Structural perturbation and enhancement of the chaperone-like activity of α-crystallin by arginine hydrochloride

VOLETY SRINIVAS, BAKTHISARAN RAMAN, KUNCHALA SRIDHAR RAO, TANGIRALA RAMAKRISHNA, AND Ch MOHAN RAO

Centre for Cellular & Molecular Biology, Hyderabad 500 007, India

(RECEIVED January 12, 2003; FINAL REVISION March 4, 2003; ACCEPTED March 11, 2003)

Abstract

Structural perturbation of α-crystallin is shown to enhance its molecular chaperone-like activity in preventing aggregation of target proteins. We demonstrate that arginine, a biologically compatible molecule that is known to bind to the peptide backbone and negatively charged side-chains, increases the chaperone-like activity of calf eye lens α-crystallin as well as recombinant human αA- and αB-crystallins. Arginine-induced increase in the chaperone activity is more pronounced for αB-crystallin than for αA-crystallin. Other guanidinium compounds such as aminoguanidine hydrochloride and guanidine hydrochloride also show a similar effect, but to different extents. A point mutation, R120G, in αB-crystallin that is associated with desmin-related myopathy, results in a significant loss of chaperone-like activity. Arginine restores the activity of mutant protein to a considerable extent. We have investigated the effect of arginine on the structural changes of α-crystallin by circular dichroism, fluorescence, and glycerol gradient sedimentation. Far-UV CD spectra show no significant changes in secondary structure, whereas near-UV CD spectra show subtle changes in the presence of arginine. Glycerol gradient sedimentation shows a significant decrease in the size of α-crystallin oligomer in the presence of arginine. Increased exposure of hydrophobic surfaces of α-crystallin, as monitored by pyrene-solubilization and ANS-fluorescence, is observed in the presence of arginine. These results show that arginine brings about subtle changes in the tertiary structure and significant changes in the quaternary structure of α-crystallin and enhances its chaperone-like activity significantly. This study should prove useful in designing strategies to improve chaperone function for therapeutic applications.

Keywords: Chaperone-like activity; α-crystallin; arginine; aminoguanidine; structural perturbation

α-Crystallin, a multimeric protein of the eye lens, is made up of two homologous gene products, αA- and αB-crystallins. αB-crystallin is expressed in significant levels in other tissues, such as heart, kidney, brain, muscle, etc., whereas αA-crystallin is expressed in trace amounts in the spleen and thymus (Bhat and Nagineni 1989; Dubin et al. 1989; Iwaki et al. 1989; Kato et al. 1991). αB-Crystallin is stress-inducible and is expressed at elevated levels under certain disease conditions (Aoyama et al. 1993; Groenen et al. 1994; Renkawek et al. 1994). αA- and αB-crystallin share structural and sequence homology with small heat shock proteins (sHSPs; Ingolia and Craig 1982; Merck et al. 1993). α-Crystallin exhibits molecular chaperone-like activity in preventing the heat-induced aggregation of other proteins (Horwitz 1992). It is now established that both αA- and αB-crystallin, either in homo-multimeric or hetero-multimeric states, exhibit molecular chaperone-like properties in preventing aggregation of other proteins (Datta and Rao

Reprint requests to: Ch Mohan Rao, Deputy Director, Centre for Cellular & Molecular Biology, Uppal Road, Hyderabad 500 007, India; e-mail: mohan@ccmb.res.in; fax: 91-40-27160591.

Abbreviations: Arg.HCl, L-arginine hydrochloride; DL-Arg.HCl, DL-arginine hydrochloride; Gdn HCl, guanidine hydrochloride; AGdn.HCl, aminoguanidine hydrochloride; Lys. 2HCl, L-lysine dihydrochloride; DTT, dithiothreitol; ANS, 8-anilino-1-naphthalenesulfonic acid; CD, circular dichroism.

Article and publication are at http://www.proteinscience.org/cgi/doi/10.1110/ps.0302003.

1999; Sun et al. 1999), protecting the enzyme activity from heat- or other stress-induced inactivation (Hook and Harding 1997; Hess and Fitzgerald 1998; Marini et al. 2000; Rajaraman et al. 2001) and in a few cases, assisting the refolding (Rawat and Rao 1998; Ganea and Harding 2000; Goenka et al. 2001; Rajaraman et al. 2001).

