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Molten-Globule State of Carbonic Anhydrase Binds to the Chaperone-like α-Crystallin*

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α-Crystallin, a multimeric protein, exhibits chaperone-like activity in preventing aggregation of several proteins. We have studied the chaperone-like activity of α-crystallin toward heat-induced aggregation of bovine and human carbonic anhydrase. Human carbonic anhydrase aggregates at 60 °C, while bovine carbonic anhydrase does not aggregate significantly at this temperature. Removal of the enzyme-bound metal ion, Zn^{2+}, by EDTA modulates the aggregation behavior of bovine carbonic anhydrase. Fluorescence and circular dichroism studies show that removal of the metal ion from the bovine carbonic anhydrase by a chelator such as EDTA enhances the propensity of the enzyme to adopt the molten-globule state. α-Crystallin binds to this state of the enzyme and prevents aggregation. Fluorescence and circular dichroism studies on the α-crystallin-enzyme complexes show that the enzymes in the complex are in the molten-globule state. These results are of relevance to the interaction of chaperones with the partially unfolded states of target proteins.

α-Crystallin is a multimeric protein present in large quantity in the eye lens. It is made up of acidic (αA) and basic (αB) subunits and is also known to be expressed in nonlenticular tissues under stress or disease (1–6). α-Crystallin is heat-stable (7) and is structurally related to small heat shock proteins (8–11). Its expression can be induced by thermal (8) or hypertonic stress (12). It interacts with membranes (13) and modulates the intermediate filament assembly (14). Horwitz (15) has shown that α-crystallin prevents the aggregation of other crystallins and enzymes. This chaperone-like activity of α-crystallin has been further studied in detail by other workers (16–23). We have earlier investigated the effect of α-crystallin on the photoaggregation of γ-crystallin (19), thermal aggregation of ζ-crystallin, dithiothreitol-induced aggregation of insulin (20), and the rapid refolding of β- and γ-crystallins (21). On the basis of these studies, we postulated that α-crystallin prevents aggregation of other proteins by providing appropriately placed hydrophobic surfaces, a structural perturbation above 30 °C enhances the chaperone-like activity of α-crystallin severalfold.

α-Crystallin from the old human lenses (24) and from the selenite-induced cataractous lenses of an animal model (25) are found to exhibit decreased chaperone-like activity. Our earlier study on refolding of crystallins at high concentrations (21) shows that β- or γ-crystallins aggregate upon refolding while α-crystallin does not aggregate. Co-refolding of β-crystallin or γ-crystallin with α-crystallin prevents the aggregation under similar conditions. All these studies provide further evidences that the chaperone-like activity of α-crystallin is important in the formation and maintenance of the transparency of the eye lens.

In the present study, we have observed that human carbonic anhydrase aggregates at 60 °C while the aggregation of bovine carbonic anhydrase is negligible at the same temperature. Removal of the protein bound Zn^{2+} ion abolishes this differential property. We have utilized this property to further investigate the chaperone-like activity of α-crystallin. The results suggest that α-crystallin binds to the molten-globule states of these proteins and prevents their aggregation. This study should prove useful in understanding of chaperone-substrate interactions.

EXPERIMENTAL PROCEDURES

Materials—Bovine carbonic anhydrase (BCA)[1] (c-3934) and human carbonic anhydrase B (HCA) (c-4396) were purchased from Sigma. BCA, as supplied by Sigma, is a mixture of the isoforms BCA-A and BCA-B. The amount of the isoform A present in the mixture from bovine erythrocytes is negligible compared to the isoform B (26). The data reported in this paper were obtained using BCA (c-3934), and similar results were obtained with bovine carbonic anhydrase B (c-2522)). 8-Anilinonaphthalene-1-sulfonic acid (ANS) was purchased from Aldrich Chemical Co. Guanidinium chloride (GdmCl) was procured from Serva, Heidelberg. α-Crystallin was purified from calf eye lenses as described in our earlier studies (19–21).

Assay for Protein Aggregation—BCA or HCA at a concentration of 0.1 mg/ml in 50 mM Tris HCl buffer (pH 7.6) containing 100 mM NaCl in the presence or in the absence of the required amount of α-crystallin was placed at 60 °C in the thermostated cuvette holder of a Hitachi F-4000 fluorescence spectrophotometer. The excitation and emission monochromators were set at 475 nm with the band passes of 1.5 nm, and the extent of light scattering was monitored with time. The temperature of the sample was maintained using a Julabo circulating water bath, and the temperature was measured using a Physitemp type-T microthermocouple placed in the sample. The buffer also contained 1 mM EDTA in experiments where the protein-bound Zn^{2+} was chelated.

