Modulation of α-crystallin chaperone activity in diabetic rat lens by curcumin

P. Anil Kumar, P. Suryanarayana, P. Yadagiri Reddy, G. Bhanuprakash Reddy

National Institute of Nutrition, Hyderabad, India

Purpose: A decline in the chaperone-like activity of eye lens α-crystallin in diabetic conditions has been reported. In this study, we investigated whether curcumin, a dietary antioxidant, can manipulate the chaperone-like activity of α-crystallin in diabetic rat lens.

Methods: A group of rats received ip injection of streptozotocin (STZ; 35 mg/kg body weight in buffer) to induce hyperglycemia, while another group of rats received only buffer as vehicle and served as control. STZ-treated rats were assigned to 3 groups and fed either no curcumin or 0.002% or 0.01% curcumin, respectively. Cataract progression due to hyperglycemia was monitored with a slit lamp biomicroscope. At the end of 8 weeks animals were sacrificed and lenses were collected. αH- and αL-crystallins from a set of pooled lenses in each group were isolated by gel filtration. Chaperone activity, hydrophobicity, and secondary and tertiary structure of αH- and αL-crystallins were assessed by light scattering/spectroscopic methods.

Results: A decrease in chaperone-like activity of αH- and αL-crystallins was observed in STZ-treated diabetic rats. The declined chaperone-like activity due to hyperglycemia was associated with reduced hydrophobicity and altered secondary and tertiary structure of αH- and αL-crystallins. Interestingly, αH- and αL-crystallins isolated from curcumin fed diabetic rat lenses had shown improved chaperone-like activity as compared to αH- and αL-crystallins from untreated diabetic rat lenses. Feeding of curcumin prevented the alterations in hydrophobicity and structural changes due to STZ-induced hyperglycemia. Modulation of functional and structural properties by curcumin was found to be greater with the αL-crystallin than αH-crystallin. Loss of chaperone activity of α-crystallin, particularly αL-crystallin, in diabetic rat lens could be attributed at least partly to increased oxidative stress. Being an antioxidant, curcumin feeding has prevented the loss of α-crystallin chaperone activity and delayed the progression and maturation of diabetic cataract.

Conclusions: We demonstrate that curcumin, at the levels close to dietary consumption, prevented the loss of chaperone-like activity of α-crystallin vis-a-vis cataractogenesis due to diabetes in rat lens.

α-Crystallin, a small heat shock protein (sHSP), constitutes the major portion of eye lens cytoplasm and its concentration in the lens can reach up to 50% of the total protein. Like other sHSP, α-crystallin displays chaperone-like activity in suppressing the aggregation of various proteins and in preventing inactivation of enzymes due to heat and other stress conditions [1-6]. Hence, in addition to providing refractive properties to the lens for focusing the image, it is believed that the molecular chaperone function of α-crystallin is essential in preventing the light scattering due to aggregation of other proteins and thus in the maintenance of lens transparency and thereby prevention of cataract [1-3]. α-Crystallin, especially αB-crystallin, is also present in various non-lenticular tissues, albeit at very low levels [7]. Both in vitro and in vivo studies established the importance of α-crystallin in the biology of the lens and in the physiology of other tissues [2,7-10]. Being a long lived protein with slow turnover, α-crystallin is known to undergo extensive posttranslational modifications (PTMs) including oxidation, mixed disulfide formation, truncation, and glycation during aging [11-13]. Moreover, the chaperone activity of α-crystallin is shown to be influenced/compromised by most of these modifications [1,14-17]. As a corollary to this, it has also been demonstrated that α-crystallin from aged lenses has decreased chaperone activity [18,19].