We have investigated earlier the chaperone-like activity of α-crystallin toward photo-induced aggregation of γ-crystallin, DTT-induced aggregation of insulin, and the refolding-induced aggregation and β-, and γ-crystallins and demonstrated that it is possible to enhance the chaperone-like activity of α-crystallin (for review, see Rao et al. 1998, 2002 and the references therein). Our results showed that a structural perturbation by temperature in α-crystallin, involving increased exposure of hydrophobic surfaces, enhanced the chaperone-like activity of α-crystallin severalfold (for review, see Rao et al. 1998, 2002). Subsequent studies from other laboratories (Das and Surewicz 1995; Palmisano et al. 1995; Borkman et al. 1996) have shown similar results. Smith et al. (1996) found that hydrophobic regions around residues 32–37 and 72–75 in αA-crystallin and 28–34 in αB-crystallin become solvent-exposed above 30°C. Sharma et al. (1997, 2000) identified specific target protein-binding regions, depending on the nature of the target proteins, which span the α-crystallin domain. Evidence has accumulated over the years that temperature regulates the chaperoning process in general through structural perturbation of the chaperone molecules, such as Hsp70 (Craig and Gross 1991; Leung et al. 1996), as well as small heat shock proteins (Rao et al. 1998; Haslbeck et al. 1999; van Montfort et al. 2001; Gu et al. 2002). Interestingly, the structurally perturbed state of Hsp 16.3 at low concentrations of guanidine hydrochloride (Gdn.HCl) has also been shown to exhibit enhanced chaperone-like activity (Yang et al. 1999).

In addition to temperature, we have observed that low concentrations of the denaturant urea also enhance activity corroborating our hypothesis (for review, see Rao et al. 1998, 2002). Gdn.HCl has been shown to enhance the chaperone-like activity of α-crystallin (Das and Liang 1997). Loss or decreased chaperone function appears to be a molecular basis for a growing number of diseases. If biologically compatible small molecules could be used to cause a structural perturbation and enhance the chaperone-like activity of α-crystallin, they would be of therapeutic significance. We, therefore, set out to investigate the effect of the biologically compatible small molecule Arg.HCl on the chaperone-like, anti-aggregating activity of α-crystallin. Guanidinium salts, including Arg.HCl, are known to bind to the peptide backbone as well as side chains of negatively charged amino acids and tryptophan (Arakawa and Timasheff 1984; Timasheff and Arakawa 1988; Lin and Timasheff 1996).

The chaperone-like activity of α-crystallin in preventing aggregation of other proteins is particularly important in the context of the eye lens in maintaining its transparency. Our results show that Arg.HCl can enhance the chaperone-like activity of bovine eye lens α-crystallin as well as the recombinant human αA- and αB-crystallins severalfold. More interestingly, it can also enhance activity of the mutant αB-crystallin (R120G-αB) that causes desmin-related myopathy and congenital cataract (Vicart et al. 1998), perhaps because of its decreased activity (Kumar et al. 1999). Our results with guanidinium compounds, such as Arg.HCl and AGdn.HCl, show that enhanced activity of α-crystallin might be attributable to altered tertiary and quaternary structure.

Results and Discussion

Structural perturbation of α-crystallin leads to a severalfold increase in its chaperone-like activity (for review, see Das and Surewicz 1995; Smith et al. 1996; Rao et al. 1998, 2002). Here, we investigate whether some biologically compatible compounds, such as Arg.HCl, AGdn.HCl, and Gdn.HCl, can achieve structural perturbation of α-crystallin and influence its chaperone-like activity. We used DTT-induced aggregation of insulin at 37°C as a model system (Fig. 1). α-Crystallin prevented the aggregation of insulin partially (~36%) at a 1:2 (wt/wt) ratio of α-crystallin:insulin (Fig. 1A). Figure 1B shows that at the same weight ratio, α-crystallin prevents the aggregation of insulin completely in the presence of 200 mM Arg.HCl. Arg.HCl alone does not prevent the aggregation of insulin; on the contrary, we observed that it leads to its significantly increased aggregation. Despite this effect of Arg.HCl on the aggregation of insulin, α-crystallin in the presence of Arg.HCl showed complete prevention of aggregation. This result shows that Arg.HCl increases the chaperone-like activity of α-crystallin significantly. The observed increase in the activity is dependent on Arg.HCl concentration, indicating a dose-dependent interaction of Arg.HCl with α-crystallin (Fig. 1C; open circles).

Eye lens α-crystallin is a heteromultimeric protein composed of two types of subunits—αA- and αB-crystallin. Both αA- and αB-crystallin form homomultimers; however, they exhibit differences in their chaperone-like activities as well as structural stability (Datta and Rao 1999; Sun et al. 1999; Reddy et al. 2000). To investigate the possible differences in the Arg.HCl-induced increase in the chaperone-like activity, we studied the effect of Arg.HCl on the activities of recombinant human αA- and αB-crystallin toward the DTT-induced aggregation of insulin. In conformity with earlier reports (Datta and Rao 1999; Sun et al. 1999; Reddy et al. 2000), we found that αB-crystallin was more effective in preventing the aggregation of insulin than αA-crystallin; it almost completely prevented the aggregation of insulin at a 1:2 (wt/wt) ratio of αB-crystallin to insulin. To see the effect of Arg.HCl on their chaperone-like

