose 6-phosphate (F6P), and carnosine (C). The asterisk indicates a statistically significant difference between α and α+F6P at 4 days (p=0.0005). The double asterisk indicates a statistically significant difference between α+F6P and α+F6P+50 mM C at 4 days (p=0.0004). The triple asterisk indicates a statistically significant difference between α+F6P and α+F6P+100 mM C at 4 days (p=0.0010).

ments. Statistical significance of differences was examined using a Student t-test, where relevant.

RESULTS

Crosslinking and chaperone activity: Electrophoresis of αL-crystallin showed the two bands of αA-crystallin and αB-crystallin at 20-21 kDa (Figure 1, lane A). F6P (100 mM) crosslinked 100 mM crystallin to form a dimer over 4 d incubation (Figure 1, lane D) but 100 mM ribose had no effect (data not shown). A band of molecular weight about 38-43 kDa appeared in the presence of F6P but it was decreased in the presence of carnosine (Figure 1, lanes D-F).



The chaperone-like activity of sugar modified α-crystallin was assayed. After four days incubation with F6P, the chaperone activity of αL-crystallin fell by 10% (Table 1). Carnosine protected αL-crystallin against this decrease induced by F6P. The protection of control αL-crystallin against aggregation of βL-crystallin was 96%, but only 85% remained in the presence of F6P after four days incubation and 96% in the presence of F6P and 50 mM carnosine (p=0.0004) and 100 mM carnosine (p=0.0010), respectively. The degree of inactivation and protection was similar after eight days. However, there was no remarkable reduction of chaperone activity induced by ribose even after 8 days (data not shown).

Incubation of αL-crystallin with 100 mM F6P and ribose at 37 °C over 15-25 days resulted in significant crosslinking in the two αL-crystallin subunit bands (Figure 2B,C, lanes 3 and 6) as shown by weakening of the αA- and αB-crystallin band with the appearance of crosslinked protein on top of the gel. The crosslinking of the αL-crystallin preparations with F6P after 25 days incubation was greater than that after 15 days (Figure 2B,C). The significant protective effect of carnosine against the crosslinking of αL-crystallin induced by

Table 2. Effect of carnosine on fructose 6-phosphate- and ribose-induced decreases in chaperone activity of αL-crystallin

αL-crystallin preparation	0 day	15 day	25 day
α	99.0±1.0	83.6±9.0	90.9± 4.7
α+F6P	98.0±0.5	2.7±1.6*	4.1± 4.8**
α+F6P+50 mM C	95.0±4.8	3.9±2.2	3.1± 2.3
α+F6P+100 mM C	93.4±5.0	3.1±2.3	13.2±13.4***
α+R	98.2±2.2	27.4±2.9#	1.6± 1.3##
α+R+50 mM C	97.1±1.0	3.2±1.1	3.1± 1.8
α+R+100 mM C	98.0±1.3	6.4±2.2	2.3± 1.3

Percent protection against aggregation after incubation for 0, 15, and 25 days for combinations of αL-crystallin (α), 100 mM fructose 6-phosphate (F6P), 100 mM ribose (R), and carnosine (C). The asterisk indicates a statistically significant difference between α and α+F6P at 15 days (p=0.0001). The double asterisk indicates a statistically significant difference between α and α+F6P at 25 days (p=0.0001). The triple asterisk indicates a statistically significant difference between α+F6P and α+F6P+100 mM C at 25 days (p=0.2565). The single sharp (hash mark) indicates a statistically significant difference between α and α+R at 15 days (p<0.0001). The double sharp indicates a statistically significant difference between α and α+R at 25 days (p<0.0001).

Figure 2. SDS-PAGE analysis of the effect of carnosine on fructose 6-phosphate- and ribose-induced modifications of αL-crystallin. Incubation of αL-crystallin with fructose 6-phosphate (F6P) and ribose over 15-25 days resulted in significant crosslinking. Carnosine appeared to have a protective effect on this crosslinking. **A**: 0 days. **B**: 15 days incubation. **C**: 25 days incubation. Lane 1: Molecular weight marker. Lane 2: Control αL-crystallin. Lane 3: αL-crystallin with 100 mM F6P. Lane 4: αL-crystallin with 100 mM F6P and 50 mM carnosine. Lane 5: αL-crystallin with 100 mM F6P and 100 mM carnosine. Lane 6: αL-crystallin with 100 mM ribose. Lane 7: αL-crystallin with 100 mM ribose and 50 mM carnosine. Lane 8: αL-crystallin with 100 mM ribose and 100 mM carnosine.

F6P and ribose was observed by SDS-PAGE analysis (Figure 2B,C, lanes 4, 5, and 8) with some protection at the higher concentration, particularly at 25 days (Figure 2C, lane 7). Ribose showed no significant crosslinking of αL-crystallin after four and eight days (results not shown). After moderate modification of αL-crystallin with F6P for four days, dimer formation was primarily seen (Figure 1, lane D). However, after longer incubation, crosslinking progressed further (Figure 2C) resulting in material unable to penetrate the gel. In a separate experiment after eight days (result not shown) there was only dimer formation similar to the four day result. There were no significant intermediates (trimers, etc.) between dimers and heavily crosslinked material on top of the gel. This may indicate that dimerization opens the structure to more extensive crosslinking. Truncation of αL-crystallin is well known but it is surprising in this work that it appears to be more pronounced after incubation with carnosine (Figure 2B,C).