GdmCl-induced Unfolding of HCA and BCA—HCA or BCA at 0.1 mg/ml concentration was incubated in 50 mM Tris HCl buffer (pH 7.6) containing various concentrations of GdmCl in the presence or in the absence of 2 mM EDTA for 2 h. Then, 10 μl of methanolic solution of 10 mM ANS was added to the samples. The fluorescence spectra were recorded with the excitation wavelength of 365 nm in a Hitachi F-4010 fluorescence spectrophotometer. The emission and excitation band passes were set at 3 and 5 nm, respectively. The emission spectra of the samples were recorded again after 14 h of incubation.

For circular dichroism (CD) studies, protein samples at the concentration of 0.5 mg/ml were used. The CD spectra were recorded using a Jasco J-20 spectropolarimeter.

Refolding of HCA and BCA from 1.5 M GdmCl—HCA or BCA at 0.1 mg/ml concentration was incubated in 50 mM Tris HCl buffer (pH 7.6) containing 1.5 M GdmCl or in the buffer containing 2 mM EDTA and 1.5 M GdmCl for 1 h. Then, 25 μl of this sample was added to 475 μl of 50

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[1] The abbreviations used are: BCA, bovine carbonic anhydrase; HCA, human carbonic anhydrase; ANS, 8-anilinonaphthalene-1-sulfonic acid; GdmCl, guanidinium chloride.

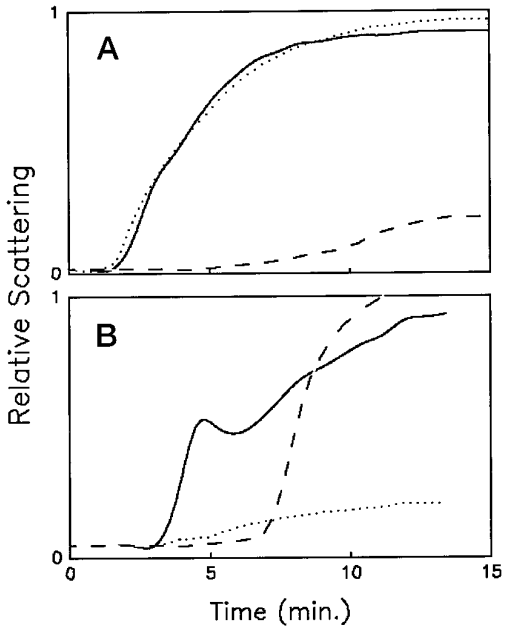

FIG. 1. **Thermally induced aggregation of (*A*) HCA and BCA (*B*) in 50 mM Tris HCl buffer of pH 7.6 containing 100 mM NaCl (····), +1 mM EDTA (——), +1 mM EDTA and 0.1 mg/ml α-crystallin (– – –).** The concentration of the enzymes was 0.1 mg/ml. The extent of aggregation was measured by monitoring the scattering of 475 nm light (see "Experimental Procedures" for details).

mM Tris HCl buffer of pH 7.6 containing 1 mM *p*-nitrophenyl acetate. The enzyme activity was measured by the rate of hydrolysis of *p*-nitrophenyl acetate by monitoring increase in the optical density at 400 nm as a function of time (27). The activity recovered on refolding was calculated with respect to the activity of the native enzymes.

ANS Binding of the Enzyme·α-Crystallin Complexes—The complexes of HCA·α-crystallin and BCA·α-crystallin were obtained by incubating the mixture of these proteins at 1:1 (w/w) ratio in 50 mM Tris HCl buffer (pH 7.6) containing 100 mM NaCl at 58 °C for 10 min. The samples were cooled to room temperature (25 °C), and 10 μl of 10 mM methanolic solution of ANS was added. The fluorescence spectra of these samples were recorded as described above.

Circular Dichroism and Fluorescence Studies on the α-Crystallin-bound Enzymes—HCA or BCA complex with α-crystallin was prepared by incubating the mixture of the enzyme (0.3 mg/ml) and α-crystallin (1 mg/ml) in 50 mM Tris HCl buffer (pH 7.6) at 60 °C for 20 min. The sample was cooled to room temperature. Near and far UV-CD spectra of the sample were recorded using a Jasco J-20 spectropolarimeter. The CD spectrum of a sample of α-crystallin (1 mg/ml) incubated at the same temperature for the same period of time was subtracted from the spectra of the complexes. The resultant spectra, thus obtained, represent the spectra of the enzymes, bound to α-crystallin.