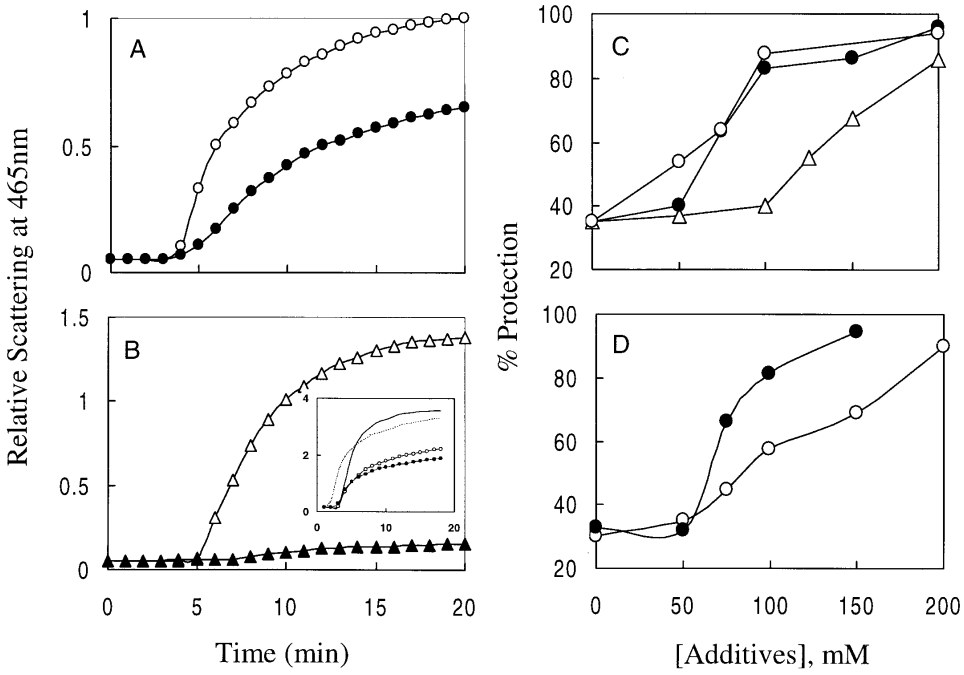

Figure 1. Effect of various additives on the chaperone-like activity of α-crystallin (0.1mg/mL) toward DTT-induced aggregation of insulin (0.2mg/mL) at 37°C. (*A*) Insulin in the absence (*open circles*) and in the presence of calf eye lens α-crystallin (*filled circles*). (*B*) Effect of Arg.HCl. Aggregation of insulin in buffer containing 200 mM Arg.HCl (*open triangles*); aggregation of insulin in the presence of α-crystallin (0.1 mg/mL) and 200 mM Arg.HCl (*filled triangles*). Symbols represent different curve types and not data points. (*Inset*) Effect of Lys.2HCl. Insulin + 100 mM Lys2HCl (*open circles*), α-crystallin + Insulin + 100 mM Lys2HCl (*filled circles*), Insulin + 300 mM Lys2HCl (solid line), α-crystallin + insulin + 300 mM Lys2HCl (broken line). (*C*) Effect of various concentrations of Arg.HCl (*open circles*), Gdn.HCl (*filled circles*), and AGdn.HCl (*triangles*). (*D*) Effect of Arg.HCl on the chaperone-like activity of recombinant human αA- and αB-crystallins toward the DTT-induced aggregation of insulin at 37°C. Percent protection offered by 0.1 mg/mL αA-crystallin (*open circles*), and 0.05 mg/mL αB-crystallin (*filled circles*), toward the aggregation of insulin (0.2 mg/mL) as a function of Arg. HCl concentration.

activities, we used a 1:2 ratio (wt/wt) for αA-crystallin to insulin, and a 1:4 (wt/wt) ratio of αB-crystallin to insulin, as αB-crystallin is more effective as a chaperone. Figure 1D shows that Arg.HCl enhanced the activity of both αA- and αB-crystallin, the increase being much sharper for αB-crystallin than for αA-crystallin. The Arg.HCl-induced enhancement of the activity of αB-crystallin is more pronounced than that of αA-crystallin.

We also tested the effect of Lys.2HCl on the chaperone-like activity of α-crystallin. Unlike Arg.HCl, Lys.2HCl did not enhance the chaperone-like activity of α-crystallin toward the DTT-induced aggregation of insulin (Fig. 1B; inset). In fact, it reduced the percent protection offered by α-crystallin from 36% in the absence of Lys.2HCl to 17% in the presence of 100 mM Lys.2HCl. At a concentration of 300 mM Lys.2HCl, the percent protection reduced further to ~8%.

Incubating α-crystallin with low concentrations of Gdn.HCl (0.8–1.0 M) increases the exposure of its hydrophobic surfaces with an accompanying enhancement in its chaperone-like activity (Das and Liang 1997). Inomata et al. (2000) showed that aminoguanidine, when fed orally to Shumiya cataract rats—a hereditary cataract model in which lens opacity appears spontaneously in the nuclear and perinuclear portions at 11–12 weeks of age—inhibited the opacification of the lens. Aminoguanidine was also found to inhibit cataract formation in diabetic rats (Swamy et al. 1996). We, therefore, compared the effect of Arg.HCl, Gdn.HCl, and AGdn.HCl on the chaperone-like activity of α-crystallin. Figure 1C shows that Arg.HCl and Gdn.HCl are comparable in their ability to enhance the activity of α-crystallin. AGdn.HCl also enhanced the activity of α-crystallin, but to a lesser extent (Fig.1C).

