Published in final edited form as:

Biochemistry. 2011 April 26; 50(16): 3279–3281. doi:10.1021/bi200091q.

Binding of gamma crystallin substrate prevents the binding of copper and zinc ions to the molecular chaperone alpha crystallin

Kalyan S. Ghosh, Ajay Pande, and Jayanti Pande*

Department of Chemistry, Life Sciences Research Building, University at Albany, State University of New York, Albany, New York 12222

Abstract

α -crystallin is a small heat shock protein and molecular chaperone. Binding of Cu^{2+} and Zn^{2+} ions to α -crystallin leads to enhanced chaperone function. Sequestration of Cu^{2+} by α -crystallin prevents metal-ion mediated oxidation. Here we show that binding of human γ D-crystallin (HGD, a natural substrate) to human α -crystallin (HAA), is inversely related to the binding of $\text{Cu}^{2+}/\text{Zn}^{2+}$ ions: Higher the amount of bound HGD, lower the amount of bound metal ions. Thus, in the aging lens, depletion of free HAA will not only lower chaperone capacity, but also lower Cu^{2+} sequestration, thereby promoting oxidation and cataract.

The crystallins constitute nearly 90% of the protein content in the eye lens. α -crystallin is a major member of this family with the β/γ crystallins being the other prominent constituents. In the mammalian lens the concentration of α -crystallin may reach up to ~50% of the total crystallin concentration (1). α -crystallin also belongs to the small heat shock protein family and is now a well-known molecular chaperone (2, 3). In this role, it has been shown to bind the β/γ -crystallins and prevent their self-aggregation under conditions that promote their unfolding (1). Analysis of bovine and human lenses shows that upon aging a significant fraction of α -crystallin is associated with the β/γ -crystallins (4). Thus it is generally acknowledged that the β/γ -crystallins are natural substrates in the chaperone function of α -crystallin (5, 6). Recently, in the *cloche* mutant of the zebrafish, it has been shown that when the concentration of α A-crystallin is limiting, the γ -crystallins form aggregates resulting in lens opacity (7). Furthermore, the opacity could be reversed by over-expressing α A-crystallin. Thus α -crystallins prevent the undesired aggregation of γ -crystallins by forming a stable complex with them (8). Such crystallin complexes have in fact been shown in human and other lenses upon aging (2, 4). Here we examine one property of human α A-crystallin (HAA), i.e. its metal-ion binding property when complexed with its substrate, human γ D-crystallin (HGD).

It is known that native α -crystallins bind Cu^{2+} and Zn^{2+} (9–12), and the chaperone activity of the protein improves upon metal-ion binding. α -crystallin is also up-regulated in lens epithelial cells upon treatment with Cu^{2+} (13). More importantly, α -crystallin has been shown to sequester Cu^{2+} ions and ameliorate cytotoxic metal-ion mediated oxidation (9). However, it is not known how the binding of the natural substrate, γ -crystallin, affects the metal-ion binding capacity of α -crystallin. In view of the significance of metal-ion binding to α -crystallin function, it is clearly important to determine whether the metal-ion binding capacity of α -crystallin is maintained once it is complexed with γ -crystallin. In this report we describe the binding of Cu^{2+} and Zn^{2+} ions to complexes of recombinant HAA and HGD

*To whom correspondence should be addressed. Phone: (518) 591-8842. Fax: (518) 442-3462. jpande@albany.edu.

SUPPORTING INFORMATION AVAILABLE Figures S1, S2, S3, S4, Table S5, and detailed Experimental procedures. This material is available free of charge via the internet at <http://pubs.acs.org>.

crystallins. HAA is the predominant form of α -crystallin (14) and HGD is one of the major γ -crystallins in the human lens. The data show that as HAA binds increasing amounts of HGD, its capacity to bind either Cu^{2+} or Zn^{2+} correspondingly decreases. The results suggest that the metal-ion binding capacity of α -crystallin may be compromised *in vivo* with aging, exacerbating oxidative insults in aging and cataract (15).

Mixtures of HAA (3 mg/ml) and HGD (1.5 mg/ml) (2:1, w/w) in 0.1M phosphate buffer, pH 7, were incubated at 50°C for 2h and 16h, and at 55°C for 9h and 16h in a shaker bath. The mixtures were chromatographed on a Superose-6 (GE Healthcare) size-exclusion chromatography (SEC) column and eluted with 0.1M phosphate buffer, pH 7 to separate the HAA-HGD complexes from unreacted HGD (Fig. 1). Under these conditions we expect complexes of HAA-HGD to form, based on the work of many investigators, for example, Wang and Spector (8). Nonetheless, to ensure that HGD alone does not form aggregates that would elute close to HAA, we ran a control experiment with HGD alone. Fig. S1 shows clearly that HGD alone, after heating, still shows the same elution profile as prior to heating.