Chronic hyperglycemia is a major determinant in the development of secondary complications of diabetes, including diabetic cataract. Studies indicate that hyperglycemia and the duration of diabetes increase the risk of development of cataract [20-22]. In view of the widespread prevalence of diabetes in developing countries [23], diabetic cataract may pose a major problem in the management of blindness. Hence, chaperone function of α-crystallin under hyperglycemic conditions is of great concern with respect to lens transparency. Indeed α-crystallin from diabetic rat and human lenses has shown a substantial loss in its chaperone function [24,25]. Furthermore, α-crystallin chaperone activity was also found to be impaired in galactosemic rat lenses [26]. These studies imply that impaired chaperone function of α-crystallin could be involved in the formation of diabetic cataract. Therefore, it is essential to investigate the ways and means by which we can maintain and/or prevent the loss of chaperone potential of α-crystallin under diabetic conditions. We reported earlier that curcumin, the active principle of turmeric and a dietary antioxidant, at very low levels in the diet, delayed cataract in rats induced by either galactose feeding or streptozotocin (STZ) treatment.
We hypothesized that delay of diabetic cataract could have been influenced by chaperone activity of α-crystallin. Therefore, in the present study we have investigated whether curcumin modulates the chaperone activity of α-crystallin in STZ-induced diabetic rat lenses.

**METHODS**

**Materials:** ANS (8-anilinonaphthalene-1-sulphonic acid), citrate synthase, curcumin, glucose-6-phosphate, glucose-6-phosphate dehydrogenase, NADP, insulin, dithiothreitol, and sodium azide were purchased from Sigma Chemical (St. Louis, MO). BCA protein assay kit was procured from Pierce (Rockford, IL). The Sephacryl S-300 HR was from Amersham Biosciences (Uppsala, Sweden).

**Experimental design:** Three-month-old male WNIN rats with an average body weight of 228 g (obtained from the National Center for Laboratory Animal Sciences, National Institute of Nutrition, Hyderabad) were used for this study. All the animals were fed on a semi synthetic AIN-93 diet [29] ad libitum throughout the study. The control rats (Group I; n=6) received 0.1 M citrate buffer pH 4.5 as vehicle, whereas the experimental rats received a single intraperitoneal injection of STZ (35 mg/kg) in the same buffer. After 72 h, fasting blood glucose levels were monitored and animals having blood glucose levels less than 145 mg/dl were excluded from the experiment and the remaining STZ treated animals were distributed into three groups. Animals in these groups received either only AIN-93 diet (Group II; n=6) or received AIN-93 diet containing 0.002% (Group III; n=6) and 0.01% curcumin (Group IV; n=6). Animal care and protocols were in accor-
dance with and approved by the Institutional Animal Ethics Committee. Animals were housed in individual cages in a temperature and humidity controlled room having a 12 h light/dark cycle. All the animals had free access to water. Food intake (daily) and body weights (weekly) were monitored.

**Slit lamp examination:** Eyes were examined every week using a slit lamp biomicroscope (Kowa Company, Ltd., Tokyo, Japan) on dilated pupil. Initiation and progression of lenticular opacity was graded into four stages as described earlier [28].

**Blood/lens collection and processing:** Blood was drawn once a week from the retro orbital plexus for glucose estimation. At the end of 8 weeks, animals were sacrificed by CO₂ asphyxiation and lenses were dissected by posterior approach and stored at -70 °C until further analysis.

**Isolation of α-crystallin:** Lenses from three rats in each group were pooled. Pooled lenses were homogenized in a buffer containing 0.025 M Tris, 0.1 M NaCl, 0.005 M EDTA, and 0.01% NaN₃, pH 8.0 (TNEN buffer) and centrifuged at 10,000x g for 30 min at 4 °C to separate water soluble and

---

**Figure 3.** Chaperone activity of α-crystallin against heat-induced aggregation of βL-crystallin. Chaperone activity of αL- (A) and αH-crystallin (B) as assessed by the suppression of heat-induced aggregation of βL-crystallin. βL-Crystallin (0.2 mg/ml in 50 mM phosphate buffer, pH 7.4) was incubated at 65 °C in the absence (trace 1) or in the presence of either αL- or αH-crystallin (0.025 mg/ml) from Group I (trace 2), Group II (trace 3), Group III (trace 4), and Group IV (trace 5). Data were the average of three chaperone assays.