To find out whether ArgHCl-induced increase in chaperone-like activity of α-crystallin is also observed with other protein aggregation systems, we have investigated the effect of α-crystallin incubated with Arg.HCl on the thermally (43°C) induced aggregation of ζ-crystallin. α-Crystallin, at a 1:4 (wt/wt) ratio of α- to ζ-crystallin, prevented the aggregation of ζ-crystallin to the extent of 53%. On preincubation with 100 mM Arg.HCl, the protective ability of α-crystallin increased to ~81%. Similar Arg.HCl-induced enhancement of the chaperone-like activity of α-crystallin is observed toward DTT-induced aggregation of α-lactalbumin at pH 6.5 (data not shown).

We have shown earlier that structural perturbation beyond 30°C increases the chaperone-like activity of α-crystallin (Rao et al. 1998, 2002). To find out if arginine can mimic the effect of elevated temperature in enhancing the chaperone-like activity of α-crystallin, we investigated the effect of Arg.HCl on the chaperone-like activity of α-crystallin at 25°C and found that at a concentration of 300 mM, Arg.HCl almost completely prevented the aggregation of insulin. AGdn.HCl also showed a similar effect on the activity of α-crystallin at 25°C, but to a lesser extent (data not shown). This result suggests that Arg.HCl can mimic temperature-effect in enhancing the chaperone-like activity of α-crystallin to some extent.

Our earlier studies (for review, see Rao et al. 1998, 2002) and studies from other laboratories (Das and Surewicz 1995; Palmisano et al. 1995; Borkman et al. 1996; Smith et al. 1996) have shown that structural perturbation of α-crystallin leads to increased exposure of hydrophobic surfaces. Hydrophobic interactions have a crucial role in the recognition between chaperones and partially unfolded proteins. We have investigated the accessibility of the hydrophobic surfaces of α-crystallin as a function of Arg.HCl concentration by solubilizing the hydrophobic fluorophore, pyrene. Pyrene is sparingly soluble in water. In the presence of α-crystallin (0.3 mg/mL), there is a 4.2-fold increase in the solubility of pyrene at 37°C. In the concentration range studied, Arg.HCl alone does not increase the solubility of pyrene significantly. Pyrene-solubilization by α-crystallin, however, is found to be significantly more in the presence of Arg.HCl than in its absence. The α-crystallin-solubilized fraction of pyrene (see Materials and Methods) increased progressively with increase in Arg.HCl concentration (Fig. 2A). These results suggest that there is an increase in the exposure of hydrophobic surfaces of α-crystallin in the presence of Arg.HCl. We have investigated this aspect further using ANS, a fluorophore that binds to and reports on the hydrophobic surfaces of a protein (Stryer 1965). In the presence of Arg.HCl, the binding of ANS to α-crystallin increased until the concentration of Arg.HCl reached 100 mM (Fig. 2B). At this concentration of Arg.HCl, there was ~38% increase in the fluorescence intensity of ANS at the emission maximum. Beyond this concentration of Arg.HCl, there was a marginal drop in the fluorescence intensity at the emission maximum (Fig. 2B). The pyrene-solubilization studies, as well as the ANS-binding studies, show that Arg.HCl increases the accessible hydrophobic surfaces of α-crystallin. We studied structural changes in α-crystallin further using intrinsic fluorescence, near- and far-UV CD spectroscopy, and glycerol density gradient centrifugation.

The intrinsic tryptophan fluorescence spectrum of α-crystallin did not change considerably in the presence of Arg.HCl, suggesting that there is no significant change in the tryptophan environment. The near-UV CD profiles of α-crystallin in the presence of increasing concentrations of