The samples of the complex prepared above were diluted 5-fold with 50 mM Tris HCl buffer (pH 7.6), and the fluorescence spectra were recorded using a Hitachi F-4000 fluorescence spectrophotometer with the excitation wavelength of 295 nm. The excitation and emission band passes were set at 5 and 1.5 nm, respectively. The fluorescence spectrum of heat-treated α-crystallin was subtracted from the fluorescence spectrum of the complex. The resultant spectrum represents the fluorescence spectrum of the enzyme bound to α-crystallin.

RESULTS AND DISCUSSION

α-Crystallin is known to prevent the aggregation of other proteins. However, little is known about the mechanistic aspects of the recognition of the partially unfolded state of proteins by α-crystallin. In order to gain better understanding of this aspect, we have studied the effect of α-crystallin in the heat-induced unfolding of human carbonic anhydrase and the homologous enzyme, bovine carbonic anhydrase. Bovine carbonic anhydrase B and human carbonic anhydrase B share about 55% sequence homology.

Fig. 1*A* shows the light scattering profile as a function of incubation time when a sample of HCA is incubated at 60 °C in the presence and in the absence of α-crystallin. As suggested by the figure, HCA aggregates quite readily at this temperature, and α-crystallin (weight ratio of 1:1) prevents this aggregation. However, BCA does not aggregate to a significant extent at this temperature (Fig. 1*B*). Interestingly, we have found that, upon addition of metal chelators such as EDTA (or ortho-phenanthroline), BCA also aggregates at 60 °C as shown in Fig. 1*B*. The light scattering as a function of incubation time indicates a biphasic aggregation behavior for BCA. Such a biphasic scattering profile is not observed for HCA. The presence of α-crystallin at the ratio of 1:1 (w/w) prevents the first phase of aggregation of BCA but not the second phase as shown in the figure. Titration with α-crystallin suggests that α-crystallin at the weight ratio of 3:1 (α:BCA) prevents both the phases of aggregation of BCA (data not shown). It is interesting to note that the threshold amount of α-crystallin required to prevent the aggregation depends on the choice of the target protein. For example, α-crystallin prevents the heat-induced aggregation of β - or γ-crystallins even at very low concentrations (15, 16). One can speculate that the apparent stoichiometry depends on (i) the extent of exposed hydrophobic surfaces on the target protein, (ii) the complementarity of the interacting surfaces of the chaperone and the target protein, and (iii) the stability of the complex.

The bivalent metal ion, Zn^{2+}, forms the prosthetic part of both the enzymes, HCA and BCA. Thus, the presence of a metal chelator such as EDTA might be expected to chelate away the metal ion from BCA, which leads to destabilization and consequent aggregation of the enzyme at 60 °C. On the other hand, EDTA does not have any significant effect on the thermal aggregation of HCA as shown in Fig. 1*A*. These results suggest that removal of the Zn^{2+} has a significant effect on the stability of BCA but not on the stability of HCA.

In order to further investigate the role of EDTA, we have chosen GdmCl-induced unfolding of these enzymes, since thermal unfolding leads to aggregation. We have studied the effect of EDTA on the GdmCl-induced unfolding of both HCA and BCA. Since hydrophobic forces play a major role in the aggregation of proteins, we have monitored the exposure of hydrophobic surfaces on GdmCl-induced unfolding of these enzymes. The hydrophobic dye, ANS, reports hydrophobic surfaces on proteins (28–30), carbohydrates (31), etc. Its fluorescence is sensitive to the polarity of its microenvironment; upon binding to the apolar surfaces, depending on the extent of hydrophobicity of the surfaces, its emission maximum is shifted to shorter wavelengths and the emission intensity is enhanced. This property of the dye has been demonstrated to identify intermediates on the unfolding pathway of proteins (30). Fig. 2 shows the fluorescence of ANS-bound HCA in the presence and in the absence of EDTA during the GdmCl-induced unfolding of the protein. The fluorescence of ANS upon binding to the native HCA (0 M GdmCl) shows an emission maximum of 525 nm (Fig. 2*A*). The emission maximum shifts to 494 nm as the concentration of GdmCl reaches 1.5 M. Further increase in the concentration of GdmCl leads to a red shift in the emission maximum. The profile of emission maximum *versus* the concentration of GdmCl shows a sharp transition at 1.5 M GdmCl. The presence of 2 mM EDTA does not have a significant effect on this profile as shown in Fig. 2*A*. The profile of emission intensity *versus* GdmCl concentration also shows a similar trend (Fig. 2*B*). Fig. 2, *C* and *D*, shows that prolonged incubation of HCA in the denaturant does not lead to any significant alteration in the profile. These results indicate that at 1.5 M GdmCl, HCA unfolds such that its hydrophobic surfaces are exposed to a greater extent compared to the native or the