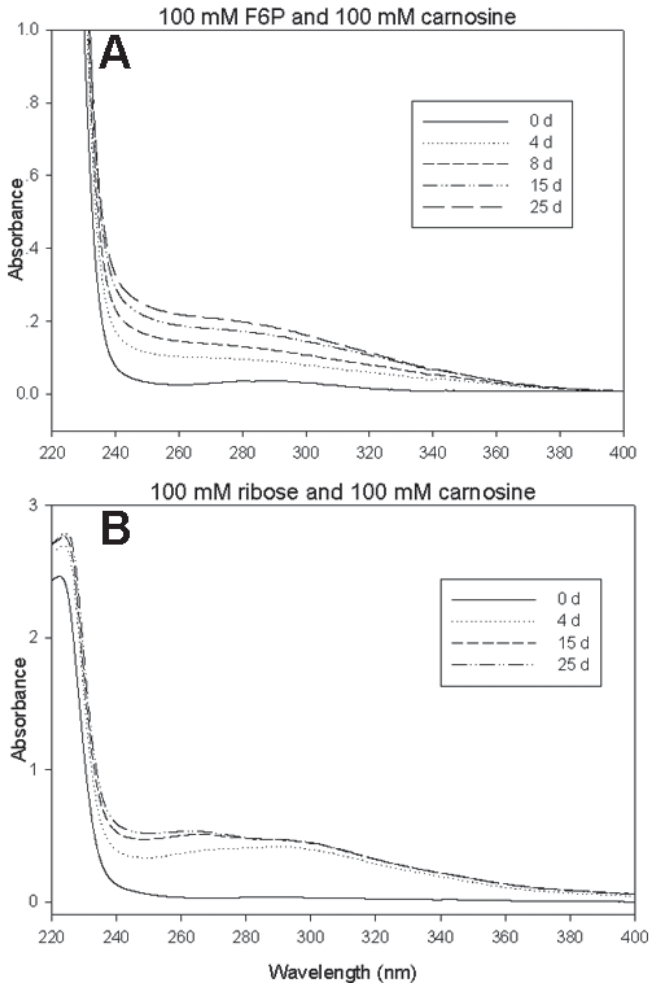


Figure 3. The absorbance spectra of fructose 6-phosphate and ribose incubated with carnosine preparations. The time-dependent increased absorbance was observed in the presence of carnosine and 100 mM fructose 6-phosphate (F6P) or 100 mM ribose. **A**: F6P and carnosine preparation after 0, 4, 8, 15, and 25 days incubation. **B**: Ribose and carnosine preparation after 0, 4, 15, and 25 days incubation.

