Differential recognition of natural and nonnatural substrate by molecular chaperone α-crystallin—A subunit exchange study

ARTICLE *in* BIOPOLYMERS · FEBRUARY 2007

Impact Factor: 2.39 · DOI: 10.1002/bip.20630 · Source: PubMed

2 AUTHORS:

Ashis Biswas
Indian Institute of Technology Bhubaneswar
35 PUBLICATIONS **430** CITATIONS
SEE PROFILE

Kali P Das
Bose Institute
100 PUBLICATIONS **1,883** CITATIONS
SEE PROFILE

Differential Recognition of Natural and Nonnatural Substrate by Molecular Chaperone α-Crystallin—A Subunit Exchange Study

Ashis Biswas,* K. P. Das
Protein Chemistry Laboratory, Department of Chemistry, Bose Institute, 93/1 A. P. C. Road, Kolkata 700 009, India

Received 8 August 2006; accepted 7 November 2006
Published online 13 November 2006 in Wiley InterScience (www.interscience.wiley.com). DOI 10.1002/bip.20630

ABSTRACT:

α-Crystallin is a molecular chaperone that recognizes proteins substrates in stress. It binds to the unstable conformer of a large variety of related or unrelated substrates and thus prevents them aggregating and holds them in a folding competent state. In this article, we have tried to critically analyze, from experimental point of view, whether α-crystallin has any preference for its natural substrates compared to the nonnatural one. Our results clearly show that α-crystallin is exceptionally active and sensitive in preventing aggregation of its natural substrates and can fully prevent such an aggregation in a substoichiometric ratio, but nonnatural substrates require a considerably higher amount of α-crystallin. Using suitable fluorescent-labeled α-crystallins and performing fluorescence resonance energy transfer experiments, we were able to determine the subunit exchange kinetics between the α-crystallin oligomers. It was found that while α-crystallin was bound to its natural substrate, the rate of subunit exchange was slightly decreased. But, when a nonnatural substrate carbonic anhydrase remained bound to the chaperone, further loss in subunit exchange rate was observed. Nonnatural substrate was found to create higher activation energy barrier for the subunit exchange reaction compared to the native substrates. Similarities in major β-sheet structure of both α-crystallin and its natural substrates may be the reason for the preference in molecular recognition in comparison with the nonnatural substrate. © *2006 Wiley Periodicals, Inc. Biopolymers 85:189–197, 2007.*

Keywords: *molecular chaperone; protein–protein recognition; chaperone; substrate recognition*

This article was originally published online as an accepted preprint. The "Published Online" date corresponds to the preprint version. You can request a copy of the preprint by emailing the Biopolymers editorial office at biopolymers@wiley.com

Correspondence to: K. P. Das; e-mail: kalipada@bosemain.boseinst.ac.in
*Present address: Department of Ophthalmology, Case Western Reserve University, Cleveland, OH-44106.
Contract grant sponsor: Council of Scientific and Industrial Research, India
Contract grant number: 37(1218)/05/EMR-II

INTRODUCTION

α-Crystallin is the major protein of the eye lens of the vertebrates. It consists of two highly homologous chains αA- and αB-crystallin, 20 kDa each. The chains associate to form a large oligomers of 30–40 subunits.[1,2] The proteins have also been found in some nonlenticular tissues[3] and have been known to be associated with some neurodegenarative disorders.[4,5] This protein is a key member of small heat shock protein family.[6] High resolution structure of this protein is lacking since neither the oligomer nor its subunits could be crystallized. An important step towards unraveling the function of α-crystallin was the discovery that this protein acts as a molecular chaperone by preventing the aggregation of substrate proteins under stress conditions.[7] This function is now believed to be crucial for the maintenance of long-term transparency of the eye lens, which is a dense mass of proteins having very little protein turnover.[1,2,6–8]

α-Crystallin prevents the aggregation of a large variety of substrates, e.g., insulin, α-lactalbumin, lysozyme, conalbumin, alcohol dehydrogenase, citrate synthase, xylose reductase, etc.[9–19] These substrates include proteins, which are of low molecular weight [e.g., insulin (6 kDa)], comparable to α-crystallin subunit molecular weight [e.g., carbonic anhydrase (29 kDa)] and high molecular weight [e.g., alcohol dehydragenase (150 kDa)]. Besides, it recognizes substrates under various stress conditions, such as heat, disulphide cleavage, UV light exposure, oxidative stress, etc.[9–16,18–21] These in vitro substrates belong to no particular category in terms of sequence or three-dimensional structure. In addition to these "nonnatural" substrates, α-crystallin prevents the aggregation of a number of its own substrates, which are present in the lens. These natural substrates include various β-crystallins, γ-crystallins, and aldose reductase. The aggregation of these natural substrates is also prevented by α-crystallin during various conditions such as thermal stress and UV-light-induced stress.[7,22,23]

This promiscuity raises questions as to how and what key features in a protein molecule it searches for recognition. However, it is generally believed that chaperones get activated when new hydrophobic sites of the substrates tend to get exposed and that hydrophobic interaction plays a major role in chaperone function; but it is not known whether such interaction is fully nonspecific. Although many of the in vitro chaperone-substrate interacting systems may involve nonspecific interactions, it is still unclear whether some specific interactions are also involved in in vivo systems. This eventually leads to the question whether α-crystallin can differentiate between its natural and nonnatural substrates by some mechanism. This question is relevant to understand chaperone-substrate recognition mechanism.

In this article, we have tried to critically analyze, from experimental point of view, whether α-crystallin has any preference for its natural substrates compared to the nonnatural one. Our results clearly show that α-crystallin is exceptionally active and sensitive in preventing aggregation of its natural substrates and can fully prevent such an aggregation in a substoichiometric ratio, but nonnatural substrates require considerably higher amount of α-crystallin. A possible mechanism by which α-crystallin may distinguish between its natural and nonnatural substrate has been suggested from subunit exchange experiments.