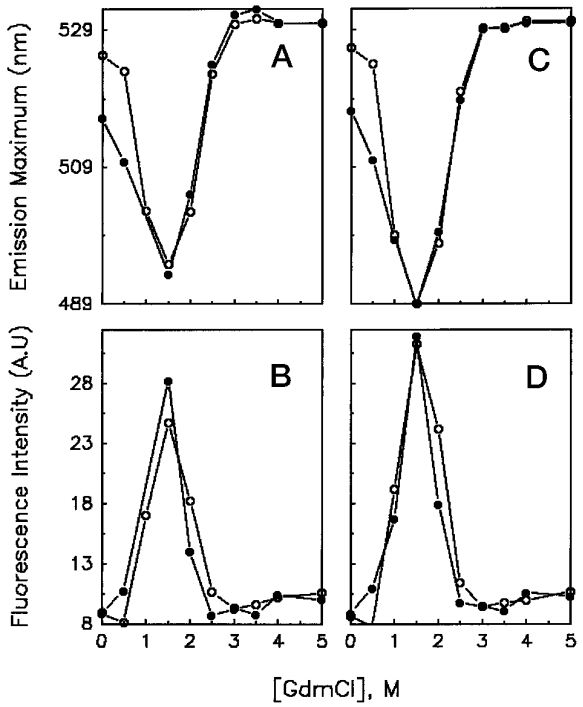

FIG. 2. **Exposure of hydrophobic surfaces of HCA upon GdmCl-induced unfolding measured by ANS binding.** Protein (0.1 mg/ml) was incubated in 50 mM Tris HCl buffer of pH 7.6 containing various concentrations of GdmCl, and 100 μM ANS was added. The fluorescence spectra were recorded after 2 h (*A* and *B*) and 14 h (*C* and *D*) of incubation. ○, buffer alone; ●, buffer containing 2 mM EDTA.

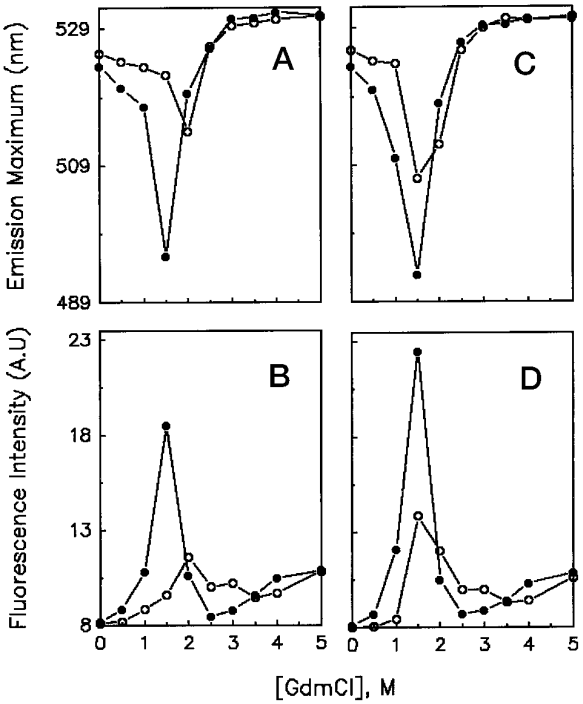

FIG. 3. **Exposure of hydrophobic surfaces of BCA upon GdmCl-induced unfolding measured by ANS binding.** Protein (0.1 mg/ml) was incubated in 50 mM Tris HCl buffer of pH 7.6 containing various concentrations of GdmCl and 100 μM ANS was added. The fluorescence spectra were recorded after 2 h (*A* and *B*) and 14 h (*C* and *D*) of incubation. ○, buffer alone; ●, buffer containing 2 mM EDTA.