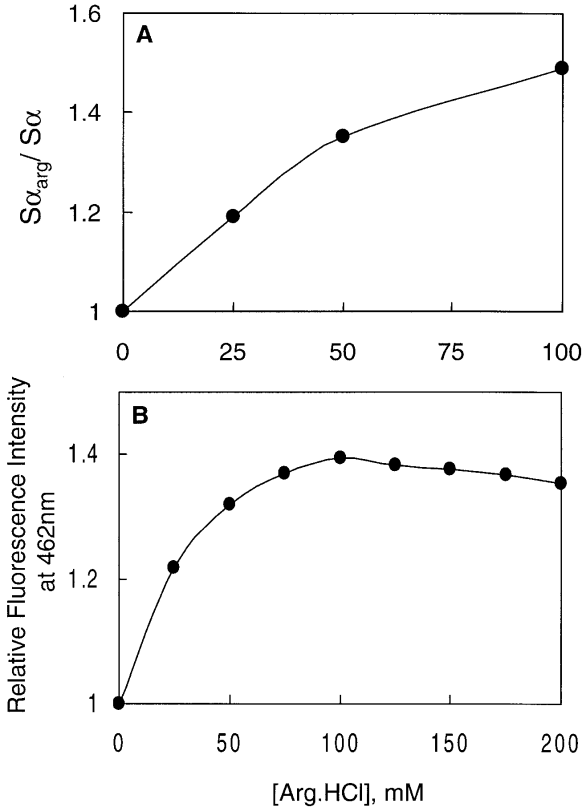

Figure 2. (*A*) Solubilization of pyrene by α-crystallin as a function of concentration of Arg.HCl at 37°C. $S_\alpha = A_\alpha - A_b$, $S_{\alpha Arg} = A_{\alpha arg} - A_{bArg}$, where A_b, A_{bArg}, A_α, and $A_{\alpha arg}$ are the absorbance values at 338 nm of the pyrene solubilized in buffer alone, buffer containing arginine, α-crystallin, and α-crystallin in the presence of arginine, respectively. S_α represents the solubility of pyrene by α-crystallin and $S_{\alpha Arg}$ represents the solubility of pyrene by α-crystallin in the presence of Arg.HCl. (*B*) Relative fluorescence intensity of ANS bound to α-crystallin in the presence of increasing concentrations of Arg.HCl at 37°C.

Arg.HCl show some differences in the region of 270–290 nm (Fig. 3A) suggesting subtle conformational changes at the tertiary structural level. As L-Arg.HCl gives a strong signal in the far-UV region below 230 nm, we used DL-Arg.HCl to perform far-UV CD studies. The far-UV CD spectra of α-crystallin in the absence and the presence of various concentrations of Arg.HCl almost overlap, with a minor change in the lower wavelength region (Fig. 3B). The minima, however, remain unchanged, suggesting that the secondary structure of α-crystallin, largely β-sheet, is not altered in the presence of DL-Arg.HCl. To assess changes in the quaternary structure of the protein, if any, we performed sedimentation experiments through a 10%–40% glycerol gradient in the absence and the presence of 300 mM Arg.HCl. Figure 4 shows significant decrease in the size of α-crystallin in the presence of Arg.HCl. We also used aldolase, catalase, and thyroglobulin as molecular mass standards and estimated the molecular mass of α-crystallin. Our results show that molecular mass of α-crystallin, which is

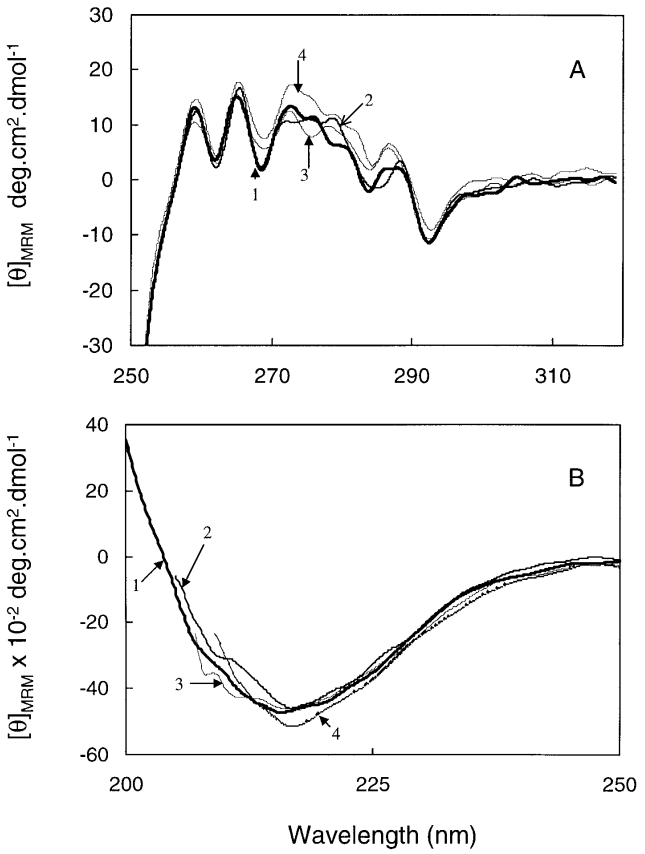

Figure 3. Circular dichroism spectra of α-crystallin in the presence and in the absence of Arg HCl at 37°C. (*A*) Near-UV CD spectra. (*B*) Far-UV CD spectra. α-Crystallin in Buffer A alone (curve *1*) and the buffer containing 100 mM (curve *2*), 200 mM (curve *3*), and 300 mM (curve *4*) Arg.HCl. To record far UV-CD spectra, DL-Arg.HCl was used. ($[\theta]_{MRM}$) Mean residue mass ellipticity.