EXPERIMENTAL

Materials

Bovine eye lenses were obtained from a local slaughterhouse and stored at -70°C. Phenyl methyl-sulfonyl fluoride (PMSF) was obtained from E. Merck, Germany, Sephacryl S-300 HR, Sephacryl S-200 HR, Sephacryl S-100 HR, carbonic anhydrase (CA), DNase, lysozyme, IPTG, and SDS were all purchased from Sigma Chemical, USA. Dithiothreitol (DTT) and all buffer salts (tris, phosphate, etc.) were from Sisco Research Laboratories, India. Lucifer yellow iodoacetamide (LYI) and 4-acetamido-4′-((iodoacetyl)amino)-stilbene-2,2′-disulfonic acid (AIAS) were obtained from Molecular Probes, USA. Other chemicals used in this study were all of analytical grade.

Preparation of Bovine β_L- and γ-Crystallin

Bovine eye lenses were homogenized on ice in 10 m*M* Tris-HCl buffer, pH 7.2 containing 100 m*M* NaCl, 1 m*M* EDTA, 0.02% sodium azide, and 0.2 m*M* PMSF, and then centrifuged at 4°C at 12,000 rpm for 45 min. The supernatant fraction was loaded onto a Sephacryl S-300 HR column (95 × 1.5 cm^2). Third peak contained high (β_H-) and the fourth peak contained low (β_L-) crystallin. Further purification was done using a Sephacryl S-200 HR column. SDS-PAGE of purified β_L-crystallin showed a single band corresponding to 20 kDa.[24,25]

For the preparation of γ-crystallin, we used the same protocol as described earlier.[25,26]

Expression and Purification of Recombinant αA- and αB-Crystallin

Plasmid DNA of human αA-crystallin construct in pAED4 vector was a gift from Dr. W. W. de Jong of Katholic University, The Netherlands. Plasmid for human αB-crystallin in pET20b+ expression vector was provided as a gift by Dr. J. Horwitz of Jules Stein Eye Institute, Los Angeles, CA. For overexpression, both the plasmids were separately introduced into *Escherichia coli* strain BL21-DE3. Cultures were grown in LB medium at 37°C with IPTG induction. Cells were centrifuged, subjected to freeze-thaw treatment, and then extracted with DNase and lysozyme. Proteins were dialyzed in 20 m*M* tris buffer, pH 7.2 containing 0.5 m*M* EDTA, and 0.5 m*M* DTT (buffer-A), concentrated in Amicon-stirred cell, applied to DEAE anion exchange column, and eluted with linear 0–0.5*M* NaCl gradients. αA- and αB-crystallin fractions were then applied to Sephacryl S-300 HR size exclusion column (1.5 cm × 90 cm) and eluted with buffer-A containing 0.1*M* NaCl. Main peak fractions were concentrated and dialyzed against buffer A or 50 m*M* phosphate buffer pH 7.2, containing 0.5 m*M* DTT and stored in aliquots at -70°C. SDS-PAGE of both αA- and αB-crystallin showed single band around 20 kDa. Concentration of recombinant proteins was determined spectrophotometrically by measuring absorbance at 280 nm using extinction coefficients of 0.83 and 0.95 (mg/ml)$^{-1}$ cm^{-1} for αA- and αB-crystallin, respectively.

Assay of Chaperone Activity

Chaperone activity of αA-crystallin was determined by a thermal aggregation assay,[24–26] using bovine γ-crystallin as natural substrate and CA as nonnatural substrate. A typical solution (600 μl) contained αA-crystallin (0.1 mg/ml) and substrate (0.2 mg/ml) in 50 m*M* phosphate buffer, pH 7.5, and 100 m*M* NaCl. The assay solution was taken in a black-masked quartz cuvette, which was placed in the spectrophotometer cell holder maintained at 65°C by a Peltier device. Apparent absorbance, as a measure of relative scattering, at

400 nm was monitored for 1 h in a kinetic mode. It was previously shown[24–26] that under the experimental conditions, the thermal aggregation reaction was complete within 1 h. Attainment of saturation scatter level or maximum scattering indicates completion of aggregation reaction. Although scattering may vary from substrate to substrate, for our purpose, comparison has been made within a subset of data for the same substrate. Experiments were carried out at different ratio between αA-crystallin and substrate, keeping the substrate concentration fixed at 0.2 mg/ml and varying the αA-crystallin concentration. Also, we did the same experiments with αB-crystallin.

Heat-Induced Association of Substrate Proteins with αA-Crystallin

Membrane Filtration Method. We incubated 0.4 mg/ml αA-crystallin with 0.4 mg/ml bovine γ-crystallin or CA in 50 m*M* phosphate buffer and pH 7.5 containing 100 m*M* NaCl at 65°C for 1 h. The solutions were cooled back to 25°C for 1 h. Unbound substrate was then separated by centrifugation at 4000*g* through 100 kDa Microcon (Amicon) filter membranes.[25] Amount of the remaining substrate protein associated with αA-crystallin was calculated from the concentration of total and free substrate, both being determined by Bradford assay using BSA as standard. For the 1:0.6 ratio (w/w) between αA-crystallin and different substrate proteins (bovine γ-crystallin or CA), we also measured the amount of substrate complexed with αA-crystallin.

