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Functional and Structural Studies of α-Crystallin from Galactosemic Rat Lenses

Fu-Yung Huang,[1] Yuh Ho, Tien-Shun Shaw, and Sheng-An Chuang
Department of Chemistry, National Cheng Kung University, Tainan, 70101, Taiwan

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Chaperone-like activity and structural changes of lens α-crystallin from rats fed with galactose at various time intervals have been studied using high-performance liquid chromatograph (HPLC), circular dichroism (CD), and 1-anilinonaphthalene-8-sulfonic acid (ANS) fluorescence emission. It was found that chaperone-like activity of α-crystallin from galactose-fed rats toward dithiothreitol (DTT)-induced insulin B aggregation started to decrease after 3 weeks and decreased significantly after 5 weeks. Consistent results were observed in lens morphology, and lens opacity slightly developed after 3 weeks and became obvious after 5 weeks. HPLC analysis for chaperone function showed that the formation of high molecular weight aggregates (HMWA) of α-/γ-crystallins decreases with the increase of galactose-feeding time, revealing that chaperone-like activity is concomitant with the formation of HMWA. Circular dichroism results showed the reduction of β-sheet structure and loss of microenvironment of aromatic-type amino acids for opaque lenses, indicating α-crystallin's secondary and tertiary structure changed with the development of the lens opacity. ANS binding site estimated by Klotz equation showed it is 1.5 times higher at room temperature and is 2.4 times higher at 58°C for age-matched normal α-crystallin than for 5-week galactose-fed lens α-crystallin, indicating opaque lens α-crystallin loses the ability to assemble into an appropriately placed hydrophobic regions. The overall results accordingly indicated that galactose-induced cataractous α-crystallin has disordered structure, leading to the loss of its chaperone-like activity.

Mammalian lens proteins consist of α-, β-, and γ-crystallins (1). α-Crystallin, a major abundant lens protein and existing in a form of oligomeric aggregate with a molecular weight of 800 kDa, is made up of two closely related polypeptides of αA- and αB-crystallins, each with a molecular weight of about 20 kDa (2, 3). α-Crystallin, especially for αB-crystallin, previously known as lens structure protein, is now identified in other tissues of human body, suggesting it possesses an alternative role (4, 5). Recently, α-crystallin has been demonstrated as a functionally important molecular chaperone against thermal denaturation, ultraviolet-induced aggregation, and oxidative stress (6–9). Despite that there is no pertinent evidence to ascertain α-crystallin's chaperone mechanism, some reports indirectly implicated that appropriately placed hydrophobic path of α-crystallin plays an indispensable and fundamental role (10, 11).

During aging and progression of diabetes, it was found that lens crystallins underwent extensive post-translational modifications, such as oxidation and glycation (12, 13), resulting in the damage of crystallin structure. α-Crystallins isolated from normal aged human lenses, cataractous human lenses, and diabetic rat lenses lost chaperone-like activity (14–16) and also *in vitro* modifications of α-crystallin resulted in the loss of chaperone-like activity (16). Oxidation-induced structural alterations in rat lens α-crystallin and its effect on α-crystallin's chaperone-like activity had been reported in our previous study (17). Galactosemic rats, which are frequently used for induction of lens cataract (18), have been found to induce dramatic imbalances in the hexose metabolism (19), the redox levels (20), and the electrolyte transportation (21), which, in turn, may result in the increase of oxidative susceptibility of lens crystallins. Since α-crystallin from galactosemic rat lens may have suffered oxidative modifications, its chaperone activity is likely to be compromised. It is thus interesting to study the relationship between the chaperone function of α-crystallin and its structural changes during galactosemic process. In this study, α-crystallin from galactose-fed rats at different time intervals were isolated and were assayed its chaperone-like activity against dithiothreitol (DTT) induced insulin B aggregation. The ability for galactosemic rat lens α-crystallin to form a high molecular weight complex with normal γ-crystallin under ther-

[1] To whom correspondence should be addressed. Fax: (886)6-274-0552. E-mail: fhuang@mail.ncku.edu.tw.

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mal insult was further analyzed by HPLC. Circular dichroism (CD) spectroscopy and fluorescence spectroscopy were employed to investigate the structural changes of α-crystallin during galactosemic experiment.