estimated to be ~700 kD in the absence of Arg.HCl, is significantly decreased in the presence of Arg.HCl (~360 kD). The half-width of the profile of α-crystallin in the presence of Arg.HCl is significantly less (6.25 *x*-axis units) than that in its absence (8.75 *x*-axis units), indicating that the polydispersity of the protein is also decreased in the presence of Arg.HCl. We also observed a similar decrease in the size of the molecule as well as the half-width of the profiles of αA- and αB-crystallin (data not shown). Therefore, our results show that Arg.HCl can bring about subtle changes in the tertiary structure and significant changes in the quaternary structure of either the homo-multimers or the hetero-multimers of αA- and αB-crystallin. Such changes also lead to increased chaperone-like activity of α-crystallin. We have also investigated the effect of aminoguanidine on the quaternary structure of α-crystallin by the glycerol gradient sedimentation. The estimated molecular mass of α-crystallin in the presence of 300 mM AGdn.HCl is 575 kD. This shows that AGdn.HCl also brings about changes in the quaternary structure and reduction in the molecular mass of α-crystallin, however, to a lesser extent compared with Arg.HCl.

Mutations in αA- and αB-crystallin at the conserved arginine residues 116 and 120, respectively, are known to affect structure and chaperone-like activity in vitro (Bova et al. 1999; Kumar et al. 1999). These mutations are known to result in congenital cataract and desmin-related myopathy (Litt et al. 1998; Vicart et al. 1998). It would be of interest to investigate in this context whether the additive Arg.HCl can restore/increase the chaperone-like activity of these mutants. Interestingly, increasing concentrations of the additive Arg.HCl increase the protective effect of R120G αB-crystallin toward the DTT-induced aggregation of insulin at 37°C, the extent of protection being ~75% at 300 mM Arg.HCl. Even 500 mM Arg.HCl did not enhance the activity of R116C αA-crystallin, however (data not shown). Earlier studies from our laboratory have shown that R120G αB-crystallin assembles into multimers with slightly increased molecular mass compared with the wild-type αB-crystallin, whereas R116C αA-crystallin forms very large (800–2000 kD), polydisperse, multimers (Kumar et al. 1999). It is not clear whether such difference in the multimeric nature of these mutants in some way leads to the observed differences in the Arg.HCl-induced effects on chaperone-like activity. As mentioned earlier, Figure 1D shows that the extent of Arg.HCl-induced increase in the chaperone activity of αA-crystallin is less than that of αB-crystallin. Therefore, these results suggest that the wild-type and mutant αA-crystallin, in general, are less sensitive toward Arg.HCl-induced changes in structure and function.

While the exact mechanism of the interaction of Arg.HCl with α-crystallin is not clear, our study shows that in the

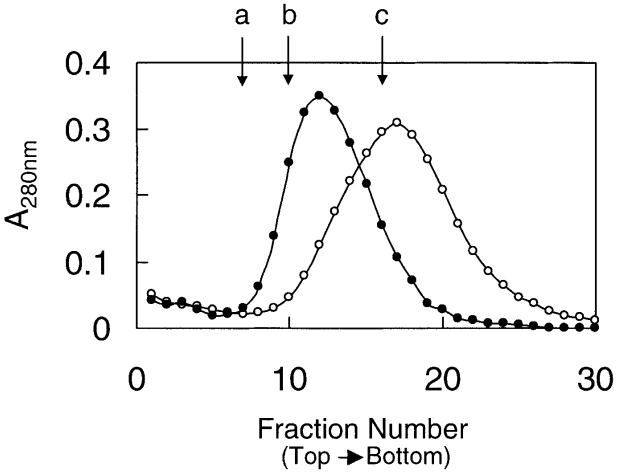

Figure 4. Sedimentation of the Arg.HCl-treated (*filled circles*) and untreated (*open circles*)α-crystallin through a linear glycerol gradient (10%–40%). The samples were incubated at 37°C for 2 h before loading on to the gradient. The positions of proteins used for standard molecular masses are also indicated. (a) Aldolase (158 kD); (b) catalase (232 kD); (c) thyroglobulin (669 kD). See Materials and Methods for details.

presence of Arg.HCl, α-crystallin undergoes subtle tertiary structural changes and significant quaternary structural changes accompanied by increase in the accessible hydrophobic surfaces of the protein. These structural changes result in a significant enhancement in the chaperone-like activity of α-crystallin. The fact that lysine hydrochloride could not enhance the chaperone-like activity of α-crystallin, whereas Arg.HCl and other guanidinium compounds, such as Gdn.HCl and AGdn.HCl, could markedly increase its chaperone-like activity (but to different extents) shows selective interaction of the guanidinium group in arginine with α-crystallin. We have also observed a change in the near-UV CD spectrum and size of α-crystallin in the presence of Lys.2HCl (data not shown), indicating that Lys.2HCl can also interact with the protein. It failed to increase the chaperone-like activity of α-crystallin, however; rather it decreased the activity. Therefore, it appears that the mode of interaction of Lys.2HCl or the consequence of the interaction with α-crystallin is either distinctly different from that of the guanidinium compounds, such as Arg.HCl and AGdn.HCl or the α-crystallin-target protein complex is unstable and aggregation-prone in the presence of Lys.2HCl. It is also known that Arg.HCl and Lys.2HCl exhibit opposite effects on the stability of globular proteins (Lin and Timasheff 1996) though both these amino acids can interact with peptide groups and negatively charged side chains of proteins (Arakawa and Timasheff 1984; Timasheff and Arakawa 1988; Lin and Timasheff 1996).