totally unfolded enzyme (at 5 M GdmCl), and the extent of this exposure is not altered significantly by the presence of EDTA. In contrast to HCA, BCA exhibits different unfolding behavior, as judged by the exposure of hydrophobic surfaces, in the presence and in the absence of EDTA as shown in Fig. 3. In the absence of EDTA, ANS reports a weak and broad transition at around 2 M GdmCl in the GdmCl-induced unfolding of BCA, and this transition shifts to 1.5 M GdmCl upon prolonged incubation (Fig. 3). However, in the presence of 2 mM EDTA, the transition becomes sharp and stronger at 1.5 M GdmCl as in the case of HCA. These results suggest that the presence of EDTA does influence the unfolding behavior of BCA; the presence of EDTA leads to the increased exposure of hydrophobic surfaces of BCA at 1.5 M GdmCl.

The sharp transition in ANS binding has been shown to indicate a molten-globule state (30). Molten-globule state is characterized by the presence of a substantial amount of secondary structure with no rigid tertiary structure of the molecule (32–36). Folding or unfolding intermediates of proteins, including the molten-globule intermediate, expose hydrophobic surfaces and have a tendency to aggregate. The partially folded or unfolded molecules expose hydrophobic surfaces which become accessible to the hydrophobic dye, ANS. In order to find out whether the unfolding intermediates of HCA and BCA obtained at 1.5 M GdmCl are indeed in the molten-globule state, we have studied the samples for secondary and tertiary structures. Fig. 4*A* shows the near UV-CD spectra of HCA in buffer, buffer containing 1.5 M GdmCl, and the buffer containing 2 mM EDTA and 1.5 M GdmCl. It is evident from the figure that in 1.5 M GdmCl HCA loses almost all its tertiary structure, and the presence of EDTA does not influence the transition significantly. Fig. 4*B* shows the presence of a substantial amount of secondary structure of HCA in the presence of 1.5 M GdmCl. Thus, at this concentration of GdmCl, HCA partially unfolds to its molten-globule state. This result is consistent with the

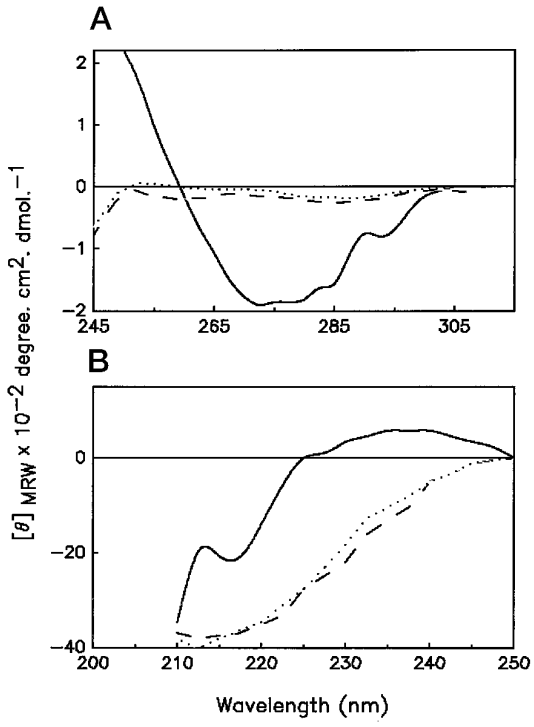

FIG. 4. **Near (*A*) and far (*B*) UV-CD spectra of HCA in 50 mM Tris HCl buffer of pH 7.6 (——), the buffer containing 1.5 M GdmCl (– – –), and the buffer containing 2 mM EDTA and 1.5 M GdmCl (····).**

earlier report (37). However, in contrast to HCA, BCA possesses a significant extent of tertiary structure in 1.5 M GdmCl in the absence of EDTA as shown in Fig. 5*A*. In the presence of 2 mM EDTA and 1.5 M GdmCl, BCA loses its native tertiary structure almost completely. Fig. 5*B* shows the far UV-CD