MATERIALS AND METHODS

Isolation of crystallins. Female Sprague–Dawley rats at the age of 3–4 weeks old were fed on a diet containing 50% galactose for 5 weeks. A control group was fed with normal diet. Two rats were sacrificed by asphyxiation in a dry ice chamber every week. The 4 lenses were homogenized in 50 mM Tris buffer (pH 7.4) containing 0.1% NaN_3 and then centrifuged at 17,000*g* for 60 min to separate the water-soluble and water-insoluble portions. The supernatant was applied to a Sephacryl S-300 column (600 × 26mm) for gel permeation. The isolated crystallins were pooled and dialyzed against water (4 × 1500 ml) at 4°C for 24 h, and stored at −70°C after lyophilization. Low molecular weight α-crystallin (α_L-crystallin) from galactose-fed rat lens and γ-crystallin from normal rat lens, a native mixture of γ_I–γ_{IV}, were used in this study. Before α-crystallin was used for chaperone-like activity assay, the purity of this concentrated sample solution was checked by isoelectronic focusing (IEF) gel electrophoresis. Protein concentration was estimated by the Bradford dye-binding assay (22).

Chaperone-like activity assay toward DTT-induced aggregation of insulin B chain. Chaperone-like activity of α-crystallins isolated form various galactosemic stages was assayed by measuring the light scattering towards DTT-induced aggregation of insulin B chain at room temperature (23). The incubation mixture contained 250 μl of insulin B (0.8 mg/ml) in the absence or presence of 500 μl of α-crystallin (1.2 mg/ml). The aggregation of insulin B chain, a control experiment, was initiated by adding a freshly prepared 50 μl of DTT (320 mM) to a 1 cm path length cuvette containing insulin B chain and the mixed solution was measured the light scattering at 360 nm by using JASCO V-550 spectrophotometer. The relative chaperone-like ability was expressed in percentage calculated by normalizing light scattering difference between the scatterings with and without the chaperone of α-crystallin to the scattering of insulin control.

HPLC analysis of α-/γ-crystallin complex. The water soluble fraction of the mixture of galactosemic lens α-crystallin (0.1 mg/ml) and native lens γ-crystallin (0.4 mg/ml), obtained after thermal denaturation (65°C for 30 min) followed by centrifugation (9000 rpm for 30 min), was analyzed by using Shimadzu LC-8A HPLC system equipped with a SPD-10 A detector and a TSK G4000 SW 7.5 × 300 mm gel filtration column. All analyses were performed at ambient temperature and eluted with 50 mM Tris-HCl buffer (pH 6.8) at a flow rate of 1 ml/min. Protein elution was monitored at 280 nm.

Measurement of CD spectra. Circular dichroism (CD) spectra of α-crystallin were obtained by following our previous report using a JASCO J-720 spectroscopy (24). The CD spectra were the average of 3 scans. Far- and near-ultraviolet (UV) CD spectra of α-crystallin were recorded at wavelengths of 200–240 nm and 250–340 nm, respectively. A cell path length of 0.1 cm and protein concentration of 0.8 mg/ml were used for far-UV CD measurement, while a cell path length of 1 cm and a protein concentration of 1 mg/ml were used for near-UV CD measurement. Spectra were expressed in terms of molar ellipticity (deg · cm^2)$dmol^{-1}$. All spectra were corrected for the baseline obtained with the buffer.

Fluorescence measurement of ANS binding to α-crystallin 1-Anilinonaphthalene-8-sulfonic acid (ANS) fluorescence was measured with a HITACHI F-2000 spectrofluorometer. Stock solutions of ANS (2×10^{-4} M) and α-crystallin (1 mg/ml) were prepared. Aliquots of ANS solution (50–250 μl) were added to 1 ml of α-crystallin solution (0.06–0.3 mg/ml). An excitation wavelength of 380 nm was used, and the fluorescence emission spectrum in the region from 400 nm to 600 nm was recorded after sample mixture was incubated for 30 minutes to stabilize the fluorescence. The ANS binding site was analyzed according to Klotz equation (25, 26), i.e., $P/xD = 1/n + k_d/nD(1 - x)$, where P and D are the protein and dye (ANS) concentrations, respectively; x is the fraction of dye (ANS) bound to α-crystallin. The number of ANS binding sites, n, and dissociation constant, k_d, were calculated from the plot of P/xD vs $1/D(1 - x)$. For analyzing temperature-induced changes of ANS fluorescence, α-crystallin/ANS solution was incubated at 58°C for 30 min, then cooled down to room temperature.