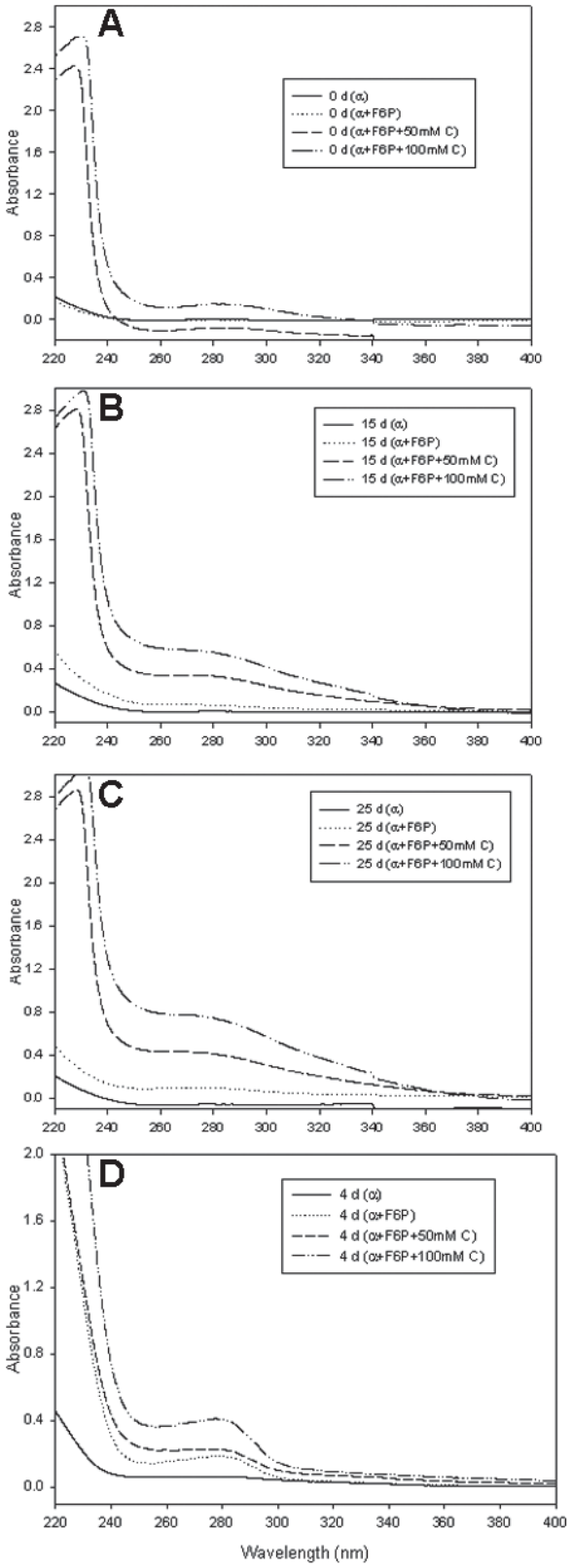


Figure 4. Absorbance spectra of fructose 6-phosphate-treated αL-crystallin. Carnosine enhanced the absorbance at 280 nm in a dose- and time-dependent manner in the presence of fructose 6-phosphate (F6P). Experiments in Panels **A-C** used 0.2 mg/ml of sample. The experiment in Panel **D** used 0.4 mg/ml of sample. **A**: 0 days. **B**: 15 days. **C**: 25 days. **D**: 4 days.

Unmodified αL-crystallin gave almost complete protection against the thermal aggregation of βL-crystallin, whereas the αL-crystallin modified by F6P showed significant loss of chaperone function after 15 days of incubation with 100 mM F6P and ribose. No protective effect of carnosine was observed in αL-crystallin preparations incubated with ribose and F6P over 15 and 25 days (Table 2). The apparent increase in protection between 15 and 25 days for αL-crystallin alone and αL-crystallin plus F6P plus 100 mM carnosine (Table 2: 83.6±9.0 to 90.9±4.7; and 3.1±2.3 to 13.2±13.4) were not statistically significant.

Overall, these observations suggest that carnosine can inhibit glycation-induced crosslinking of α-crystallin and protect its loss of chaperone activity induced by F6P. It cannot protect against ribose at an early stage. When the chaperone activity was extensively decreased, carnosine was not able to prevent the loss of chaperone activity induced by glycation.

We should note here that a remarkable color change of the αL-crystallin preparations with carnosine was observed. After 24 h incubation, αL-crystallin preparations with 100 mM

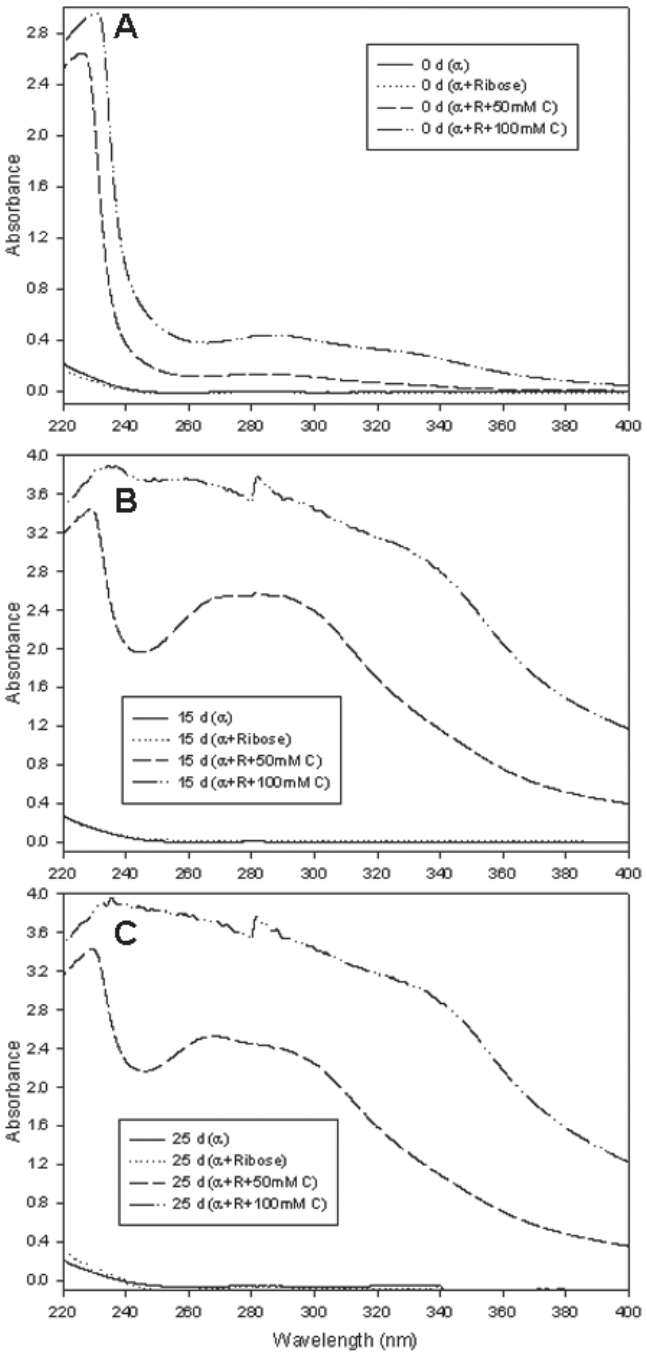


Figure 5. Absorbance of ribose-treated αL-crystallin samples at various wavelengths. Carnosine significantly enhanced the absorbance at 280 nm in a dose- and time-dependent manner in the presence of ribose. Each experiment used 0.2 mg/ml of sample. **A**: 0 days. **B**: 15 days. **C**: 25 days.