**Figure 4.** Chaperone activity of αL-crystallin against heat-induced aggregation of CS or DTT induced aggregation of insulin. Chaperone activity of αL-crystallin as assessed by the suppression of heat induced aggregation of CS (A) or DTT induced aggregation of insulin (B). CS (0.05 mg/ml) or insulin (0.40 mg/ml) was incubated in the absence (trace 1) or presence of αL-crystallin from Group I (trace 2), Group II (trace 3), Group III (trace 4), and Group IV (trace 5). The CS and insulin assays used 0.025 and 0.50 mg/ml αL-crystallin, respectively. Data were the average of three chaperone assays.
water insoluble fractions. The water soluble fraction was applied onto a 90 cmx2.5 cm Sephacryl S-300 HR column for separating the crystallins [30]. The column was equilibrated and proteins were eluted with TNEN buffer. Fractions corresponding to αH- and αL-crystallins were pooled separately. The purity of pooled crystallins was assessed by SDS-PAGE and they were dialyzed against water and stored at -20 °C until further use. Protein concentration was determined by the BCA kit method and crosschecked by absorption at 280 nm.

Chaperone activity assays: Chaperone activity of α-crystallin (both αH- and αL-crystallin) was assessed using two different assay systems; aggregation and enzyme inactivation. Aggregation assays were done by measuring the ability of α-crystallin in suppressing either the heat-induced aggregation of βL-crystallin (purified from control rat lenses) at 60 °C, citrate synthase (CS) at 45 °C, or DTT-induced aggregation of insulin at 25 °C. The aggregation of protein due to heat or DTT denaturation in the absence and presence of α-crystallin was performed essentially as described previously [4] by monitoring the absorption at 360 nm as a function of time using a Cary100 spectrophotometer. The potential of αH- and αL-crystallin to prevent the heat inactivation of glucose-6-phosphate dehydrogenase (G6PD) was monitored by measuring the residual activity in the absence and presence of α-crystallin as described earlier [5].

Fluorescence measurements: Fluorescence measurements were performed using a Jasco spectrofluorometer (FP-6500; Tokyo, Japan). For all measurements, 0.15 mg/ml protein in 20 mM sodium phosphate buffer, pH 7.2 was used. Intrinsic tryptophan fluorescence was recorded by exciting at 280 nm and following the emission between 310-390 nm. Fluorescence of 8-anilino-1-naphthalene-sulfonic acid (ANS) bound to α-crystallin was measured by excitation at 390 nm and following the emission between 450 and 600 nm. For this, α-crystallin was incubated with 50 µM ANS for 30 min at room temperature and the fluorescence of protein bound dye was measured. The spectra were corrected with appropriate protein and buffer blanks.

Circular dichroism studies: Far and near UV CD spectra were recorded at room temperature using a Jasco J-810 spectropolarimeter. All spectra were an average of six accumulations and recorded using cells of 0.1 and 0.5 cm path length, respectively, for far and near UV CD. All spectra were corrected for the respective blanks. Protein concentration used for far and near UV was 0.15 and 1.5 mg/ml, respectively.

Figure 5. Chaperone activity of αL-crystallin in enzyme inactivation assays. Protection of heat-induced inactivation of glucose-6-phosphate dehydrogenase at 42 °C by αL-crystallin. Bar 1 is G6PD alone, bars 2-5, are G6PD plus αL-crystallin from Groups I-IV, respectively. Data were the mean of three chaperone assays.

Figure 6. Hydrophobicity of α-crystallin. Hydrophobicity of αL- (A) and αH-crystallin (B) as assessed by ANS fluorescence. Traces 1-4 correspond to αL- or αH-crystallin from Groups I-IV, respectively. Data were the average of three assays.
RESULTS

Food intake and body weights: There was an increase in the food intake in all the diabetic groups (II-IV) compared to the control group (Group I, Figure 1A). Despite the increased food intake, the body weight of Group II animals was decreased (194 g), when compared to the controls (385 g). However, the decrease in body weight due to hyperglycemia was not ameliorated by treatment with curcumin (Figure 1B).