Growing evidences suggest that some small molecules modulate chaperone function (Clark and Huang 1996; Voziyan et al. 2000; Diamant et al. 2001; Song and Chuang 2001; Tieman et al. 2001). Osmolytes, such as glycinebetaine, proline, and glycerol, have been shown to activate the molecular chaperones GroEL, DnaK, and ClpB (Diamant et al. 2001). The cellular metabolite pantethine has been shown to increase the anti-aggregation activity of α-crystallin (Clark and Huang 1996). Osmolytes, such as sarcosine, potassium glutamate, and trimethylamine-N-oxide, can significantly enhance the folding yields of chaperone-bound glutamine synthetase (Voziyan et al. 2000). Some osmolytes can themselves enhance the folding of the target proteins and also help in release and folding of chaperone-bound target proteins (Voziyan et al. 2000; Song and Chuang 2001; Tieman et al. 2001). Synergistic effects are also observed in some cases (Voziyan et al. 2000). In such a situation, finding out the specific effect(s) of osmolytes on target protein, chaperone, and the chaperone-target protein complex individually becomes difficult. Our study shows that the insulin aggregation system is particularly useful in this context. Arginine is known to reduce aggregation and enhance the refolding yields of some enzymes (Rudolph and Lilie 1996). Our results, however, show that arginine does not decrease the aggregation of insulin and in fact increases the aggregation of insulin. The ability of α-crystallin to prevent this aggregation is increased in the presence of arginine. Our results also show that arginine can bring about changes in tertiary and quaternary structure of α-crystallin leading to increased exposure of hydrophobic surfaces. These results, therefore, suggest that the arginine-induced changes in α-crystallin are important factors, which contribute to the observed arginine-induced enhancement in the chaperone-like activity.

Therefore, our study shows that Arg.HCl, a biologically compatible amino acid, can significantly improve the chaperoning function of α-crystallin by specific interactions with the protein. Our study also demonstrates that aminoguanidine (AGdn.HCl), which has been shown previously to inhibit lens opacification in animal models (Swamy et al. 1996; Inomata et al. 2000), also increases the chaperone-like activity of α-crystallin. Aminoguanidine, lysine, and other amino acids can inhibit glycation and therefore confer protection against diabetes-induced lens opacification (Swamy et al. 1996; Sulochana et al. 1998). It is also possible that the enhancement of chaperone-like activity of α-crystallin is one of the probable mechanisms, if not the sole mechanism, involved in the inhibition of lens opacification by aminoguanidine in some animal models. Arginine might confer beneficial effects against lens opacification because, as our results show, it is more effective in increasing the chaperone-like activity of α-crystallin than aminoguanidine. We are currently testing this possibility in animal models. In addition, arginine may offer other beneficial effects, as it is the substrate for enzymatic production of nitric oxide and is believed to confer cardiovascular protection (Suematsu et al. 2001). It exhibits antioxidant properties in scavenging superoxide radicals (Haklar et al. 1998). Therefore, Arg.HCl is one of the promising agents to exhibit pleotropic beneficial effects in general health and disease resistance.

Materials and methods

Materials

Arg.HCl, DL-Arg.HCl, AGdn.HCl, Lys.2HCl, bovine insulin, and pyrene were obtained from Sigma Chemical Company. ANS was obtained from Aldrich Chemical Company. Gdn.HCl was purchased from Serva, Germany. All other chemicals used in the studies were of Analytical Reagent grade.

Methods

Preparation of α-crystallin and ζ-crystallin

α-Crystallin was purified from calf eye lenses as previously described (Raman & Rao 1994). Recombinant human αA- and αB-crystallins were prepared by cloning, overexpression in *Escherichia coli* and purification as described earlier (Kumar et al. 1999).

ζ-Crystallin was purified from guinea pig eye lenses as described by Rao and Zigler (1992).

Assays for chaperone-like activity of α-crystallin

Aggregation of insulin was initiated by reducing the disulfide bonds as described below. All assays to measure chaperone-like activity were carried out in 10 mM phosphate buffer, pH 7.4, containing 100 mM NaCl (Buffer A). The buffer alone or the buffer containing α-crystallin (0.1 mg/mL), αA-crystallin (0.1 mg/mL) or αB-crystallin (0.025 mg/mL) and the required amount of Arg.HCl was equilibrated at 37°C for 3 min with constant stirring in the sample holder using a Julabo thermostated water bath. Insulin (0.2 mg/mL) was then added and reduction of insulin was initiated by adding 20 μL of 1 M DTT to 1.2 mL of the sample. The extent of aggregation of insulin was measured as a function of time by monitoring 90° scattering at 465 nm using a Hitachi F-4000 fluorescence spectrophotometer. The excitation and emission band passes were set at 3 nm.