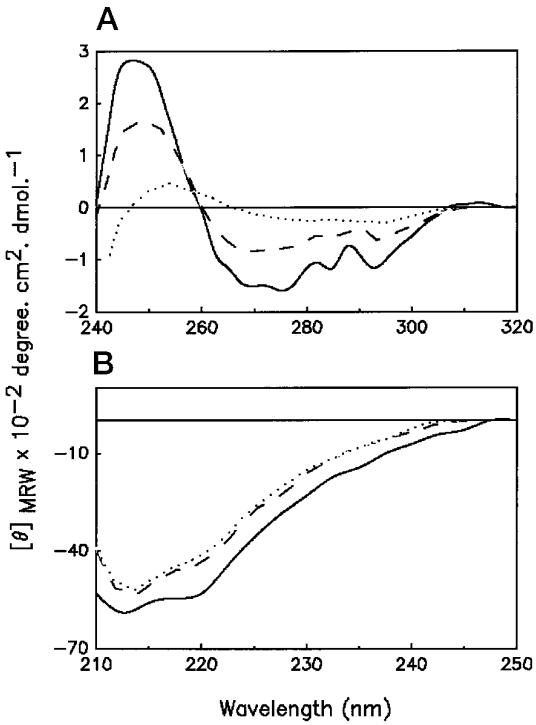

FIG. 5. **Near (*A*) and far (*B*) UV-CD spectra of BCA in 50 mM Tris HCl buffer of pH 7.6 (——), the buffer containing 1.5 M GdmCl (– – –), and the buffer containing 2 mM EDTA and 1.5 M GdmCl (····).**

spectrum of this sample indicating the presence of native-like secondary structure. ANS-binding data described above is consistent with these findings. All these results suggest that the transition to the molten-globule state of BCA is greatly influenced by the presence of metal chelator such as EDTA. In other words, removal of Zn^{2+} ion alters the stability of the enzyme and its unfolding properties, increasing the propensity of the enzyme to adopt the molten-globule state. The molten-globule states of both human and bovine carbonic anhydrases have been known for long time (37–39). However, the propensity of its formation, as modulated by the Zn^{2+} ion, has not been recognized earlier. The unfolding conditions used in the present study could distinguish the differences in the propensity of BCA to form the molten-globule state on removal of the Zn^{2+} ion.

Many metal ion-binding proteins have been shown to unfold through the molten-globule state. α-Lactalbumin, the well studied protein for its three-state unfolding properties, binds Ca^{2+} and removal of this ion does affect the stability and the propensity of this protein to adopt the molten-globule state (40, 41). The homologous enzyme, lysozyme, which does not bind Ca^{2+}, has not been shown to exhibit similar equilibrium molten-globule states. However, it has been shown to exhibit the kinetic molten-globule state (42, 43). The trifluoroethanol-induced partially unfolded state (44) and the partially folded state obtained prior to the formation of disulfide bonds on the refolding pathway (45) have properties similar to molten-globule state. Equine lysozyme which binds Ca^{2+} (46) and engineered human lysozyme whose residues 76–102 have been replaced with the Ca^{2+} binding loop (residues 72–97) of α-lactalbumin (47), have been shown to readily adopt the molten-globule state. Apomyoglobin readily exhibits the three-state unfolding transition (48, 49). Our results suggest that the removal of Zn^{2+} does alter the propensity of BCA to adopt the molten-globule state. The apparent difference between BCA and HCA in this context can be explained as follows. The Zn^{2+}

TABLE I
Refolding of HCA and BCA denatured in 1.5 M GdmCl either in the presence or in the absence of 2 mM EDTA

The activity of the native enzymes has been taken as 100%.

Sample	% activity
HCA (native) + 2 mM EDTA	100
BCA (native) + 2 mM EDTA	97
HCA + 1.5 M GdmCl[a]	13
HCA + 1.5 M GdmCl + 2 mM EDTA[a]	16
BCA + 1.5 M GdmCl[a]	62
BCA + 1.5 M GdmCl + 2 mM EDTA[a]	32

[a] The samples were subjected to refolding as described under "Experimental Procedures." The % activity represents the activity recovered after refolding.