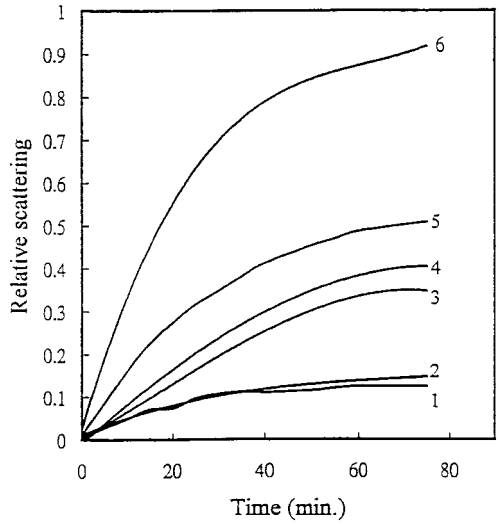

FIG. 1. Protection of DTT-induced insulin B aggregation with or without the presence of α-crystallin obtained from various galactosemic stages. Curves 1–6 represent α-crystallins from 1-, 2-, 3-, 4-, and 5-week galactosemic rat lenses, respectively. Curve 6 is as a reference, without the protection of α-crystallin. The assay was carried out at room temperature.

RESULTS AND DISCUSSION

Chaperone-like Activity of α-Crystallin from Various Galactose-Fed Times

It was found that the lens morphology of galactose-fed rat started to develop opaque after three weeks and became dense opaque after 5 weeks. The α-crystallins obtained from these galactosemic rat lenses were assayed for the chaperone-like activity towards DTT induced insulin B aggregation at room temperature (Fig. 1). Upon reduction of the disulfide bond by DTT without the addition of α-crystallin, insulin B chain aggregated and formed water-insoluble particle as illustrated by the increasing scattering (curve 6). By the addition of α-crystallins obtained from 1- or 2-week galactose-fed rat lenses, the development of turbidity was suppressed (curves 1, 2). When α-crystallins from 3-, 4- and 5-week galactose-fed rat lenses were added to the insulin solutions, the decreased chaperone-like activity was more obvious as demonstrating by increased turbidity in curves 3, 4, and 5. These results are in consistent with the development of lens cataract ob-

