

Carnosine disaggregates glycated α -crystallin: an in vitro study

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Abstract

Protein glycation, which promotes aggregation, involves the unwanted reaction of carbohydrate oxidation products with proteins. Glycation of lens α -crystallin occurs in vivo and may contribute to cataractogenesis. Anti-glycation compounds such as carnosine may be preventive, but interestingly carnosine reverses lens opacity in human trials. The mechanism for this observation may involve carnosine's ability to disaggregate glycated protein. We investigated this hypothesis using glycated α -crystallin as our in vitro model. Methylglyoxal-induced glycation of α -crystallin caused aggregation as evidenced by increased 90° light scattering. After addition of carnosine, light scattering returned to baseline levels suggesting that the size of the glycation-induced aggregates decreased. Additionally, carnosine 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. Comparatively, guanidine-HCl and urea had no effect. Our data support the hypothesis that carnosine disaggregates glycated α -crystallin.

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Cataracts are insoluble aggregates that result from dysfunctional protein interactions leading to increased lens opacity [1]. The aggregates consist predominantly of α -crystallin (α CRY)¹ [2] in age-related cataracts [3]. The cause of protein insolubility—and hence lens opacity—remains poorly understood. However, we know that chemical modifications of α CRY such as protein glycation play an important and pivotal role [4–7]. Glycation leads to protein crosslinking [8], which may act as a nucleation site causing further aggregation [9]. Glycating agents such as fructose and methylglyoxal (MG) [10–12] form advanced glycation endproducts (AGEs) on α CRY and contribute to cataract formation [12]. Carnosine, which is an endogenous histidine dipeptide, prevents protein modification [13–15]. Carnosine reacts with oxygen radicals [16], lipid peroxidation products [17], and carbohydrate oxidation products [18]. These observations suggest that carnosine may prevent cataracts.

However, there is evidence suggesting that carnosine reverses lens opacity in humans [19]. The mechanism for this observation may involve carnosine's ability to disaggregate glycated protein. We previously observed [20] that carnosine increases the unfolding and hydration of glycated protein, proposing that carnosine may play a role in the removal of aggregated proteins. Other chemicals (tetracyclines, nitrophenols, and benzoic acid derivatives) act as fibril disruptors [21,22] using the transthyretin aggregation model. The endogenous carnosine and these other synthetic chemicals may have chaotropic properties analogous to that of urea, which is a commonly used denaturant. The current study investigated the hypothesis that carnosine is a disaggregation agent using glycated α -crystallin as our in vitro model.

Materials and methods

Materials

Bovine lens α CRY, methylglyoxal (MG), 2,4-dinitrophenylhydrazine (DNPH), L-carnosine, and guanidine-HCl (Gdn-HCl) were obtained from Sigma

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¹ Abbreviations used: α CRY, α -crystallin; MG, methylglyoxal; Gdn-HCl, guanidine-HCl; g- α CRY, glycated α -crystallin; DNPH, 2,4-dinitrophenylhydrazine; rfu, relative fluorescence units.

Chemical (St. Louis, MO), labeled α CRY (Alexa Fluor 488) from Molecular Probes (Eugene, OR), and urea was from Matheson, Coleman and Bell (currently EMD Chemicals, Gibbstown, NJ). All other chemicals were of reagent grade or better.

Incubation conditions

Different amounts of α CRY were incubated in a 100 mM sodium phosphate buffer (pH = 7.4) with various concentrations of MG for 0–24 h at 24–37 °C prior to dialysis. This approach was chosen in order to demonstrate that the aggregation–disaggregation dynamics can be observed under diverse glycosylating conditions. Samples were dialyzed (1:20,000) against a 100 mM sodium phosphate buffer (pH = 7.4) containing 0.25 mM sodium azide using Spectra/Por dialysis membranes, MWCO 6-8000 (Spectrum Laboratories, Rancho Dominguez, CA). Following dialysis, samples were analyzed for AGEs and for changes in aggregation and peptide chain mobility. The effects of carnosine on glycosylated α CRY (g- α CRY) were examined as described below.