Onset and progression of cataract: There was a delay in progression of hyperglycemia-induced cataract due to treatment with curcumin. At the end of 8 weeks, while most of the lenses (65%) in Group II developed mature cataract, the percent of mature cataract lenses were decreased to 43 and 33% in Group III and Group IV, respectively. The data thus suggest that curcumin delayed maturation of diabetic cataract.

Gel filtration profile of α-crystallins: There was a marked difference in the relative distribution of α-crystallins between the groups, the αH-crystallin peak was elevated and the αL-crystallin was decreased in Group II rat lens compared to Group I rat lens (Figure 2). Feeding curcumin (Group III and IV) reverted the altered distribution of profile αH- and αL-crystallins in a dose dependent manner (Figure 2).

Chaperone activity of αH- and αL-crystallins: As shown in Figure 3A, αL-crystallin from Group II rat lens showed a 50% decrease in chaperone activity in suppressing the heat-induced aggregation of βL-crystallin when compared to the activity of αL-crystallin from Group I. Interestingly, the chaperone-like activity of αL-crystallin from Group III and IV was higher than Group II rat lenses, though not identical to the Group I rat lens (Figure 3A). Strikingly, aggregation kinetics of βL-crystallin displayed longer lag time in the presence of αL-crystallin from Group III and IV compared to αL-crystallin from Group II (Figure 3A). Usually αH-crystallin has been treated as a modified version of αL-crystallin due to various insults. Therefore, we have also assessed the ability of αH-crystallin to suppress the aggregation of other proteins. As expected, the αH-crystallin chaperone potential was lower than that of αL-crystallin in Group I (Figure 3). Furthermore, αH-crystallin from Group II rat lens showed decreased protection against heat-induced aggregation of βL-crystallin as compared to that of Group I (Figure 3A). Moreover, αH-crystallin from Group III and IV showed no improvement in its chaperone ability over Group II (Figure 3B). Likewise, similar results were observed with the CS and insulin aggregation assay for αL-crystallin (Figure 4). We have also assessed the ability of αH- and αL-crystallins from control, untreated and curcumin treated diabetic rat lenses to protect heat-induced inactivation of G6PD. Similar to aggregation assays, the ability of αL-crystallin from Group II to prevent heat-induced inactivation of G6PD was declined as compared to Group I (Figure 5). Furthermore, in contrast to the partial protection in aggregation assays, αL-crystallin from Group III and IV rat lens exhibited a remarkable protection against G6PD inactivation (Figure 5). However, αH-crystallin from either untreated or curcumin treated rats showed no significant protection of G6PD when compared to αH-crystallin from control rats (data not shown).

Figure 7. Secondary structure of αL-crystallin. Secondary structure of αL-crystallin from different groups was assessed by far UV CD spectroscopy using 0.10 mg/ml protein in 20 mM sodium phosphate buffer (pH 7.2).

Figure 8. Intrinsic tryptophan fluorescence spectra of αL-crystallin. Traces 1-4 correspond to αL-crystallin from Groups I-IV, respectively.
Surface hydrophobicity: Numerous studies indicated that surface-exposed hydrophobic sites on α-crystallin could be playing a critical role in chaperone activity [4,31-34]. In the present study we have investigated surface hydrophobicity as a function of binding of a hydrophobic probe (ANS) to α-crystallin. α-L-crystallin from Group II showed lesser ANS binding when compared with αL-crystallin from Group I (Figure 6A), which correlated well with the decreased chaperone activity of αL-crystallin from Group II. Furthermore, improved chaperone activity of αL-crystallin from Group III and IV is also reflected in increased ANS binding when compared to that of αL-crystallin from Group II (Figure 6A). However, the relative ANS binding for αH-crystallin from all groups is not only lower as compared to αL-crystallin from respective groups, but there was a marginal improvement in ANS binding due to curcumin treatment (Figure 6B).