Fluorescence Labeling of Recombinant αA-Crystallin with LYI and AIAS

The cysteine residue at position 131 in recombinant human αA-crystallin was covalently labeled with the fluorescence probes, AIAS and LYI, separately by incubating the protein samples (1 mg/ml) in 50 m*M* phosphate buffer, pH 7.5 containing 100 m*M* NaCl with 250 μ*M* of the probes at 37°C for 18 h. The covalently labeled αA-crystallin was freed from unreacted reagents by passing through Sephadex G-25 column (2.0 cm × 25 cm) equilibrated with 100 m*M* NaCl, 2 m*M* DTT, 50 m*M* sodium phosphate, and pH 7.5. The void volume fractions, which contained labeled protein, were pooled and dialyzed against 50 m*M* phosphate buffer, pH 7.5 for 24 h. Percentage labeling of AIAS to αA-crystallin was calculated from the absorbance of αA-crystallin and AIAS at 278 and 335 nm, respectively. Percentage labeling of LYI to αA-crystallin was also calculated from the absorbance of αA-crystallin and LYI at 278 and 435 nm, respectively. The concentrations of LYI and AIAS were determined from their absorption spectra using molar extinction coefficients of 13,000 and 35,000 cm^{-1} M^{-1} at 435 and 335 nm, respectively.[27]

Measurements of Subunit Exchange Rate

The subunits of α-crystallin associate to form multimeric units. However, this association process is a dynamic one and different oligomeric units can exchange subunits among them. Thus, when oligomers of αA-crystallin tagged with AIAS is mixed with αA-crystallin oligomers tagged with LYI, subunit exchange brings both the tagged αA-crystallin subunits in the same oligomer. The excitation maxima of AIAS- and LYI-labeled αA-crystallin were at 335 and 435 nm, respectively, and the emission maxima at 415 and 525 nm, respectively. The significant overlap of the emission spectrum of the AIAS fluorophore with the absorption spectrum of LYI fluorophore indicates that they are excellent donor–acceptor pair for fluorescence resonance energy transfer (FRET).[27] This energy transfer efficiency is dependent upon the distance between the two fluorophores. Subunit exchange brings the fluorophores tagged to αA-crystallin close enough for the energy transfer to take place. Subunit exchange experiment was carried out by mixing equal volumes of 0.4 mg/ml AIAS-labeled αA-crystallin and 0.4 mg/ml LYI-labeled αA-crystallin at 37°C in 50 m*M* phosphate buffer, pH 7.5 containing 2 m*M* DTT, and 100 m*M* NaCl. At different time intervals, 20 μl of the reaction mixture was removed and diluted 100-fold with the same buffer. The fluorescence spectrum of different samples was taken from 360 to 600 nm at room temperature using a Hitachi F-4500 spectrofluorimeter. The excitation wavelength was 335 nm. The bandpass of both excitation and emission monochromators was 5 nm each. The intensity at 415 nm was determined. The subunit exchange rate was calculated from the equation[27]:

$$F(t)/F(0) = \tilde{A} + \tilde{B}\exp(-k_r t) \quad (1)$$

where $F(t)$ is the fluorescence intensity at 415 nm at different time interval, $F(0)$ the fluorescence intensity at 415 nm at $t = 0$, and k_r is the subunit exchange rate constant. The constants, A and B were determined using the conditions where $\tilde{A} + \tilde{B} = 1$ at $t = 0$ and $\tilde{A}$ is the fluorescence intensity at $t = \infty$. The rate constant k_r was determined by nonlinear regression analysis of the data using Microcal Origin 6.0 software.

Effect of Bound Substrates on the Subunit Exchange Rate of αA-Crystallin

The effect of bound natural substrate and nonnatural substrates on the subunit exchange rate of recombinant human αA-crystallin was determined by the complexation of bovine β_L-crystallin or γ-crystallin (natural substrate) or CA (nonnatural substrate) with αA-crystallin under thermally stressed condition. The complex was allowed to form by incubating different concentrations separately of each substrate protein with both 0.4 mg/ml AIAS-tagged and LYI-tagged αA-crystallin at 65°C for 1 h. The solutions were cooled back to 25°C for 1 h. Then, we mixed equal amounts of AIAS-labeled and LYI-labeled αA-crystallin containing the bound substrate and measured the subunit exchange rate at 42, 45 and 48°C, respectively. We also studied the subunit exchange kinetics of unbound αA-crystallin (heated at 65°C for 1 h and cooled back to 25°C for 1 hr) at 37, 42, and 45°C, respectively.

RESULTS

Assay of Chaperone Activity

To compare the chaperoning efficiency of αA-crystallin against protection of aggregation of its natural and nonnatural substrates, we undertook thermal aggregation experiments using γ-crystallin and CA as natural and nonnatural substrate, respectively. Both the substrates were assayed at