Assay for AGEs

AGE formation was determined by measuring absorbance at 350 nm [23] using an Ultrospec 4000 spectrophotometer (Amersham Biosciences, Piscataway, NJ). Fluorescence due to AGEs [24] was measured using an LS50B fluorometer (Perkin–Elmer, Shelton, CT). Spectral settings were as follows: excitation wavelength was 335 nm (slit = 5 nm) scanning speed was 600 nm/min, and the recording range was 350–600 nm (slit = 5 nm). Additionally protein carbonyl content which represents modified protein [25] was determined by a spectrophotometric assay [26] using the carbonyl reagent DNPH. Samples containing 75 μ g g- α CRY protein were incubated in 0.1% DNPH (2 N HCl) for 1 h at room temperature. Carrier protein (2 mg α CRY) was added and precipitated with 20% trichloroacetic acid. Samples were kept on ice for 30 min and centrifuged at 14,000g for 5 min using a Jouan A14 centrifuge (Winchester, VA). The pellet was washed 3 \times with ethanol/ethyl acetate (1:1 v/v), resuspended in a 133 mM Tris buffer (pH = 7.4) containing 8 M Gdn–HCl and 13 mM EDTA, and read at 365 nm using an Ultrospec 4000 spectrophotometer.

Measurement of α CRY aggregation

Glycation-induced opacity was used as an indicator of α CRY aggregation. Opacity was calculated from measurements of light transmittance (wavelength was set at 595 nm) using a Bio-Rad microplate reader model 550 (Hercules, CA). Data were normalized and con-

verted to percent opacity (100 – % transmittance). Glycation-induced 90° light scattering was also used to determine aggregation dynamics. g- α CRY samples (450 μ g protein) were placed in an LS50B fluorometer with constant stirring and measurements were taken at the following settings: excitation at 450 nm emission at 450 nm (slit = 2.5 nm) cutoff at 430 nm, and integration times at 10 s. Fluorescent-labeled α CRY was also used to measure aggregation. Following MG-induced glycation and dialysis to remove unbound MG g- α CRY versus control samples (200 μ g protein) were mixed with Alexa Fluor 488 labeled α CRY (20 μ g protein) and read in an LS50B fluorometer under constant stirring with excitation set at 496 nm emission at 518 nm (slits = 2.5 nm), and integration times at 10 s. Incorporation of labeled α CRY monomers into oligomeric complexes would follow the expected reversible mono-to oligo-dynamics hence allowing assessment of the effects of aggregation–disaggregation on fluorescence emission of incorporated label. Solvent accessibility rules apply and the fluorescence maximum changes as a function of accessibility to solvent. Measurements were taken before and after addition of carnosine.

Measurement of peptide chain mobility

We determined the degree of peptide chain mobility by measuring the polarization of specific fluorophores. Conformational mobility is inversely related to polarization of bound fluorescent compounds. Fluorescence polarization of intrinsic fluorophores (i.e., tryptophan residues) of g- α CRY samples (0.375 mg protein/mL) was measured before and after addition of carnosine. The settings were 290 nm excitation (slit = 5 nm) and 335 nm emission (slit = 5 nm) with integration times of 10 s using an LS50B fluorometer. Additionally fluorescence polarization of glycation-induced fluorophores (i.e., AGEs) of g- α CRY samples was measured before and after addition of carnosine. The settings were excitation at 335 nm (slit = 5 nm) and emission at 420 nm (slit = 5 nm) with integration times of 10 s. In both sets of fluorescence polarization experiments the common denaturants Gdn–HCl and urea were compared with carnosine.

Results

Glycated α CRY: AGE formation and protein aggregation

Consistent with previous studies [10,12,27,28], we detected AGEs following incubation of α CRY with MG (98 mM, 24 h, 37 °C). Peak fluorescence emission of g- α CRY and unmodified α CRY was 187.8 rfu (relative fluorescence units) and 9.8 rfu, respectively (ex: 335 nm; em: 420 nm). Carbonyl content of g- α CRY was

41.3 nmol/mg protein and the AGE-specific absorbance (350 nm) was 0.13 AU/mg protein. Even after brief incubation with lower MG concentrations (1.25 mM, 140 min, 37 °C) we observed a 5-fold increase in the AGE-related rfu of g- α CRY compared with unmodified α CRY (data not shown). These observations suggest that α CRY is readily glycosylated by MG.

To assess glycation-induced aggregation, we measured real time changes in 90° light scattering of α CRY following addition of MG (5 mM, 0–30 min, 37 °C). The observed increase in light scattering appeared biphasic, suggesting a gradual increase in aggregate size over the first 20 min followed by a more rapid rate of aggregation. Light scattering values from $t_{0\text{min}}$ to $t_{30\text{min}}$ at 5 min intervals were 136 ± 2 , 159 ± 1 , 164 ± 2 , 179 ± 1 , 196 ± 2 , 199 ± 0.3 , 348 ± 1 , and 546 ± 6 . MG (3–7 mM, 0–200 min, 24 °C) also exhibited a concentration-dependent increase in opacity of α CRY samples (data not shown) suggesting that glycation-induced aggregation occurs under these conditions.