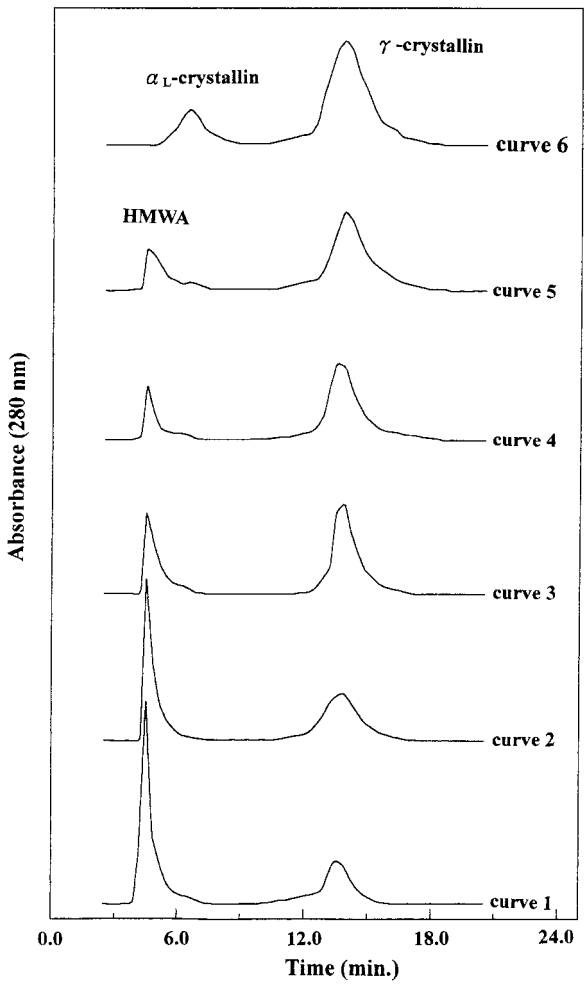

FIG. 2. HPLC gel filtration profiles of various α-/γ-crystallin mixtures after being thermally incubated. Curves 1–5 show chromatograms of α-crystallins from 1-, 2-, 3-, 4-, and 5-week galactose-fed rat lens mixed with normal γ-crystallin, respectively. Curve 6 shows gel filtration profile of normal α-crystallin and γ-crystallin without thermal denaturation and as a reference.

served during the 5-week galactosemic periods, a direct evidence showing that the loss of α-crystallin chaperone function results in the formation of cataract.

HPLC Analysis of Thermally Denatured α-/γ-Crystallin Mixture

In order to see whether the decrease of the chaperone-like activity towards DDT-induced insulin B aggregation (as shown in Fig. 1) of α-crystallin from galactose-fed lenses at various time intervals results in the decrease of high molecular weight aggregates (HMWA) formation, the mixtures of α-crystallin from galactose-fed lenses and normal γ-crystallin were subjected to HPLC analysis after been thermally denatured. Figure 2 presents the HPLC analysis profiles of various α-/γ-crystallin mixtures after thermal denaturation. Curves 1, 2, 3, 4, and 5 are the chromatograms of α-crystallin from 1-, 2-, 3-, 4-, and 5-week galactose-fed rat lenses mixed with normal γ-crystallin, respectively. Curve 6 is the gel filtration profile of the mixture of normal α-/γ-crystallins without thermal denaturation, no HMWA peak being observed, and is as a reference. There is an unambiguous formation of HMWA and the amount of which gradually decreases while the amount of γ-crystallin gradually increases as shown from curve 1 to 5. These results clearly indicate that the HMWA consists of a α-/γ-crystallin complex and the ability to form this HWMA decreases with the increase of galactose-feeding time. It has been reported that thermally denatured α-crystallin is capable of self-aggregating to form a higher molecular weight aggregate (27). In the preparation of α-/γ-crystallin mixtures for HPLC analysis there was observable light scattering turbidity for the thermally incubated mixtures of α-crystallins from 3-, 4-, and 5-week galactose-fed rat lenses with normal γ-crystallin, whereas no light scattering turbidity was observed for the thermally incubated mixtures of α-crystallins from 1- and 2-week galactose-fed rat lenses with normal γ-crystallin. Curves 3, 4, and 5 show detectable amount of α-crystallin. Thus, HMWA formed in curves 3, 4, and 5 consists mostly of self-aggregated α-crystallins than α-/γ-crystallin complex. And the decrease of the amount of HMWA is mainly resulted from the reduction of α-crystallin's chaperone-like activity. This in turn shows that chaperone function is a factor leading to the formation of α-/γ-crystallin complex. Accordingly, the results from Figs. 1 and 2 are consistent and indicate that α-crystallins obtained from 1- and 2-week galactose-fed lenses, clear lenses, still have the ability to complex with normal γ-crystallin to form HMWA as to prevent them from precipitation under thermal insult, whereas α-crystallins obtained from 3-, 4-, and 5-week galactose-fed lenses, opaque lenses, failed to do so.

Secondary and Tertiary Structural Characterizations of Cataractous α-Crystallins

Figure 1 and 2 show α-crystallins from 3-, 4-, and 5-week galactose-fed rat lenses lose their chaperone-like activity. It is thus interesting to characterize their structures. Figure 3 shows the near-UV CD spectra of α-crystallin isolated from galactose-fed rat lens. The spectra of α-crystallin from 1- and 2-week galactose-fed rat lenses (curves 1 and 2), which is very similar to that of normal α-crystallin (not shown), show a transition, derived from tryptophan, at around 290 nm and four positive bands from aromatic side chains centered at 254, 262, 270, and 287 nm. These characteristic features are resulted from the interactions between the amino acid residues of tyrosine, tryptophan, and phenylalanine, especially the μ–μ interaction of tyrosine-tryptophan coupling (28). The CD spectra of curves 3