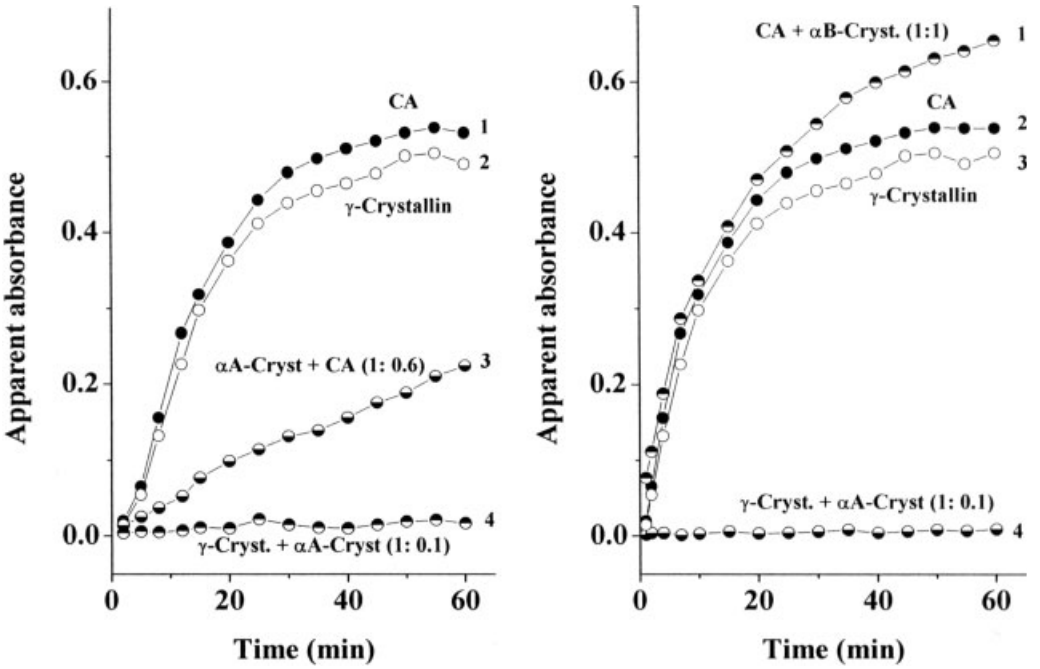

FIGURE 1 Chaperone activity of α-crystallin against natural and nonnatural substrate. Carbonic anhydrase was used as a nonnatural substrate and γ-crystallin was used as a natural substrate and the thermal assay for both substrates was carried out at 65°C. Left panel: αA-crystallin and right panel for αB-crystallin. Concentration of carbonic anhydrase or γ-crystallin in all the experiments were 0.2 mg/ml and the ratio of substrate to chaperone as shown in figure are on (w/w) basis.

65°C, keeping their concentration fixed at 0.2 mg/ml. The concentration of αA-crystallin was varied to change the weight ratio between substrate protein and chaperone αA-crystallin. Aggregation profiles for CA are shown in Figure 1 (left panel). In absence of αA-crystallin, CA completely aggregated within 60 min (Trace 1). At 1:0.6 ratio (w/w) between αA-crystallin and CA, aggregation decreased to $\sim$40% (Trace 3). For complete prevention of aggregation, it required a ratio of 1:1 between CA and αA-crystallin (data not shown). The aggregation profiles of γ-crystallin at the temperature (65°C) were also shown in Figure 1 (left panel). In this case, even at a ratio of 1:0.1 between γ- and αA-crystallin, complete protection of aggregation was observed (Trace 4). In short, there seems to be a clear preference for αA-crystallin to become super efficient in preventing the aggregation of its own substrate, even at a very low substoichiometric ratio, while a stoichiometric weight ratio was clearly found to be not enough for the complete prevention of its nonnatural substrate.

It was known from earlier studies[28] that with rise of temperature above 60°C, chaperone activity of αA-crystallin increased whereas that of αB-crystallin decreased. We assayed the chaperone activity of αB-crystallin also at 65°C against natural substrate γ-crystallin and nonnatural substrate CA. The results were shown in Figure 1B (right panel). We find that at 1:1 ratio between CA and αB-crystallin, no protection against aggregation was obtained (Trace 1). It may be recalled that αB-crystallin at 65°C tended to aggregate slowly.[26] The higher value of the scattering compared to control observed in presence of αB-crystallin is because of the additional scattering contribution from the chaperone. Interestingly, when γ-crystallin was used as a substrate, full protection against aggregation was observed even at 1:0.1 ratio between γ- and αB-crystallin (Trace 4). This clearly reflects that αB-crystallin has extraordinary affinity for γ-crystallin and its aggregation is prevented with higher efficiency when compared with that of a nonnatural substrate such as CA. The super specificity of αA- or αB-crystallin towards β_L-crystallin (data not presented here) showing that this phenomenon applies to all natural substrate of α-crystallin.

Heat-induced Association of Substrate Proteins with αA-Crystallin

Binding was assayed by membrane filtration method.[25] When a mixture of αA-crystallin homo-oligomer and monomeric γ-crystallin, 0.4 mg/ml each, was incubated at 65°C and the mixture was passed through a 100 kDa membrane, we found about 85% of the γ-crystallin was bound to αA-crystallin (Table I). Even when the ratio between αA- and γ-crystallin is 1:2 (w/w), we found that $\sim$80% γ-crystallin was

Table I Percentage of Substrate Binding to αA-Crystallin at 65 °C

System Studied	αA:Substrate (w/w)	Amount of Bound Substrate (%)
Human αA- + γ-crystallin	1:0.6	90.2
	1:1	84.8
	1:2	80.1
Human αA-crystallin + CA	1:0.6	71.9
	1:1	68.2
	1:2	–

complexed with αA-crystallin (Table I). But for CA (nonnatural substrate), we found that at 1:1 ratio (w/w), only 68% remained bound to αA-crystallin. This value is considerably less compared to binding of γ-crystallin (85%) under similar condition. The percentage binding increased marginally to 72%, when 1:0.6 ratio (w/w) between αA-crystallin and CA was used.

Subunit Exchange in αA-Crystallin

Recombinant human αA-crystallin has two cysteine residues at positions 131 and 142. Among two cysteine residues, Cys^{131} is fully exposed and Cys^{142} is buried. Percentage labeling of AIAS and LYI to αA-crystallin revealed that an average of 1 mol of fluorophore was covalently attached to 1 mol of αA-crystallin subunit, which means that only the fully exposed Cys^{132} residue was covalently modified. The elution volumes of gel-filtration profiles of unlabeled, AIAS-labeled, and LYI-labeled αA-crystallin were very similar indicating that the modification of Cys^{131} did not perturb the oligomeric size of αA-crystallin (data not shown).