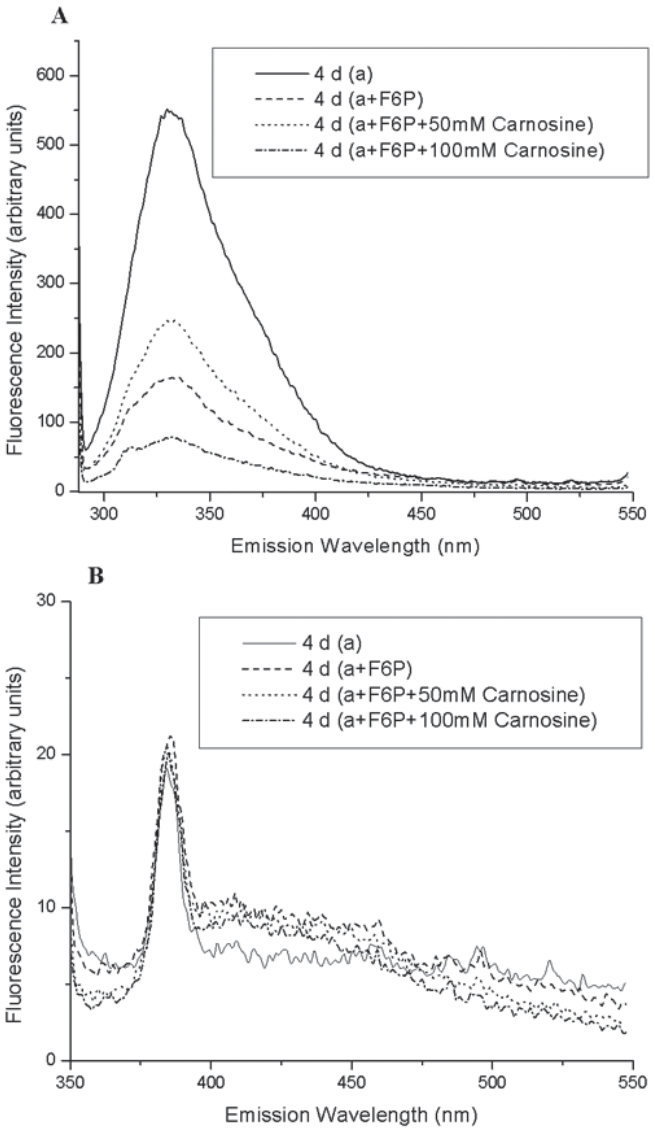


Figure 6. Tryptophan and nontryptophan fluorescence of αL-crystallin incubated with fructose 6-phosphate. Carnosine protected the loss of tryptophan and nontryptophan fluorescence of αL-crystallin induced by fructose 6-phosphate (F6P). Each experiment used 0.04 mg/ml of sample. **A**: Tryptophan. **B**: Nontryptophan.

ribose and 50 mM carnosine became light yellow, whereas in the presence of 100 mM carnosine and 100 mM F6P, the solution became light yellow over four days. Prolonged incubations of carnosine with ribose produced brown solutions. Moreover, the protein became very brown even after being extensively dialyzed when the incubation period increased in the presence of ribose and 100 mM carnosine. It seems that carnosine may react with sugars directly, particularly with ribose, and this probably results in formation of colored compounds. This hypothesis was further investigated by incubation of sugars with carnosine only. Absorbance spectra of carnosine solutions after incubation with F6P and ribose are shown in Figure 3. The increased absorbance was time-dependent in the presence of carnosine and 100 mM F6P or 100 mM ribose, supporting the idea that an adduct formed between carnosine and F6P or ribose.

Fluorescence measurements: The absorbance of the F6P- and ribose-treated αL-crystallin samples at various wavelengths (220-400 nm) is shown in Figure 4 and Figure 5. The absorbance at 280 nm was slightly increased in the presence of F6P and ribose. The addition of carnosine enhanced the absorbance in a dose- and time-dependent manner, especially with ribose.

Tryptophan fluorescence is an indicator of the modification of the environment of tryptophan residues. Nontryptophan fluorescence indicates the formation of new structures. Tryptophan and nontryptophan fluorescence were measured in F6P- and ribose-treated αL-crystallin solution with or without carnosine to identify conformational changes and fluorophore formation (Figure 6, Figure 7, Figure 8). Tryptophan fluorescence was decreased after only four days incubation with F6P (Figure 6A) and decreased further in eight days (data not shown), indicating conformational change. However, the loss of tryptophan fluorescence was less in the presence of 50 mM carnosine, supporting a protective effect. Carnosine at 50 mM seemed to protect against this but at 100 mM there was a greater loss of fluorescence. This is possibly due to quenching by the increased absorbance at 280 nm decreasing the amount of incident light reaching tryptophan residues and hence decreasing the emitted light (Figure 4D). In the 15 day and 25 day incubations, carnosine enhanced the loss of tryptophan fluorescence (Figure 7A,B). Nontryptophan fluorescence was increased slightly by F6P with little modulation by carnosine (Figure 6B). Ribose, unlike F6P, had relatively little effect on the tryptophan fluorescence of αL-crystallin at four and eight days. The nontryptophan fluorescence increased only at eight days with ribose, but increased much more when carnosine was also present (data not shown).