in HCA does not impart significant stability to the enzyme and it might be loosely bound at 1.5 M GdmCl. Therefore, the transition to molten-globule state of HCA is not greatly affected by the metal ion. On the other hand, the Zn^{2+} in BCA might be bound relatively strongly, imparting stability to the enzyme at 1.5 M GdmCl. Therefore, removal of the metal ion destabilizes BCA and affects the transition of the enzyme to the molten-globule state quite significantly. This is readily seen in the refolding of these enzymes from 1.5 M GdmCl. These enzymes bind Zn^{2+} at the active site, and the metal ion is required for the catalytic activity (50). The activities recovered upon refolding these enzymes, denatured in 1.5 M GdmCl either in the presence or in the absence of 2 mM EDTA, are listed in Table I. Refolding of HCA denatured in 1.5 M GdmCl either in the presence or in the absence of EDTA leads to a poor recovery of activity (13–17%). Refolding of BCA from 1.5 M GdmCl yields 62% recovery of the activity. However, refolding of BCA denatured in 1.5 M GdmCl in the presence of 2 mM EDTA yields only 32% of the active enzyme. These results can be explained on the basis of the ease with which the Zn^{2+} ion can be removed from these enzymes at 1.5 M GdmCl. The recovery of activity would depend on the efficiency of recapturing the metal ion by the enzyme molecule during its folding from the molten-globule state to the native state. In the case of HCA, the metal ion is removed easily at 1.5 M GdmCl, and the efficiency of recapturing the metal ion is poor. On the other hand, BCA binds the metal ion relatively strongly at 1.5 M GdmCl and hence the higher recovery of activity upon refolding from its molten-globule state. EDTA scavenges the metal ion, resulting in poor recovery of the activity. We have also found that incubation of native HCA or BCA in 2 mM EDTA does not lead to either significant loss of activities of the enzymes (Table I) or any alteration of their tertiary and secondary structures (data not shown).

As mentioned earlier, removal of Zn^{2+} by EDTA accelerates the aggregation of BCA through a biphasic transition upon thermally induced unfolding of the enzyme, and α-crystallin prevents this aggregation (see Fig. 1). GdmCl-induced unfolding of the enzyme suggests that removal of the metal ion increases its propensity to adopt the molten-globule state. These results, taken together, suggest that α-crystallin might bind to the molten-globule state of the enzyme and prevent its aggregation. This is further supported by the study on the exposed hydrophobic surfaces on the BCA·α-crystallin and HCA·α-crystallin complexes. Rao *et al.* (18) have shown that α-crystallin forms a stable complex with carbonic anhydrase upon heat denaturation of the enzyme. In our present study, the enzyme·α-crystallin complex was obtained by incubating these proteins at 58 °C for 10 min and cooling the sample to room temperature (25 °C). The exposed hydrophobic surfaces of this sample were studied by ANS binding. Heat-stable α-crystallin (7) incubated at 58 °C for 10 min and cooled to room temperature does not show significant alteration in binding to

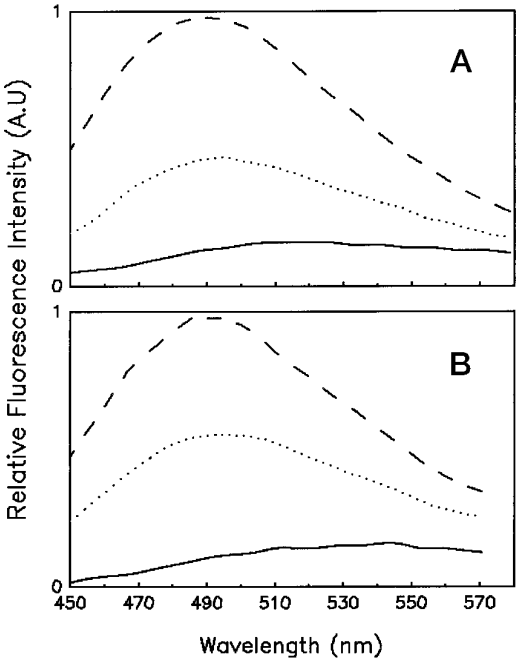

FIG. 6. **Fluorescence of ANS bound to: *A*, HCA alone (——), the mixture of HCA and α-crystallin (····), and HCA·α-crystallin complex (– – –); *B*, BCA alone (——), the mixture of BCA and α-crystallin (····), and BCA·α-crystallin complex (– – –).**