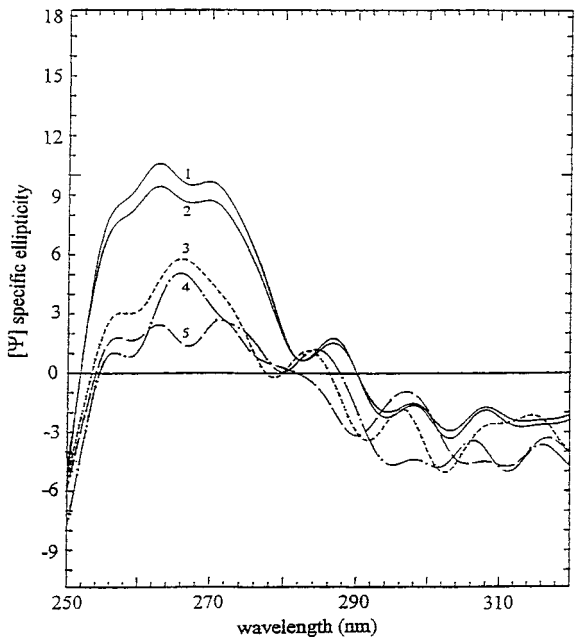

FIG. 3. The near-UV CD spectra of α-crystallins from various galactosemic stages. Curves 1–5 show the spectra of α-crystallins from 1-, 2-, 3-, 4-, and 5-week galactosemic rat lenses, respectively.

and 4 of Fig. 3 indicate that the vibronic vicinal interactions of tyrosine and tryptophan residues gradually decrease, characterized by the decreased CD intensity and by the blue-shift of a positive peak due to tryptophan residue from 287 nm to 284 nm. The near-UV CD spectrum of α-crystallin from 5-week galactose-fed rat lenses, curve 5, shows three bands similar to those observed for α-crystallin from 1- and 2-week galactose-fed rat lenses; however, the transition at 290 nm was blue-shifted to 281 nm, and the nearby positive peak due to tryptophan residue was diminished. The decrease of CD intensity is obvious with the increase of galactose feeding time. Chakrabarti *et al.* (29) and Harding *et al.* (30) reported that sugar induced the increase of near-UV CD intensity of bovine lens α-crystallin, which is totally opposite as we observed in this study; i.e., galactosemic rat lens α-crystallin showed decrease intensity. In their studies, the α-crystallin had been modified with glucose-6-phosphate (G6P) and suggested the increase of CD band intensity is due to the interactions of glycosylated G6P with protein, whereas there was no glycated α-crystallin detected in this study. Our study reveals that α-crystallins from galactose-fed rat lenses gradually lose its tertiary structure as to lose the noncovalent μ–μ interaction between aromatic side chains, then resulted in the decrease of CD intensity. The far-UV CD region provides information about secondary structure of protein (31). Figure 4 shows the far-UV CD spectra of α-crystallin obtained from various galactosemic stages. The negative band within the spectrum is centered at 219 nm, suggesting that β-sheet is a major secondary structure of α-crystallin. As the feeding of galactose proceeded, the CD intensity decreased and the band of 219 nm was shifted for rats fed with galactose more than three weeks. The trend of the changed CD pattern is similar to the results obtained from the titration of guanidine hydrochloride to α-crystallin (not shown), which indicates that the secondary structure of α-crystallin had gradually lost during the development of cataract. Interestingly, Chakrabarti *et al.* (29) and Harding *et al.* (30) found no gross alterations of secondary structure for glycosylated bovine lens α-crystallin and dissociation study of native and glycated bovine lens α-crystallin by Facchiano *et al.* (32) showed gycation acts on protein by altering its charge distribution, all of which indicating *in vitro* sugar modified α-crystallin results in the protein conformational changes. Chiou *et al.* reported that glycosylation might not be responsible for the sugar-induced cataract (33). Accordingly, α-crystallin from *in vivo* galactosemic study shows different structural alterations from that of *in vitro* glycated α-crystallin and results in both the secondary and tertiary structural changes leading to the formation of cataract.