Buffer A either in the absence or in the presence of α-crystallin (0.025 mg/mL) and required concentrations of Arg.HCl was incubated at 43°C for 3 min in the thermostated cuvette holder of the fluorescence spectrophotometer. ζ-Crystallin was then added to a final concentration of 0.1mg/mL. The extent of aggregation was monitored as a function of time by measuring the scattering of 465 nm light as described above.

The percentage protection was calculated using the equation $([I_c - I_\alpha]/I_c) \times 100$, where I_c and I_α are the intensities of light scattered by control (target protein alone) and by the sample (plus α-crystallin), respectively. In the cases where additives were added, the I_c was the scattering intensity of the control in the presence of the additive and I_α, the scattering intensity of this sample with additive in the presence of α-crystallin.

Solubilization of pyrene

α-Crystallin solution (0.3 mg/mL in Buffer A) in the absence or the presence of varying concentrations of Arg.HCl was stirred with 1 mM pyrene suspension at 37°C for 30 min. The mixture was centrifuged at 14,000 rpm for 5 min to remove the unsolubilized pyrene. Optical density of the supernatant was measured at 338 nm in a Hitachi-330 UV-Visible spectrophotometer. Solubility of pyrene in Arg.HCl solutions alone was also measured using the above procedure. The solubilization of pyrene by α-crystallin alone or by α-crystallin in the presence of varying concentrations of Arg.HCl was obtained after correcting for the appropriate blanks. To assess the extent of increase in pyrene solubilization by α-crystallin in the presence of Arg.HCl, the ratio of pyrene solubilized by α-crystallin in the presence of a given Arg.HCl concentration ($S_{\alpha arg}$) to pyrene solubilized by α-crystallin alone (S_α) was plotted as a function of Arg.HCl concentration.

Circular dichroism studies

Circular dichroism spectra were recorded using a Jasco J-715 Spectropolarimeter. All spectra are the average of four accumulations. Far- and near-UV CD spectra of α-crystallin (1.5 mg/mL) in the absence and the presence of various concentrations of Arg.HCl were recorded at 37°C using thermostated 0.01 cm and 1 cm pathlength cuvettes, respectively. All spectra were corrected for the respective blanks. As L-arginine hydrochloride shows a large CD signal below 230 nm, we used DL-Arg.HCl to record the far-UV CD spectra of α-crystallin.

Fluorescence studies

Fluorescence spectra were recorded using a Hitachi F-4000 Fluorescence Spectrophotometer equipped with a thermostated cuvette holder. All the studies were performed at 37°C. All spectra were recorded in the corrected spectrum mode. The intrinsic tryptophan fluorescence spectra of α-crystallin (0.2 mg/mL) in Buffer A alone or in the buffer containing the required concentrations of Arg.HCl were recorded by exciting the sample at 295 nm with the excitation and emission band passes set at 3 nm.

To study the binding of the hydrophobic probe, ANS, to α-crystallin, 12 μL of 10 mM methanolic solution of ANS was added to 1.2 mL of a 0.5 mg/mL solution of α-crystallin in Buffer A alone or in the buffer containing the required concentrations of Arg.HCl. The samples were excited at 365 nm and emission spectra recorded from 400–600 nm at 37°C. The excitation and emission band passes were set at 3 and 5 nm, respectively. All spectra were corrected for the respective blanks. Spectra were recorded in the corrected spectrum mode.

Glycerol gradient centrifugation

Glycerol gradient centrifugation was carried out essentially as described by Lambert et al. (1999). α-Crystallin, αA- or αB-crystallin (2 mg/mL) was incubated in 50 mM Tris HCl buffer (pH 7.4) containing 100 mM NaCl, 1 mM EDTA alone or in the presence of 300 mM Arg.HCl for 2 h at 37°C. The samples (0.5 mL) were loaded on top of a 12 mL linear gradient of glycerol (10%–40%) made in 50 mM Tris-HCl buffer, pH 7.4, containing 100 mM NaCl and 1 mM EDTA. Arg.HCl-treated α-crystallin samples were run in the gradient also containing 300 mM Arg.HCl. The tubes were centrifuged for 18 h at 30,000 rpm in a Beckman SW41 rotor at 4°C. Fractions (0.3 mL) were withdrawn from the top using a Haake-Buchler Auto Densi-Flow IIC gradient former/remover and optical density at 280 nm of the fractions were measured using a Shimadzu UV- 1601 spectrophotometer. To estimate the molecular masses, of the α-crystallin samples, proteins with defined molecular masses, such as thyroglobulin (669 kD), catalase (232 kD), and aldolase (158 kD) were used. We have also studied the effect of aminoguanidine on the size of α-crystallin using the similar procedure described above, except the incubation time was 20 min.

Acknowledgments

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