In αL-crystallin from F6P-treated solution after 15 days incubation, there was an approximately three fold loss of tryptophan fluorescence (Figure 7A), under conditions where F6P had little effect on the absorbance of αL-crystallin (Figure 4A). Therefore the change in fluorescence spectrum is probably attributable to a change in conformation induced by F6P. Similar changes were seen at 25 days (Figure 7B). Tryptophan fluorescence was even more decreased in the presence of carnosine but this could be due to quenching. Nontryptophan fluo-

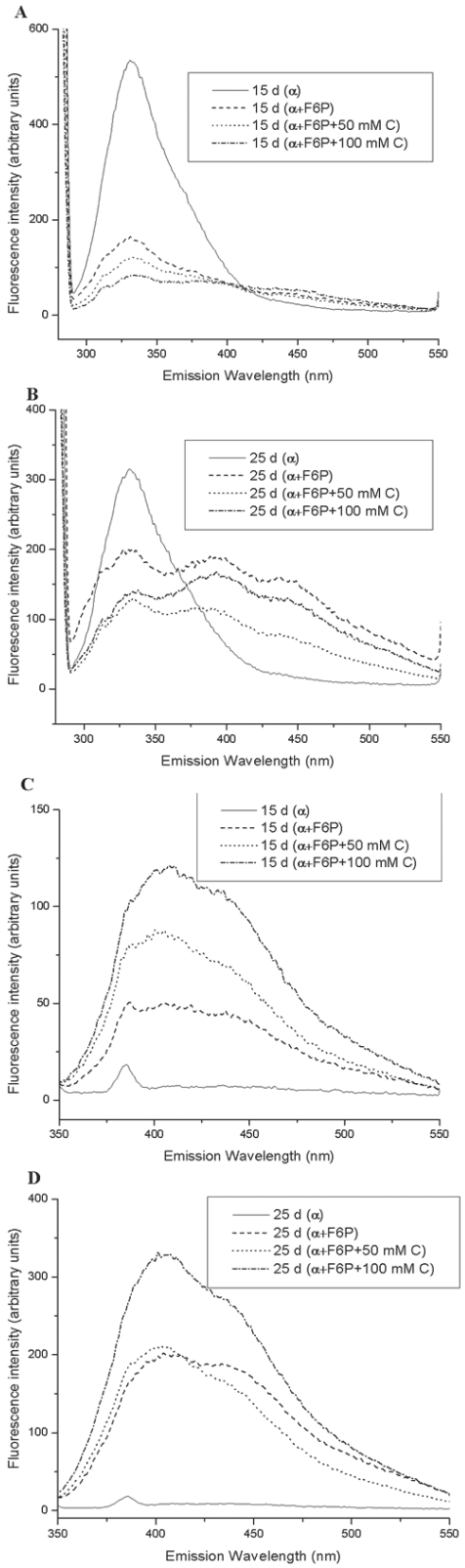


Figure 7. Tryptophan and nontryptophan fluorescence of αL-crystallin modified by fructose 6-phosphate. Carnosine enhanced the loss of tryptophan fluorescence. Nontryptophan fluorescence was continuously increased with a shift to longer wavelengths in the presence of fructose 6-phosphate (F6P) alone, and more so with carnosine. **A**: 15 days tryptophan. **B**: 25 days tryptophan. **C**: 15 days nontryptophan. **D**: 25 days nontryptophan.