Indirectly Probing the Quaternary Structure of Cataractous α-Crystallins

ANS, essentially nonfluorescent in aqueous solution and becoming fluorescent when bound to the hydrophobic area of macromolecule, has been widely employed to probe α-crystallin's quaternary structure (26, 34, 35). Figure 5a shows ANS fluorescence spectra for being in complex with α-crystallin or being in buffer

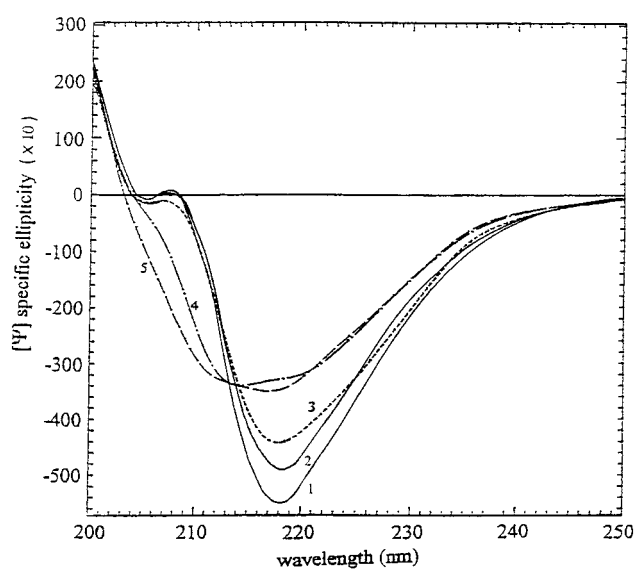

FIG. 4. The far-UV CD spectra of α-crystallins from various galactosemic stages. Curves 1–5 show the spectra of α-crystallins from 1-, 2-, 3-, 4-, and 5-week galactosemic rat lenses, respectively.

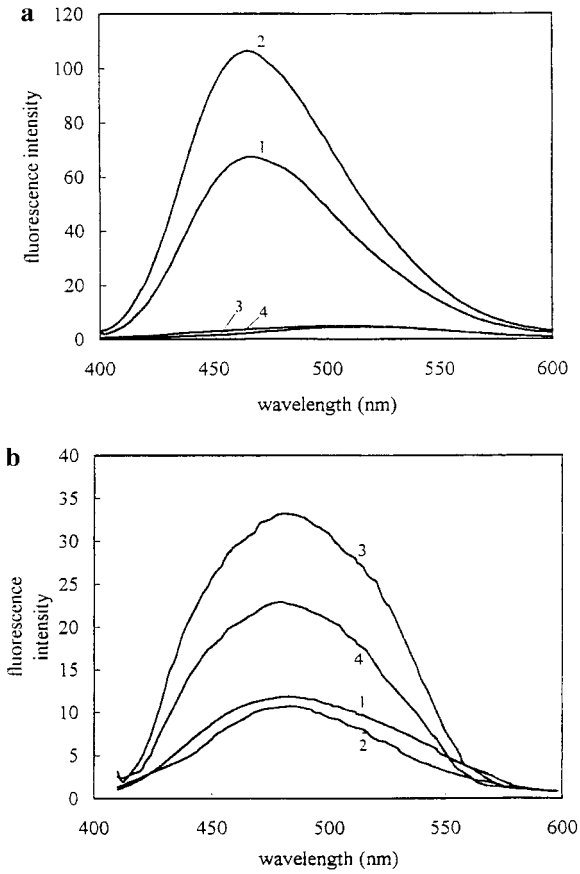

FIG. 5. ANS fluorescence spectra. (a) 1, ANS in α-crystallin; 2, ANS in α-crystallin preincubated at 58°C; 3, α-crystallin alone as a control; 4, ANS alone as a control. (b) 1, ANS in normal α-crystallin; 2, ANS in α-crystallin from 5-week galactose-fed rat lenses; 3, ANS in normal α-crystallin preincubated at 58°C; 4, ANS in cataractous α-crystallin preincubated at 58°C.