Structural alterations: To understand further the mechanism for the altered chaperone-like function of α-crystallin in diabetic rat lens and its modulation by curcumin, we have monitored secondary and tertiary structural states of αL-crystallin by CD and fluorescence spectroscopy. As shown in Figure 7, α-crystallin from Group I has a maximum negative ellipticity around 217 nm, typical of β-sheet structure as reported previously by many investigators [2,16,32]. However, the far UV CD signal for α-crystallin isolated from diabetic rat lens decreased, indicating altered secondary structure and the results are consistent with findings of altered secondary structure of α-crystallin of the galactosemic rat lens [26]. A loss of intensity in tryptophan fluorescence indicated altered tertiary structure of α-crystallin due to hyperglycemia (Figure 8). Furthermore, changes in near UV CD spectra of αL-crystallin from Group II, particularly in the aromatic region (not shown), also suggest conformational changes at the tertiary structural level due to hyperglycemia. Although curcumin treatment at both the levels has not affected the altered secondary structure due to hyperglycemia in a significant way (Figure 7), curcumin mediated modulation to altered tertiary structural changes were quite noticeable (Figure 8).

DISCUSSION
Diabetes is a known risk factor for cataract formation. In view of the prevailing and predicted outbreak of diabetes in developing countries like India [23,35], diabetic cataract may become a leading cause of blindness, along with senile cataract. α-Crystallin is believed to be the key structural and functional element for maintaining the transparency of the lens. Moreover, it is known that the chaperone activity of α-crystallin is compromised in various types of cataract, including diabetic cataract. Further, various modifications, which are accelerated in diabetes, are shown to decrease the molecular chaperone activity of α-crystallin. On the other hand, a number of studies reported that agents like antioxidants, aldose reductase inhibitors, and antilysing agents slow down the progression of diabetic complications, including cataract [36-39]. Earlier we demonstrated that feeding of curcumin delayed the progression of galactose-induced and STZ-induced cataracts in rats [27,28]. However, it is not known whether the prevention or delay of diabetic cataract by these agents is mediated through the modulation of α-crystallin chaperone-like activity.

In the present study we demonstrated that in STZ-induced diabetic cataract, α-crystallin exhibited diminished chaperone activity, which was prevented, at least partially, by dietary curcumin. The appearance of αH-crystallin fraction upon preparative gel filtration in Group I suggests age related changes to α-crystallin, since these animals were more than 5 months old at the end of experiment. An increased αH-crystallin fraction in Group II rat lens indicate that these changes are accelerated under diabetic conditions. Further, the αH-crystallin fraction of Group I differs from Group II with regard to structure and function. Moreover, the improvement in structural and functional properties of αH-crystallin due to curcumin feeding is not as significant with that of αL-crystallin.

Previous studies have shown that some small molecules can modulate α-crystallin chaperone activity in vitro by affecting either the crystallin or aggregating substrate [40-42]. Therefore, we investigated whether curcumin influences α-crystallin chaperone activity by direct interaction with the protein. However, in vitro studies indicate that curcumin did not affect the chaperone activity of α-crystallin directly (not shown) and suggest an indirect modulation in vivo. Recently, it was shown that curcumin inhibits formation of amyloidβ oligomers and fibrils and its antioxidant potential was thought to be responsible for this effect [43]. We have shown that oxidative stress may be a predominant mechanism in STZ-induced hyperglycemia in rats as there was increased lipid peroxidation and protein carbonyl content, decreased glutathione, and altered activities of antioxidant enzymes leading to cataract formation [28]. Moreover, the antioxidant effect of curcumin was attributed for the delayed progression of STZ-induced cataract in rats [28]. Further, food and vitamin restriction was also shown to influence the chaperone like activity of α-crystallin and aggregation of other crystallin in rats [30]. Thus, one of the possible explanations for the modulatory effect of curcumin on α-crystallin chaperone activity in diabetes could be decreased oxidative stress by curcumin in hyperglycemia. Earlier reports on disrupted α-crystallin chaperone activity due to oxidative stress [1,44] lend support to our explanation.

ACKNOWLEDGEMENTS
This work was supported by grants from the Department of Science & Technology, and Life Sciences Research Board, DRDO. PAK acknowledges Council of Scientific and Industrial Research for the research fellowship.

REFERENCES
29. Reeves PG. Components of the AIN-93 diets as improvements in the AIN-76A diet. J Nutr 1997; 127:838S-841S.