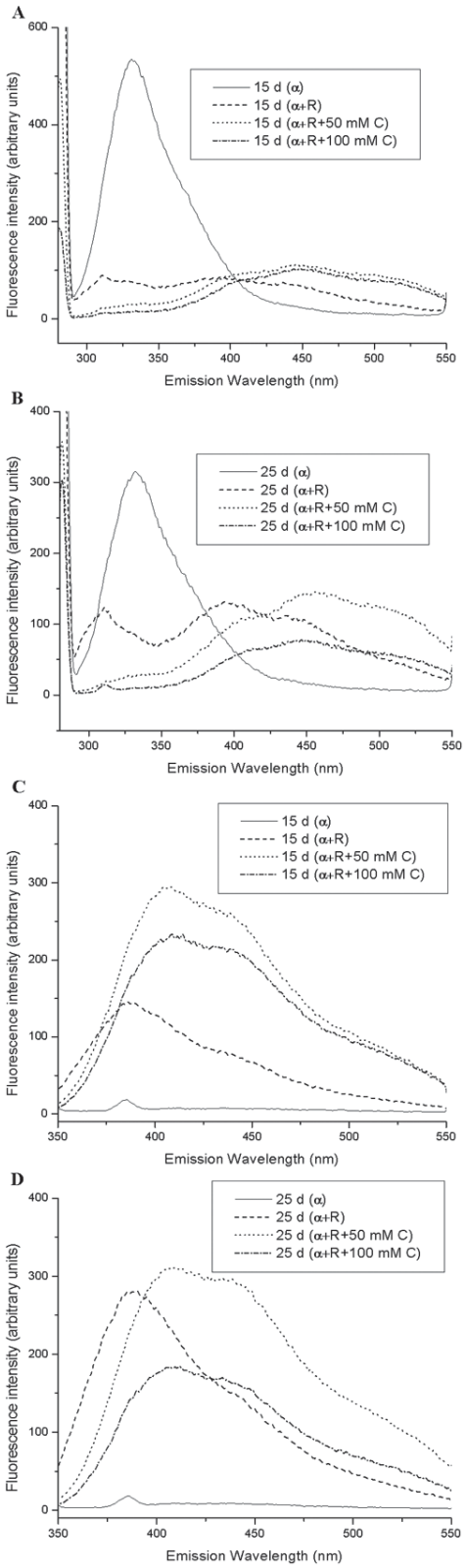


Figure 8. Tryptophan and nontryptophan fluorescence of αL-crystallin modified by ribose. Carnosine enhanced the loss of tryptophan fluorescence. Nontryptophan fluorescence was remarkably increased in the presence of ribose with a shift to longer wavelengths (approximately 410 nm) when carnosine was also present. **A**: 15 days tryptophan. **B**: 25 days tryptophan. **C**: 15 days nontryptophan. **D**: 25 days nontryptophan.

rescence was continuously increased with a shift to longer wavelengths (approximately 410 nm) in the presence of F6P alone, and more so with carnosine (Figure 7C,D), continuing to increase to 25 days, suggesting the formation of several adducts with different structures and fluorescent characteristics.

There was a significant loss of tryptophan fluorescence with a shift to longer wavelengths (approximately 400-500 nm) in αL-crystallin from ribose-treated solution after 25 days incubation with or without carnosine (Figure 8A,B) under conditions where ribose alone had no effect on absorbance (Figure 5). Thus, ribose induced conformational changes in αL-crystallin. Nontryptophan fluorescence was remarkably increased in the presence of ribose with a shift to longer wavelengths (approximately 410 nm) when carnosine was also present (Figure 8C,D). There was more fluorescence intensity in the presence of 50 mM carnosine than with 100 mM carnosine, probably due to the loss of protein from solution indicated by the significant precipitation that was observed during incubation with high concentration of carnosine.

Above all, it is clear that tryptophan fluorescence in F6P- and ribose-treated αL-crystallin was lost, indicating the modification of the environment of the tryptophan residues. Nontryptophan fluorescence was increased with a shift to longer wavelengths (approximately 400-450 nm), indicating formation of new fluorophores. The 50 mM carnosine level seems to inhibit the decrease of tryptophan fluorescence induced by F6P at four days, supporting the results on decreased chaperone activity (Table 1) and the crosslinking in SDS-PAGE analysis (Figure 1), but at 100 mM and at longer times, carnosine had a deleterious effect. This effect presumably results from the extensive adduct formation (see previous observations), which in turn, reacts with crystallin or sugar and speeds up aggregations.

DISCUSSION

The results presented here provide evidence to support the hypothesis that carnosine can protect against the loss of chaperone activity of α-crystallin induced by glycation, in addition to describing the effects of carnosine on prevention of crosslinking. This effect may exhibit therapeutic potential.

The present results showed that carnosine inhibited the crosslinking of αL-crystallin induced by F6P and ribose, and protected αL-crystallin against its decreased chaperone activity induced by F6P. However, it failed to protect the more extensive modifications by sugar. The tryptophan fluorescence intensity of αL-crystallin modified by F6P and ribose was significantly decreased, whereas its nontryptophan fluorescence was increased with a shift to longer wavelengths in a time- and dose-dependent manner, suggesting that new fluorophores are formed [34]. Crosslinked proteins interfere with tissue function and act as trapping material, causing aggregation. Carnosine may dissociate thermally-induced aggregates by promoting hydration [32] or disaggregate glycated α-crystallin [17]. Amino acid sequence of carnosine is similar to Lys-His, whereas the amino groups of lysine residues are the primary targets by glycation. The dipeptide reacts rapidly with reduc-

ing sugars [15]. Therefore, carnosine has the potential to react with sugars.

Protein glycation, which promotes aggregation, involves the unwanted reaction of carbohydrate oxidation products with proteins. It has been shown by Seidler et al. [17] that carnosine disaggregated methylglyoxal-induced glycation of α-crystallin and decreased tryptophan fluorescence polarization of glycated α-crystallin, suggesting that carnosine increased peptide chain mobility, which may contribute to the controlled unfolding of glycated protein. The results described in this paper suggest that carnosine also reacts directly with F6P and ribose and results in formation of new adducts (Figure 4, Figure 5, Figure 7B, Figure 8B). Removal of reactive sugars may be responsible for the protective effect of carnosine on chaperone activity. Carnosine may suppress the deleterious effects of a reactive carbonyl group intracellularly by reacting with sugars to form protein/carbonyl/carnosine adducts similar to the reported aldehyde adducts [33] and carnosine-glyceraldehyde 3-phosphate adduct [34]. Its protection against glycation-induced inactivation of esterase and catalase has been described recently [21,22].