When 0.4 mg/ml AIAS-labeled and 0.4 mg/ml LYI-labeled αA-crystallin were mixed at 37°C, we found that the fluorescence intensity of the both fluorophore was markedly altered (Figure 2). Because of the energy transfer between AIAS and LYI, we observed a time-dependent decrease in AIAS emission intensity at 415 nm and a concomitant increase in LYI fluorescence intensity at 525 nm. The decrease in AIAS fluorescence intensity was completed in 4 h at 37°C (Figure 3A). We also plotted the increase in LYI fluorescence intensity at 525 nm as a function of time in Figure 3B. The declining AIAS intensity or the increasing LYI intensity was fitted to the equation $F(t)/F(0) = \tilde{A} + \tilde{B}\exp(-k_r t)$ to obtain the subunit exchange rate constant (k). Both the data showed very similar value of 0.054 min^{-1} for the subunit exchange rate constant. Since both measurements gave identical exchange rate constant, all subsequent data fitting was obtained by

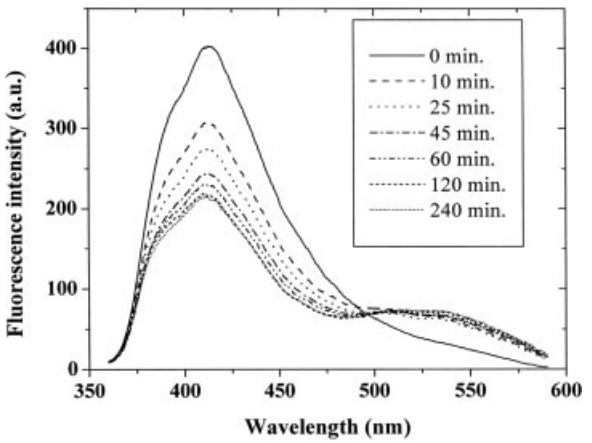

FIGURE 2 Time-dependent changes in the emission spectrum of AIAS labeled αA-crystallin due to subunit exchange. The emission spectra of αA-crystallin were recorded at different times (1) 0; (2) 10; (3) 25; (4) 45; (5) 60; (6) 120; (7) 240 min after mixing equal amount of AIAS-labeled and LYI-labeled αA-crystallin (0.4 mg/ml) at 37°C. The fluorescence spectrum of different samples was taken from 360 to 600 nm at room temperature (25°C). The excitation wavelength was 335 nm.

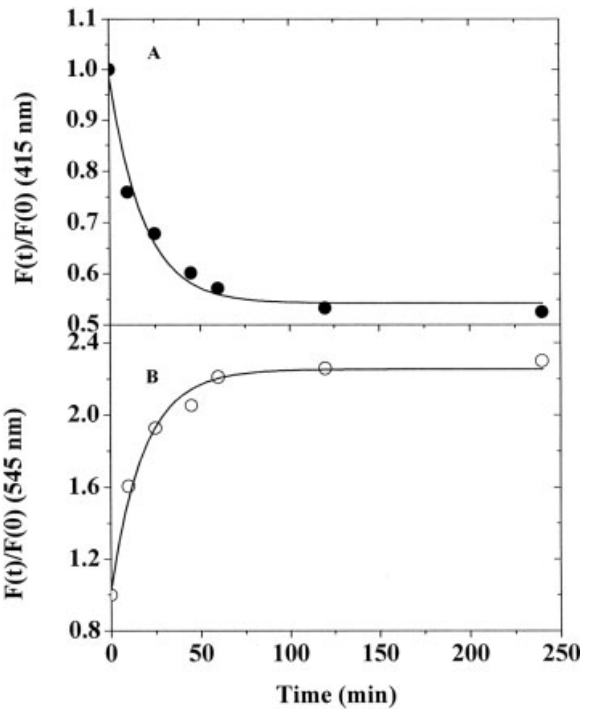

FIGURE 3 Time-dependent changes in emission intensity due to subunit exchange. (A) Decrease in fluorescence intensity at 415 nm as a function of time. (B) Increase in fluorescence intensity 545 nm as a function of time. First, equal amount of AIAS-labeled and LYI-labeled αA-crystallin (0.4 mg/ml) was mixed at 37°C. Then, at different time intervals, fluorescence spectrum of different samples was taken from 360 to 600 nm at room temperature (25°C). The excitation wavelength was 335 nm. The curves were fitted to the equation $F(t)/F(0) = \tilde{A} + \tilde{B}\exp(-k_r t)$.

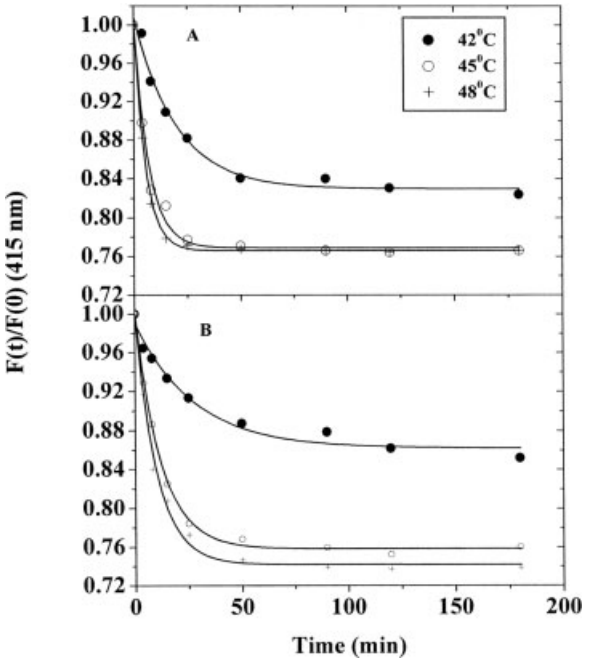

FIGURE 4 Effect of temperature on the subunit exchange of substrate bound αA-crystallin. (A). Measurements of subunit exchange between γ-crytallin bound AIAS-labeled and γ-crytallin bound LYI-labeled αA-crystallin at 42 (●), 45 (O), and 48°C (+). (B) Measurements of subunit exchange between CA bound AIAS-labeled and CA bound LYI-labeled αA-crystallin at 42 (●), 45 (O), and 48°C (+). First, complex was allowed to form by incubating separately different concentrations of each substrate protein with both 0.4 mg/ml AIAS-tagged and LYI-tagged αA-crystallin at 65°C for 1 h. The solutions were cooled back to 25°C for 1 h. Then, equal amounts of AIAS-labeled and LYI-labeled αA-crystallin were mixed containing the bound substrate and measured relative emission intensity at 415 nm at different time intervals.

using only the AIAS fluorescence intensities as a function of time.