Effects of carnosine on glycation-induced aggregation

Carnosine reversed the glycation-induced increase in 90° light scattering (Fig. 1). Light scattering initially increased following incubation with MG (molar ratio of 25:1 MG to α CRY; 8 mM, 24 h, 24 °C). When carnosine (50 mM) was added to these g- α CRY samples, light scattering decreased down to baseline levels, suggesting that carnosine promoted disaggregation. Addition of carnosine to unmodified α CRY was without effect.

Opacity of α CRY samples also initially increased following incubation with MG (7.5 mM, 2 h, 24 °C) ($P < 0.025$, one-tailed t test) and then decreased back down to control levels after addition of carnosine (42 mM) (Fig. 2). Non-specific absorbance at 595 nm

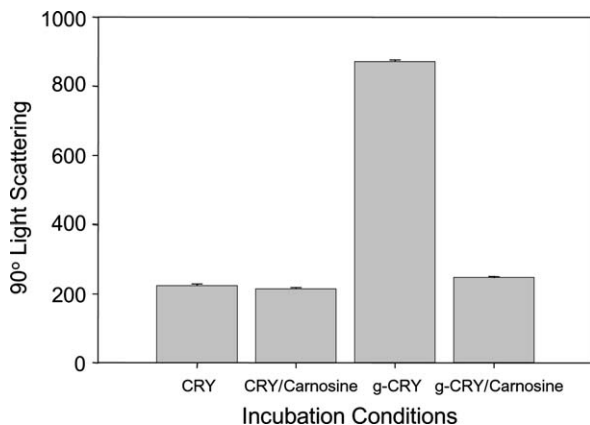


Fig. 1. Carnosine reverses MG-induced changes in 90° light scattering. Light scattering was measured before and after addition of carnosine (50 mM) to samples of α CRY and g- α CRY, which was prepared at a 25:1 MG to α CRY ratio. Data represent $M \pm SD$ of multiple readings from two experiments.

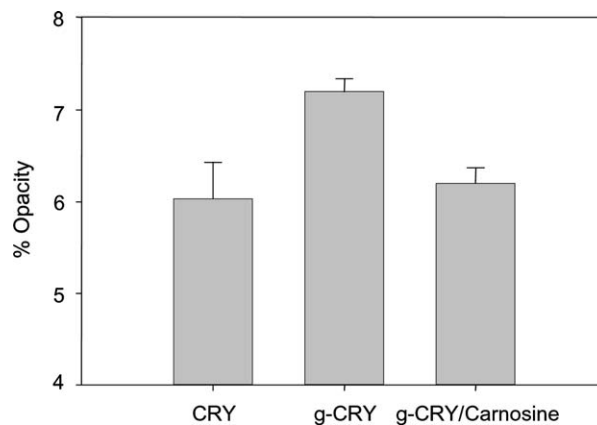


Fig. 2. Carnosine reverses MG-induced changes in opacity. Opacity of g- α CRY was measured before and after addition of carnosine (42 mM) and compared with control α CRY. Data represents $M \pm SD$ of multiple readings from a representative experiment.

was used to measure light transmittance, and thus avoiding interference by AGEs, which do not absorb at this wavelength.

In order to further assess carnosine's effect on g- α CRY aggregates, we mixed fluorescent-labeled α CRY with g- α CRY and unmodified samples and measured the spectra of the tagged fluorophore. g- α CRY samples (2.5–7.5 mM, 22 h, 36 °C) showed a concentration-dependent increase in fluorescence intensity compared with unmodified α CRY (Fig. 3A) suggesting that g- α CRY is aggregated causing the labeled α CRY to be sequestered from solvent. Addition of carnosine (10 mM) resulted in a decrease in fluorescence intensity of the tagged α CRY (Fig. 3B). These observations suggest that carnosine effectively disrupted α CRY-derived aggregates.

Effects of carnosine on peptide chain mobility of g- α CRY

Since MG-treated α CRY has fluorescent AGEs, we tested the effects of carnosine on AGE fluorescence polarization assessing peptide chain mobility. Additionally, carnosine was compared with common denaturants, Gdn-HCl and urea. We observed that carnosine decreased AGE fluorescence polarization (ex 335 nm; em 420 nm) at a much lower concentration than did either Gdn-HCl or urea (Fig. 4). Control samples represent g- α CRY that was prepared by incubating α CRY with MG (98 mM, 24 h, 37 °C) prior to addition of denaturant (control was 0.362 ± 0.002). Carnosine also affected fluorescence polarization of native tryptophans measured at excitation 290 nm and emission 335 nm (Fig. 5). Carnosine (200 mM) decreased tryptophan fluorescence polarization of g- α CRY samples ($P < 0.0005$, one-tailed t test). Using the same concentrations neither Gdn-HCl nor urea had an effect.