However, the higher concentration of carnosine had a deleterious effect on chaperone activity of α-crystallin after a longer time of incubation. It is present in several mammal tissues (e.g., muscle and brain) at high concentration (up to 20 mM in humans) [12]. In an in vitro study, 1-10 mM carnosine can protect the inactivation of enzymes [21] and protect cultured rat brain endothelial cells against malondiadehyde (MDA)-induced toxicity [26], whereas 50 mM carnosine disaggregates glycated α-crystallin [17], inhibits MDA-induced protein modification (formation of crosslinks and carbonyl groups) [26], and forms new adducts [34]. Therefore, it may point to the possibility that a higher dose of carnosine reacts with sugars and a sugar phosphate extensively resulting in accumulation of new adducts, which in turn, further react and aggregate crystallin. This may be deleterious to the ability to protect against thermal-induced aggregation of βL-crystallin presented as chaperone activity. The potential biological and therapeutic significance of these observations need to be explored further.

There appear to be at least two factors playing major roles in the chaperone-like activity of α-crystallin: one is the hydrophobicity of the exposed protein surface and the other is its structural stability [35]. It is possible that the modifications described in this paper decreased the stability of αL-crystallin so that it dissociated at the raised temperature of the chaperone assay. Although crosslinking appeared to have a deleterious effect on chaperone function after some modifications [9], in others extensively crosslinked αL-crystallin retained its ability to chaperone [3]. More recently, the question has been explored first that the crosslinked α-crystallin was able to inhibit the thermally induced precipitation of β-crystallin and appeared to be more effective than the native protein under the same conditions [36]. Furthermore, the native oligomeric state of α-crystallin may not be essential for its ability to suppress nonspecific aggregation [37]. In both studies, α-crystallin maintained chaperone function. Neither subunit exchange nor the native oligomeric state is essential for chaperone function. Therefore, the response of crosslinked α-crystallin to temperature needs to be further elucidated.

To conclude, this is the first report describing the protective effect of carnosine on glycation-induced decreased chaperone activity of α-crystallin. In addition, our results further support the notion that carnosine can disaggregate crosslinking mediated by glycation and possible adduct formation among carnosine and sugar, and a sugar phosphate. These results shed new light on the properties of carnosine and have important implications for understanding the mechanism by which carnosine may be of benefit in preventing lens opacity in humans.

ACKNOWLEDGEMENTS

The authors gratefully acknowledge the Wellcome Trust for financial support.

REFERENCES

1. Horwitz J. Alpha-crystallin. Exp Eye Res 2003; 76:145-53.
2. Harding JJ. Viewing molecular mechanisms of ageing through a lens. Ageing Res Rev 2002; 1:465-79.
3. Derham BK, Harding JJ. Effects of modifications of alpha-crystallin on its chaperone and other properties. Biochem J 2002; 364:711-7.
4. Hook DW, Harding JJ. Protection of enzymes by alpha-crystallin acting as a molecular chaperone. Int J Biol Macromol 1998; 22:295-306.
5. Derham BK, Harding JJ. Alpha-crystallin as a molecular chaperone. Prog Retin Eye Res 1999; 18:463-509.
6. Ghosh JG, Estrada MR, Clark JI. Interactive domains for chaperone activity in the small heat shock protein, human alphaB crystallin. Biochemistry 2005; 44:14854-69.
7. Augusteyn RC. alpha-crystallin: a review of its structure and function. Clin Exp Optom 2004; 87:356-66.
8. Avilov SV, Aleksandrov NA, Demchenko AP. Quaternary structure of alpha-crystallin is necessary for the binding of unfolded proteins: a surface plasmon resonance study. Protein Pept Lett 2004; 11:41-8.
9. van Boekel MA, Hoogakker SE, Harding JJ, de Jong WW. The influence of some post-translational modifications on the chaperone-like activity of alpha-crystallin. Ophthalmic Res 1996; 28[Suppl]:32-8.
10. Satish Kumar M, Mrudula T, Mitra N, Bhanuprakash Reddy G. Enhanced degradation and decreased stability of eye lens alpha-crystallin upon methylglyoxal modification. Exp Eye Res 2004; 79:577-83.
11. Hook DW, Harding JJ. The effect of modification of alpha-crystallin by prednisolone-21-hemisuccinate and fructose 6-phosphate on chaperone activity. Dev Ophthalmol 2002; 35:150-60.
12. Hipkiss AR. Carnosine, a protective, anti-ageing peptide? Int J Biochem Cell Biol 1998; 30:863-8.
13. Hobart LJ, Seibel I, Yeargans GS, Seidler NW. Anti-crosslinking properties of carnosine: significance of histidine. Life Sci 2004; 75:1379-89.
14. Mehta AD, Seidler NW. Beta-alanine suppresses heat inactivation of lactate dehydrogenase. J Enzyme Inhib Med Chem 2005; 20:199-203.
15. Hipkiss AR, Michaelis J, Syrris P. Non-enzymatic glycosylation of the dipeptide L-carnosine, a potential anti-protein-cross-linking agent. FEBS Lett 1995; 371:81-5.