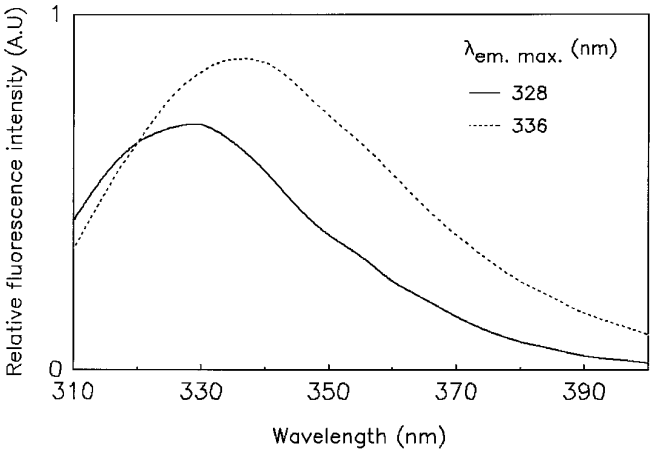

FIG. 7. **Fluorescence spectra of HCA (——) and α-crystallin-bound HCA (– – –) (see "Experimental Procedures" for details).** The excitation and emission band passes were set at 5 and 1.5 nm, respectively. The excitation wavelength was set at 295 nm.

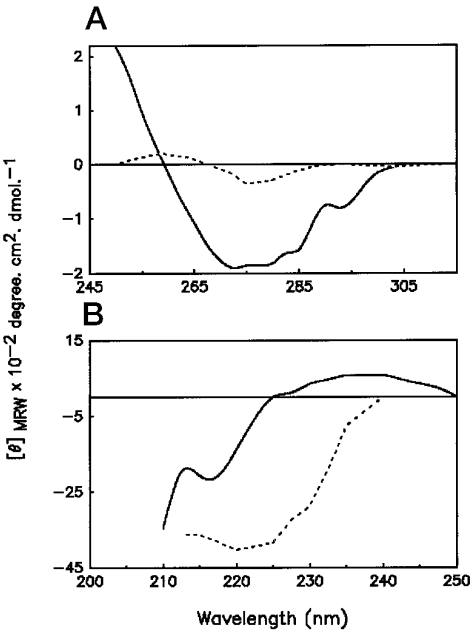

FIG. 8. **Near (*A*) and far (*B*) UV-CD spectra of HCA (——) and α-crystallin-bound HCA (– – –) (see "Experimental Procedures" for details).**

ANS (data not shown). Fig. 6 shows that the accessible hydrophobic surfaces of the enzyme·α-crystallin complex are significantly greater than the mixture of the enzyme and α-crystallin. Similar experiments have been performed to suggest that the chaperonin, GroEL, binds to the molten-globule state of proteins (51).

In order to confirm whether carbonic anhydrase bound to α-crystallin is indeed in the molten-globule state, we further investigated the conformation of HCA or BCA bound to α-crystallin by intrinsic fluorescence and circular dichroism (see "Experimental Procedures" for details). Fig. 7 shows the intrinsic fluorescence of HCA and the HCA bound to α-crystallin. The HCA bound to α-crystallin exhibits the fluorescence spectrum with an emission maximum of 336 nm while the native HCA shows the emission maximum of 328 nm. This shows that the tryptophans of α-crystallin-bound HCA are relatively more exposed to the solvent as compared to that of the native enzyme. The completely unfolded enzyme emits at about 350 nm (data not shown). Near UV-CD spectrum of α-crystallin-bound HCA shows almost no tertiary structure (Fig. 8*A*) while the far UV-CD spectrum of α-crystallin-bound HCA shows that the intermediate bound to α-crystallin possesses a substantial amount of secondary structure (Fig. 8*B*). A comparison of Figs. 8 and 4 suggests that the secondary structure of the intermediate of HCA bound to α-crystallin is similar to the molten-globule state of the enzyme in 1.5 M GdmCl. Similar results were obtained for the α-crystallin-bound BCA (data not shown). All these results clearly demonstrate that the molten-globule state of carbonic anhydrase binds to the chaperone-like α-crystallin.

The molten-globule state of proteins is thought to be involved in a variety of cellular processes such as membrane translocation of proteins (52, 53) and chaperone-assisted protein folding (51). GroEL has been shown to bind to the molten-globule state of rhodanese and chicken dihydrofolate reductase (51). The chaperones of the hsp70 family have been thought to bind to nascent polypeptide chains in their extended conformation (54). Our results suggest that the molten-globule state of carbonic anhydrase binds to α-crystallin.

We conclude that Zn^{2+} modulates the stability of bovine carbonic anhydrase and the propensity of the enzyme to adopt the molten-globule state. α-Crystallin binds to the molten-globule state of BCA and HCA and prevents their aggregation. These results should prove useful in understanding of chaperone-substrate protein interactions.

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