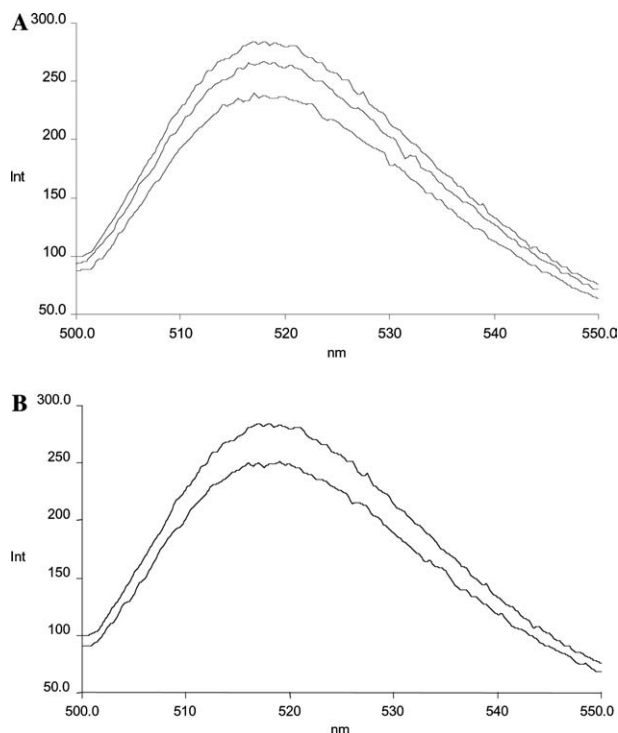


Fig. 3. Aggregation–disaggregation dynamics as measured by Alexa Fluor-labeled α CRY fluorescence emission. (A) Emission spectra (excitation 496 nm) of control α CRY (lower tracing) were compared to those of g- α CRY prepared at 2.5 mM MG, 22 h, 36 °C (middle tracing) and g- α CRY prepared at 5.0 mM MG, 22 h, 36 °C (upper tracing). (B) Emission spectra (excitation 496 nm) of g- α CRY prepared at 5.0 mM MG, 22 h, 36 °C (upper tracing) were compared with those of a g- α CRY sample containing 10 mM carnosine (lower tracing).

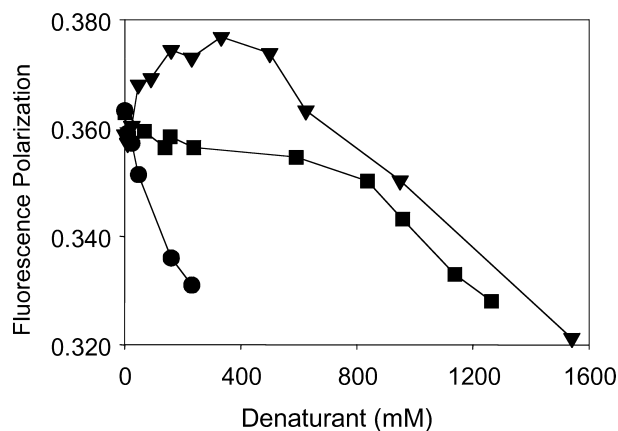


Fig. 4. Effects of carnosine and denaturants on polarization of AGE fluorophores in g- α CRY. Various concentrations of carnosine (circles), Gdn-HCl (triangles), and urea (squares) were added to samples of g- α CRY prior to measuring fluorescence polarization (ex 335 nm; em 420 nm).

Discussion

The current study presents evidence to support the hypothesis that carnosine is an effective disaggregation

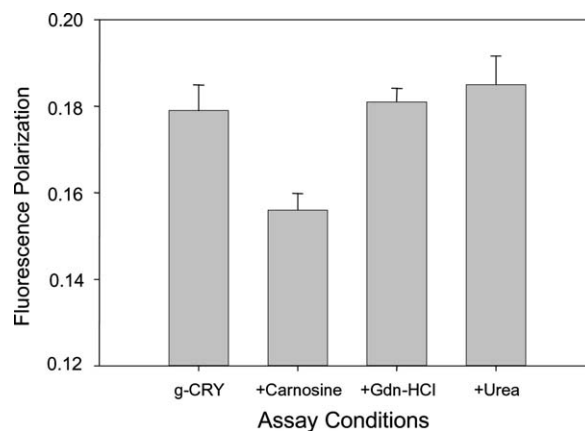


Fig. 5. Effects of carnosine and denaturants on fluorescence polarization of tryptophan residues in g- α CRY. Carnosine, Gdn-HCl, and urea (each 200 mM) were added to g- α CRY prior to measuring fluorescence polarization (ex 290 nm; em 335 nm).

agent, which may eventually exhibit therapeutic potential. We observed that carnosine dispersed glycation-induced α CRY aggregates, presumably by directly affecting protein–protein interaction and conformational mobility. These observations using α CRY, which provides the lens with refractive properties [29], corroborates previous work involving a catalytic protein, aspartate aminotransferase [20].