solution alone. ANS displays little fluorescence in buffer solution alone (curve 4). With the addition of α-crystallin, ANS fluorescence intensity increased significantly and the maximum wavelength of fluorescence emission was blue-shifted from 523 nm to 463 nm (curve 1). When solution of α-crystallin/ANS was heated to 58°C, then cooled down to room temperature, ANS fluorescence intensity increased compared to that of ANS with α-crystallin without being heated up to 58°C (curve 2). This result indicates that more ANS binds to α-crystallin as the temperature increases, suggesting a greater exposure of hydrophobic regions. Figure 5b shows fluorescence spectra of ANS in normal α-crystallin and in α-crystallin obtained from 5-week galactose-induced lenses, dense opaque lenses. With the same concentration, ANS fluorescence intensities observed in normal α-crystallin and in cataractous α-crystallin show no difference (curves 1 and 2), indicating that α-crystallin from cataractous lens does not show significant difference in exposed hydrophobic region when compared with normal α-crystallin. After the temperature was lifted up to 58°C, then cooled down to room temperature, fluorescence of ANS in normal α-crystallin was obviously greater than that in cataractous α-crystallin (curves 3 and 4, respectively). To obtain further information about the surface hydrophobicity of α-crystallin, we analyzed ANS binding sites by using the Klotz equation (25, 26), which indicated that the value of n and k_d obtained at 58°C are larger than that obtained at room temperature for normal and cataractous α-crystallins (Table 1). The ANS binding sites for normal α-crystallin show an increase of 2.2 times ($P < 0.01$), whereas it is only a 42% increase ($P > 0.1$) for 5-week galactosemic α-crystallin, upon heat treatment at 58°C. It was found that the n value obtained at room temperature is 55% higher for normal α-crystallin than for 5-week galactosemic α-crystallin, which is statistically significant ($P < 0.05$). However, the dissociation constant of normal α-crystallin is 17% higher than cataractous α-crystallin, which is not statistically significant ($P > 0.5$). These results indicate that the ANS binding site is statically much less significant for 5-week galactosemic α-crystallin than for normal α-crystallin when being thermally activated at 58°C. Sharma *et al.* reported that *in vitro* glycation of bovine α-crystallin with ascorbate for 4 weeks displayed a 25% decrease in ANS fluorescence at ambient temperature and a decrease in chaperone-like activity and suggested the involvement of glycation site as well as ANS binding site in chaperone-like activity display (36). In this study, we found that 5-week galactosemic rat lens α-crystallin showed 35% decrease in ANS binding sites and almost totally loss of activity and no glycated α-crystallin was detected, thus our results suggest that glycation is only a factor leading to the loss of ANS binding site and glycation site may not necessarily involves in chaperone-like activity. We also found that after being thermally activated, ANS binding site is about 2.4 times higher for normal lens α-crystallin than for cataractous lens α-crystallin, implicating normal and cataractous α-crystallin subunits show different assembling behavior. Since ANS-detectable hydro-

TABLE 1

The Number of ANS Binding Sites (n) and Dissociation Constant (k_d) of Rat Lens α-Crystallin

	Normal*		Galactose-fed†	
	R.T.	58°C	R.T.	58°C
$n \times 10^2$ (mmol/g)	1.88 ± 0.54	4.17 ± 1.10	1.21 ± 0.32	1.72 ± 0.41
$k_d \times 10^3$ (mM)	1.87 ± 0.56	4.30 ± 1.90	1.59 ± 0.72	2.30 ± 1.16

* and † are mean value ± SD of five and three measurements, respectively.

phobicity in α-crystallin has been related to its chaperone-like activity and chaperone-like activity assay is performed at higher temperature (usually >55°C), thus, ANS fluorescence study indicates normal α-crystallin subunits are capable of assembling into a proper quaternary structure to function as a molecular chaperone, whereas galactosemic cataractous α-crystallin subunits are not. Thus, it is the secondary, tertiary and quaternary structural changes that lead to the loss of chaperone-like activity as to cause the formation of cataract in galactosemic experiment.

In summary, in this study, the onset of galactose-induced cataract was observed after being fed with 50% galactose for 3 weeks and the results of chaperone activity assay and of CD spectra were found concurrently change with the development of cataract. Thus, it is likely that α-crystallin was modified through oxidation to lose its ordered structure leading to the decrease of chaperone-like activity. Our results here provide not only a pertinent evidence to illustrate the decline of α-crystallin's chaperone-like activity during the development of galactose-induced cataract by the characterization of secondary, tertiary, and quaternary structure, but also show a plausible explanation for the reduction of α-crystallin's chaperone-like activity.

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