16. Brownson C, Hipkiss AR. Carnosine reacts with a glycated protein. Free Radic Biol Med 2000; 28:1564-70.
17. Seidler NW, Yeargans GS, Morgan TG. Carnosine disaggregates glycated alpha-crystallin: an in vitro study. Arch Biochem Biophys 2004; 427:110-5.
18. Ukeda H, Hasegawa Y, Harada Y, Sawamura M. Effect of carnosine and related compounds on the inactivation of human Cu,Zn-superoxide dismutase by modification of fructose and glycolaldehyde. Biosci Biotechnol Biochem 2002; 66:36-43.
19. Kang JH, Kim KS, Choi SY, Kwon HY, Won MH, Kang TC. Protective effects of carnosine, homocarnosine and anserine against peroxyl radical-mediated Cu,Zn-superoxide dismutase modification. Biochim Biophys Acta 2002; 1570:89-96.
20. Stvolinskii SL, Fedorova TN, Yuneva MO, Boldyrev AA. Protective effect of carnosine on Cu,Zn-superoxide dismutase during impaired oxidative metabolism in the brain in vivo. Bull Exp Biol Med 2003; 135:130-2.
21. Yan H, Harding JJ. Carnosine protects against the inactivation of esterase induced by glycation and a steroid. Biochim Biophys Acta 2005; 1741:120-6.
22. Yan H, Harding JJ. Protective effect of carnosine against inactivation of enzymes induced by glycation and a steroid: relevance to cataract. Am J Ophthalmol 2005; 139(3):S55.
23. Swearengin TA, Fitzgerald C, Seidler NW. Carnosine prevents glyceraldehyde 3-phosphate-mediated inhibition of aspartate aminotransferase. Arch Toxicol 1999; 73:307-9.
24. Babizhayev MA, Deyev AI, Yermakova VN, Semiletov YA, Davydova NG, Kurysheva NI, Zhukotskii AV, Goldman IM. N-Acetylcarnosine, a natural histidine-containing dipeptide, as a potent ophthalmic drug in treatment of human cataracts. Peptides 2001; 22:979-94.
25. Babizhayev MA, Yermakova VN, Sakina NL, Evstigneeva RP, Rozhkova EA, Zheltukhina GA. N alpha-acetylcarnosine is a prodrug of L-carnosine in ophthalmic application as antioxidant. Clin Chim Acta 1996; 254:1-21. Erratum in: Clin Chim Acta 1997; 259:199-201.
26. Hipkiss AR, Preston JE, Himswoth DT, Worthington VC, Abbot NJ. Protective effects of carnosine against malondialdehyde-induced toxicity towards cultured rat brain endothelial cells. Neurosci Lett 1997; 238:135-8.
27. Lee JW, Miyawaki H, Bobst EV, Hester JD, Ashraf M, Bobst AM. Improved functional recovery of ischemic rat hearts due to singlet oxygen scavengers histidine and carnosine. J Mol Cell Cardiol 1999; 31:113-21.
28. Slingsby C, Bateman OA. Rapid separation of bovine beta-crystallin subunits beta B1, beta B2, beta B3, beta A3 and beta A4. Exp Eye Res 1990; 51:21-6.
29. Yan H, Harding JJ, Hui YN, Li MY. Decreased chaperone activity of alpha-crystallin in selenite cataract may result from selenite-induced aggregation. Eye 2003; 17:637-45.
30. Laemmli UK. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 1970; 227:680-5.
31. Horwitz J. Alpha-crystallin can function as a molecular chaperone. Proc Natl Acad Sci U S A 1992; 89:10449-53.
32. Yeargans GS, Seidler NW. Carnosine promotes the heat denaturation of glycated protein. Biochem Biophys Res Commun 2003; 300:75-80.
33. Hipkiss AR, Brownson C, Carrier MJ. Carnosine, the anti-ageing, anti-oxidant dipeptide, may react with protein carbonyl groups. Mech Ageing Dev 2001; 122:1431-45.
34. Seidler NW, Shokry SS, Nauth J. Properties of a glycation product derived from carnosine. J Biochem Mol Biol Biophys 2001; 5:153-162.
35. Liao JH, Lee JS, Chiou SH. Distinct roles of alphaA- and alphaB-crystallins under thermal and UV stresses. Biochem Biophys Res Commun 2002; 295:854-61.
36. Augusteyn RC. Dissociation is not required for alpha-crystallin's chaperone function. Exp Eye Res 2004; 79:781-4.
37. Horwitz J, Huang Q, Ding L. The native oligomeric organization of alpha-crystallin, is it necessary for its chaperone function? Exp Eye Res 2004; 79:817-21.

The print version of this article was created on 27 Mar 2006. This reflects all typographical corrections and errata to the article through that date. Details of any changes may be found in the online version of the article.