Effect of Bound Substrates on the Subunit Exchange Rate of αA-Crystallin

We incubated separately AIAS- and LYI-labeled αA-crystallin with different substrate proteins (bovine γ-crystallin or CA) in 1:1 ratio (w/w) at 65°C for 1 h. After complex formation, the solutions were cooled to room temperature. Initially, we tried to measure the subunit exchange kinetics both for γ-crystallin bound and CA bound αA-crystallin at 37°C, but the exchange kinetics was found to be very slow in both cases (data not shown). This showed that binding of substrates lowered the subunit exchange rate. Reasonable rate of subunit exchange of αA-crystallin with bound substrate was observed at 42°C. The data were plotted as the fluorescence intensity of AIAS at 415 nm against time in Figure 4. In the same figure, the kinetic profiles at two more temperatures namely 45 and 48°C are also shown. It can be seen that the amount of decrease in AIAS fluorescence intensity was more, when αA-crystallin was complexed with its natural substrate γ-crystallin compared to nonnatural substrate CA (Figures 4A and 4B). For example, $F(t)/F(0)$ attained steady value at 0.84 for bound γ-crystallin system at 42°C, whereas the value for CA bound system remained 0.89. At 45°C, $F(t)/F(0)$ for γ-crystallin bound αA-crystallin attained steady value in less than 25 min compared to 40 min for the corresponding CA system. It is also noticed that steady values for γ-crystallin bound system reached earlier than CA bound system (Figures 4A and 4B). The subunit exchange profiles for αA-crystallin bound to natural substrate γ-crystallin and nonnatural substrate CA at the three temperatures are shown in Panels A and B, respectively (Figure 4). The subunit exchange rate constant of unbound αA-crystallin at 37, 42, and 45°C was 0.054, 0.152, and 0.246 min^{-1}, respectively (Table II). The subunit exchange rate constant of γ-crystallin bound αA-crystallin at 42, 45, and 48°C was 0.048, 0.144, and 0.166

Table II Subunit Exchange Rate Constant of αA-Crystallin in Absence and Presence of Bound Substrate (at ratio 1:1, w/w) at Different Temperatures

System Studied	Temperature at Which Exchange Kinetics Was Done (°C)	Subunit Exchange Rate Constant (min^{-1})
Human αA	37	0.054
	42	0.152
	45	0.246
Human αA- + γ-crystallin	42	0.048
	45	0.144
	48	0.167
Human αA + CA	42	0.024
	45	0.084
	48	0.101
Human αA- + β_L-crystallin	45	0.137

Table III Subunit Exchange Rate Constant of αA-Crystallin in Presence of Different Amount of Substrate at 45°C

System Studied	αA:Substrate (w/w)	Subunit Exchange Rate Constant (min^{-1})
Human αA + γ-crystallin	1:0.6	0.134
	1:1	0.144
	1:2	0.166
Human αA + CA	1:0.6	0.082
	1:1	0.084
	1:2	–

min^{-1}, respectively, whereas the subunit exchange rate constant of CA bound αA-crystallin at 42, 45, and 48°C was 0.024, 0.84, and 0.101 min^{-1}, respectively (Table II). This suggests that the subunit exchange kinetics was comparatively more reduced upon the binding of CA to αA-crystallin than the binding of γ- to αA-crystallin. We also measured the subunit exchange kinetics at 45°C at varied chaperone to substrate ratio. These subunit exchange rate constants were listed in Table III. The activation energy was calculated by Arrhenius equation:

$$\ln(k_r) = \ln A - E_a/RT \quad (2)$$

where k_r is the subunit rate constant, A the preexponential factor, T the absolute temperature in Kelvin scale, and E_a the activation energy for subunit exchange. The activation energy for subunit exchange for native, γ-crystallin bound, and CA bound αA-crystallin obtained from the plot of ln (k_r) against $1/T$ (Figure 5) was given in Table IV. It was found that in absence of any bound substrate, the activation energy of αA-crystallin subunit exchange was 158 kJ/mol. On binding with its natural substrate γ-crystallin, the activation energy of subunit exchange was increased by ~22%, whereas with CA, the enhancement of activation energy was ~33%.