Glycation-induced dysfunctional protein interactions cause aggregation, which increase lens opacity [30–32]. These aggregates contain α CRY [2,33] and promote cataract formation [4,5]. While models of prevention are important, we chose to investigate a corrective mechanism that may be applicable following aggregate formation.

Corroborating many previous studies [10,12,27,28], we observed that MG glycated α CRY. Concentrations of MG used in the current study allowed the reactions to occur in measurable time scales, which under in vivo conditions may require months to years. Glycation affects protein structure [34–37] and promotes protein crosslinking [8] and aggregation [9]. In our model we observed that glycation of α CRY increased light scattering (Fig. 1) and decreased light transmittance (Fig. 2) consistent with protein aggregation. Glycation-induced crosslinking may be occurring only within normal oligomeric complexes and not between oligomers that ultimately form aggregates. This would be presumably due to the closer proximity of the α CRY monomers in the complex.

In human trials carnosine reverses the lens opacity associated with cataracts [19]. The authors of that study suggested that carnosine’s anti-glycation properties are responsible for reversal of lens opacity. We proposed and the current study investigated a novel alternate hypothesis that involves carnosine having disaggregation properties.

We observed that carnosine returned the light scattering of aggregated α CRY to baseline levels, suggesting that the size of the glycation-induced aggregates decreased (Fig. 1). This suggests that carnosine dissociates glycation-induced aggregates but not normal oligomeric complexes presumably by disrupting complementary binding between AGE-related hydrophobic patches on neighboring oligomers [20]. We saw a similar effect of carnosine on aggregated α CRY using light transmittance (Fig. 2) and Alexa Fluor-labeled α CRY (Fig. 3B). In comparing carnosine with commonly used chaotropic agents, such as Gdn-HCl and urea, we found that carnosine was considerably more effective at increasing the conformational mobility of aggregated α CRY as evidenced by carnosine-induced decrease in polarization of AGE fluorophores (Fig. 4) and native tryptophans (Fig. 5). The apparent increase in fluorescence polarization of AGEs with lower concentrations of Gdn-HCl but not with urea (Fig. 4) is presumably due to the ionic nature of Gdn-HCl [38].

The literature contains evidence, which support carnosine as a protective agent. Carnosine protects against glycation-induced loss of enzyme activity [13], prevents neural cell toxicity [17], and protects tissues against ischemic [39] and thermal [40] injury. Carnosine directly reacts with free radicals [16] and oxidized carbohydrates [18], suggesting a logical role as an anti-oxidant and as an anti-glycation agent.

Alternately, the literature also suggests that carnosine may have additional properties. Carnosine reduces lens opacity [19], speeds wound healing [41], and interferes with β -amyloid peptide aggregation [9]. These events represent molecular interventions that occur following chemical insult. The solubilization of aggregated protein may contribute in part to removal of damaged protein, particularly those chemically modified. Carnosine may assist in the unfolding of damaged proteins and in the solubilizing of precipitated protein aggregates. An additional mechanism may involve playing a role in protein de-oligomerization. Elucidation of the diverse possible mechanisms by which damaged protein is removed or made less cytotoxic is crucial to understanding the potential utility of carnosine and carnosine-derivatives as therapeutic agents.

In summary we observed that MG-induced glycation of α CRY caused aggregation. After addition of carnosine, the size of the glycation-induced aggregates decreased. Additionally, carnosine decreased tryptophan fluorescence polarization of glycated α CRY, suggesting that carnosine increased conformational mobility. The aggregation-disaggregation dynamics observed in this study reflect to some degree the lens opacity occurring in cataracts, but we recognize that there are some limitations. The model may not fully represent the complexities of cataract formation, which involves complex mixtures of diverse proteins. Nonetheless, there is evi-

dence to support the significant contribution of protein glycation to aggregation in cataractogenesis [31,32] and amyloidosis [42]. Our findings that carnosine disaggregated glycated α CRY suggest a potential therapeutic role for carnosine.

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