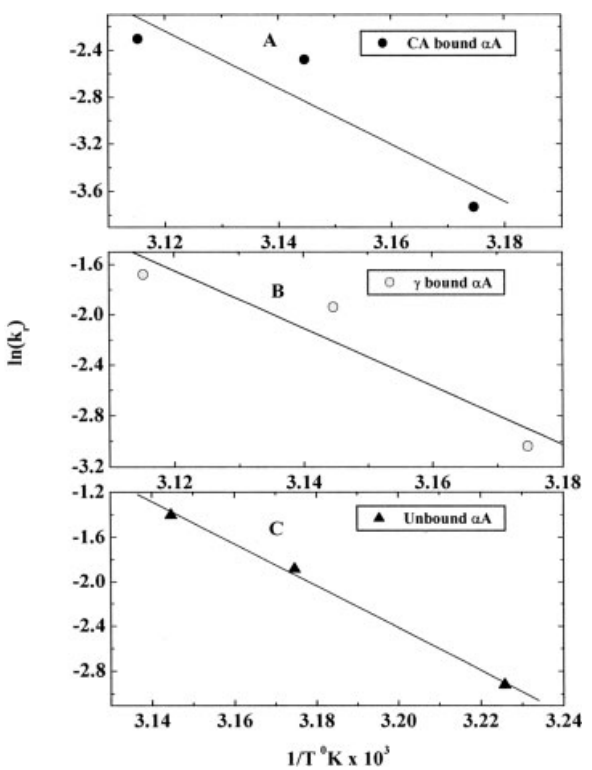

FIGURE 5 Determination of the activation energy of subunit exchange of αA-crystallin in absence and presence of different bound substrates. Arrhenius plot of the subunit exchange reaction of native αA-crystallin (Panel A), γ-crystallin bound αA-crystallin (Panel B), and CA bound αA-crystallin. The complex was allowed to form by incubating different concentrations separately of each substrate protein with both 0.4 mg/ml AIAS-tagged and LYI-tagged αA-crystallin at 65°C for 1 h. The solutions were cooled back to 25°C for 1 h. Then, equal amounts of AIAS-labeled and LYI-labeled αA-crystallin were mixed containing the bound substrate and measured the subunit exchange rate at 42, 45, and 48°C, respectively. The rate constants at respective temperature were obtained by $\ln(k_r) = \ln A - E_a/RT$. The activation energy (E_a) was determined from the slope.

Table IV Activation Energy (E_a) for the Subunit Exchange Reaction of Recombinant Human αA-Crystallin in Absence and Presence of Bound Substrate (at Ratio 1:1, w/w)

Bound Substrate	Activation Energy (kJ/mol)
None	158
Bovine γ-crystallin	193
Carbonic anhydrase	210

DISCUSSION

The results presented earlier clearly reveal that despite its ability to prevent aggregation of structurally and functionally unrelated proteins, the chaperone α-crystallin can preferentially recognize its own substrates and is extremely effective in preventing their aggregation even when present in extremely low concentration compared to the concentration of its natural substrates. This observation indicates that a separate mechanism may be operative in the recognition between α-crystallin and its own substrate. The results also indicate that subunit exchange may play an important role in aggregation prevention against thermal stress, and such a mechanism may be responsible for the discrimination of α-crystallin between its natural and nonnatural substrate.

It is still debated whether molecular chaperone like function involve specific interaction. Hydrophobic interaction is crucial to such function and it is known that hydrophobic interaction is generally nonspecific in nature. That is why chaperones usually recognize a large number of related or unrelated substrates. But, we had shown earlier that ionic interactions are also involved in controlling chaperone functions of α-crystallin.[25] Physical basis of specific interactions on the protein surface has recently become the subject of intense experimental and theoretical studies.[29–32] The concept of protein interaction hot spots has been put forward.[29] It has been proposed that only a small set of hot spot residues may contribute to binding free energy and the presence of

hot spots are general characteristics of protein–protein interfaces.[30,31] It has been suggested that conserved polar residues constitute hot spots but many interaction hot spots involve hydrophobic or large aromatic residues as well.[29,32] Such specific interactions may be responsible for the action of α-crystallin against β- and γ-crystallin, while the nonspecific interaction may be responsible for its chaperone-like activity against other substrates. For nonnatural substrates, relatively higher amount of α-crystallin is required for aggregation prevention. Insulin requires 5–6 times its own concentration (w/w) of α-crystallin for complete prevention.[9,33] It is true that thermal assay usually requires relatively less amount of α-crystallin because of its higher activity at high temperature,[10,22,34] but it still requires approximately a ratio of 1:1 (w/w) between substrate and α-crystallin for nearly full protection (data not shown). However, nearly complete prevention of aggregation of β- and γ-crystallin by substoichiometric amount of α-crystallin clearly indicates a different type of interaction and specificity. Binding assay data presented here show higher amount of binding to the chaperone to γ-crystallin compared to CA treated under identical condition. The data clearly indicate a different mechanism of chaperone action in both the cases.

Subunit exchange of α-crystallin has been suggested to have direct link with the chaperone function of α-crystallin.[25] As subunit exchange increases with the rise of temperature, the chaperone activity also increases. Thus, chaperone activity is believed to be linked to the dynamics of the exchange process. Any process that is supposed to decrease the dynamics of this exchange process will decrease the chaperone activity. Substrate binding to α-crystallin slows down the exchange reaction.[27] We have chosen natural and nonnatural substrates of comparable molecular weight to that of the subunit of α-crystallin. Our data show that the binding of CA substantially slows down the subunit exchange process compared to the binding of γ-crystallin and the rate constant for the subunit exchange process for the previous situation becomes nearly half of the later (Table II). Binding of CA raises the activation energy of the exchange process more than γ-crystallin (Table IV). Complete prevention of aggregation of β- and γ-crystallin with 1/10th the amount of α-crystallin indicates that such a process cannot be explained by static-binding method, which would require at least one α-crystallin subunit for one molecule of substrate. We speculate that the interaction between α-crystallin and its own substrate may involve a very dynamic process in which given α-crystallin subunit, which is exchanging between different oligomers, may interact with its crystallin substrate for an extremely short duration before interacting with another substrate molecule. Because of the extremely short residence time of the α-crystallin molecule on the substrate, it will be able to interact dynamically with a large number of substrate molecules in short time. This average time difference for a subunit of α-crystallin between moving from one substrate to another may be sufficiently short compared to the time required for collision between two substrate molecules causing initiation for aggregation. For nonnatural substrates, a less dynamic process takes place where the substrate has a significantly high residence time on the chaperone surface. In this case, lack of sufficient amount of chaperone would cause aggregation.

The basis for such a selection by α-crystallin is not obvious from our data. We believe that the similarity of the secondary structure of α-, β-, and γ-crystallin may be one of the reasons for such a distinctive behavior. CD and FTIR data have revealed that all the three proteins have very similar secondary structure dominated by significant amount ($\sim$50%) β-sheet structure and very little α-helical structure.[28,35,36] Perhaps, it is the similarity in sheet-like structures of both the substrate and the chaperone that provide much less barrier to the subunit exchange facilitating the dynamic interaction mechanism. Nonnative substrates having higher amount of helical structures may provide greater obstruction for the β-sheet rich α-crystallin subunit exchange and may thus lead to higher residence time of the chaperone in bound complex and hence favoring a static binding mechanism for the nonnative substrate.

AB was recipient of Bose Institute fellowship.

REFERENCES

1. Groenen, P. J. T. A.; Merck, K. B.; de Jong, W. W.; Bloemendal, H. Eur J Biochem 1994, 225, 1–19.
2. Horwitz, J. Invest Ophthalmol Vis Sci 1993, 34, 10–21.
3. Srinivasan, A. N.; Nagineni, C. N.; Bhat, S. P. J Biol Chem 1992, 267, 23337–23341.
4. Iwaki, T.; Kume-Iwaki, A.; Liem, R. K. H.; Goldman, J. E. Cell 1989, 57, 71–78.
5. Sax, C. M.; Piatigorsky, J. Adv Enzymol 1994, 69, 153–201.
6. Narberhaus, F. Microbiol Mol Biol Rev 2002, 66, 64–93.
7. Horwitz, J. Proc Natl Acad Sci USA 1992, 89, 10449–10453.
8. Derham, B. K.; Harding, J. J. Prog Retin Eye Res 1999, 18, 463–509.
9. Farahbakhsh, Z. T.; Huang, Q.-L.; Ding, L.-L.; Altenbach, C.; Steinhoff, H.-J.; Horwitz, J.; Hubbell, W. L. Biochemistry 1995, 34, 509–516.
10. Das, K. P.; Surewicz, W. K. FEBS Lett 1995, 369, 321–325.
11. Lindner, R. A.; Kapur, A.; Carver, J. A. J Biol Chem 1997, 272, 27722–27729.
12. Abgar, S.; Yevlampieva, N.; Aerts, T.; Vanhoudt, J.; Clauwaert, J. Biochem Biophys Res Commun 2000, 276, 619–625.
13. Mchaourab, H. S.; Dodson, E. K.; Koteiche, H. A. J Biol Chem 2002, 277, 40557–40566.

14. Carver, J. A.; Aquilina, J. A.; Copper, P. G.; Wikkiams, G. A.; Truscott, R. J. W. Biochim Biophys Acta 1994, 1204, 195–206.
15. Rajaraman, K.; Raman, B.; Rao, C. M. J Biol Chem 2001, 497, 118–123.
16. Rawat, U.; Rao, M. J Biol Chem 1998, 273, 9415–9423.
17. Borkman, R. F.; Knight, G.; Obi, B. Exp Eye Res 1996, 62, 141–148.
18. Muchowski, P. J.; Clark, J. I. Proc Natl Acad Sci USA 1998, 95, 1004–1009.
19. Wang, K.; Spector, A. J Biol Chem 1994, 269, 13601–13608.
20. Wang, K.; Spector, A. Invest Ophthalmol Vis Sci 1995, 36, 311–321.
21. Ho, Y.; Lai, C. L.Huang, F. Y. J Chin Chem Soc 1998, 45, 285–292.
22. Raman, B.; Rao, C. M. J Biol Chem 1994, 269, 27264–27268.
23. Marini, J.; Bucchioni, L.; Voltarelli, N.; Del Corso, A.; Murh, U. Biochem Biophys Res Commun 1995, 212, 413–420.
24. Bhattacharyya, J.; Das, K. P. Biochem Mol Biol Int 1998, 46, 249–258.
25. Biswas, A.; Das, K. P. J Biol Chem 2004, 279, 42648–42657.
26. Saha, S.; Das, K. P. Proteins 2004, 57, 610–617.
27. Bova, M. P.; Ding, L. L.; Horwitz, J.; Fung, B. K. J Biol Chem 1997, 272, 29511–29517.
28. Reddy, G. B.; Das, K. P.; Petrash, J. M.; Surewicz, W. K. J Biol Chem 2000, 275, 4565–4570.
29. Li, W.; Keeble, A. H.; Giffard, C.; James, R.; Moore, G. R.; Kleanthous, G. J Mol Biol 2004, 337, 743–759.
30. Scott, J. K.; Huang, S. F.; Gangadhar, B. P.; Samoriski, G. M.; Clapp, P.; Gross, R. A.; Taussiq, R.; Smrcka, A. V. EMBO J 2001, 20, 767–776.
31. Clackson, T.; Wells, J. A. Science 1995, 267, 383–386.
32. Kortemme, T.; Baker, D. Proc Natl Acad Sci USA 2002, 99, 14116–14121.
33. Das, K. P.; Petrash, J. M.; Surewicz, W. K. J Biol Chem 1996, 271, 10449–10452.
34. Raman, B.; Ramakrishna, T.; Rao, C. M. FEBS Lett 1995, 365, 133–136.
35. Surewicz, W. K.; Olesen, P. R. Biochemistry 1995, 34, 9655–9660.
36. Das, K. P.; Choo-Smith, L. P.; Petrash, J. M.; Surewicz, W. K. J Biol Chem 1999, 274, 33209–33212.

Reviewing Editor: Nils Walter