Metal-ion complexes of proteins were made as follows: A 10-fold molar excess of an aqueous solution of either CuCl_2 or ZnSO_4 was added to HAA or HAA-HGD in 0.1M phosphate buffer, pH 7 with constant stirring while maintaining the pH at 7. The precipitated copper and zinc hydroxides were removed and the solutions containing the Cu^{2+} and Zn^{2+} complexes were passed through a Superose-6 SEC column to remove free metal salts. To determine the concentration of bound Cu^{2+} and Zn^{2+} in the protein complexes, protein solutions were mixed with a solution of 4M guanidinium hydrochloride and incubated at room temperature for 5 min. A freshly prepared solution of 4-(2-pyridylazo) resorcinol (PAR) (100 μM) was then added and absorbances at 514 nm (for Cu^{2+}) and 500 nm (for Zn^{2+}) were measured immediately (16). This method has been used successfully by Rasia et al. (17) to determine Cu^{2+} binding to α -synuclein. Standard solutions of copper and zinc salts were used to calibrate the amount of metal ions. Direct determination of the HAA-bound Cu^{2+} ions was achieved by a second method which is based on the fact that the HAA- Cu^{2+} complex has an absorption band around 600 nm, with a corresponding positive CD band around 575 nm (Fig. S2). Ellipticity values of the Cu^{2+} complexes of HAA-HGD at 575 nm are a direct measure of their bound- Cu^{2+} concentration, and are plotted in Fig. 2.

When a mixture of HAA and HGD is heated above 50°C, increasing amounts of HGD binds to HAA. Fig. 1 shows the separation of the HAA-HGD complexes from free HGD using SEC. By varying the incubation time and temperature, we produced HAA-HGD complexes containing different weight% of bound-HGD. Thus, heating HGD and HAA at 50°C for 2 hours, gave a complex containing 4.3% (wt/wt) of HGD. The proportion of HGD increased to 19.9% (wt/wt) by heating at 55°C for 16 h. Increased binding resulted in the shift of the higher molecular weight peak in Fig. 1 to the left, with a corresponding decrease in the height of the remaining unbound-HGD peak in Fig. 1. In contrast to the peak showing unbound HGD in Fig. 1, the high molecular weight HAA-HGD complexes show variable widths and peak heights, which suggests that a different distribution of hetero-oligomers are formed at each temperature and incubation time.

Following SEC separation, the complexes were reacted with Cu^{2+} and Zn^{2+} salts. Native HAA and HGD were also reacted with both metal ions in parallel and served as controls. The concentration of bound Cu^{2+} and Zn^{2+} in HAA, HGD and HAA-HGD were determined by using PAR (16). HGD itself shows significant Cu^{2+} binding (Fig. S3), but negligible Zn^{2+} binding. Therefore in calculating the amount of Cu^{2+} bound to HAA-HGD complexes, the contribution of Cu^{2+} bound to HGD was subtracted from the total bound Cu^{2+} at each condition. Besides this, Cu^{2+} binding of HAA-HGD was also monitored directly by CD spectroscopy in the visible spectral region (12). Since the Cu^{2+} complex with HGD alone

does not show CD (or absorption) in the visible region, the ellipticity at 575 nm for Cu^{2+} complexes of HAA-HGD is only due to the Cu^{2+} bound to HAA, and not to HGD. In Fig. 2 we plot the corrected concentration of bound metal ions as a function of the weight percentage of HGD in the complexes. Clearly, the decrease in Cu^{2+} binding to the HAA-HGD complex, as observed by CD, agrees well with that observed using the PAR dye. This is further elaborated in Fig. S4. The data clearly show that as the amount of bound HGD increases, the amount of bound Cu^{2+} and Zn^{2+} decrease, revealing an inverse linear relationship i.e. increased binding of HGD proportionally lowers the binding of metal ions to HAA. A similar decrease in Cu^{2+} binding is also observed in HAA upon binding human βB1 -crystallin (HBB), as described in the Experimental procedures of the Supporting Information. Taken together these data suggest that binding of substrates to HAA prevents the binding of metal ions.

To eliminate the possibility that heating HAA at 50/55°C for extended periods of time might have resulted in lowered ion-binding, we performed another control experiment and measured metal ion-binding using HAA alone under identical temperatures and incubation times as those used for the HAA-HGD complexes. Fig. 3 shows that in fact, *higher* amounts of both Cu^{2+} and Zn^{2+} ions bind to HAA under these conditions than they do at room-temperature (22°C). Thus, if preheated HAA is taken as a control, we observe a more pronounced lowering in the metal-ion binding capacity of HAA-HGD.

It is known that upon aging the native crystallins in the lens are gradually depleted and replaced by *hetero*-oligomeric crystallin complexes. Such complexes comprising α -crystallin and members of the β/γ family have been found in the water-soluble as well as water-insoluble protein fractions obtained from aged and cataractous human lenses (4, 18). In this report, we have modeled such complexes *in vitro* using HAA and HGD crystallin, and shown that both Zn^{2+} and Cu^{2+} ion binding drops significantly and almost linearly, when HAA binds β/γ crystallin substrates.

Fig. 2 shows that around 10.1 μmoles of Zn^{2+} and 14.7 μmoles Cu^{2+} bind to about 30.5 μmoles of HAA in the absence of HGD. Thus, the Zn^{2+} to HAA ratio (~1:3) is comparable to the Cu^{2+} to HAA ratio (1:2). Since HGD-binding lowers both Cu^{2+} and Zn^{2+} binding in a similar manner (Fig. 2), it is tempting to suggest that the two ions may bind *independently* at the same site on HAA. This has been shown for some proteins that bind both Cu^{2+} and Zn^{2+} ions (19, 20). The recent X-ray crystal structure of a truncated form of bovine α -crystallin (tr-BAA) with bound Zn^{2+} ions (21) shows that the Zn^{2+} binding site is formed by three different monomer chains of tr-BAA, while each tr-BAA monomer binds three Zn^{2+} . On this basis, the overall stoichiometry $[\text{Zn}^{2+}]/[\text{tr-BAA}]$ in these crystals would be 1:1. In our study, with *full-length* HAA in solution, we obtain only a third of that ratio. This difference may be either due to the particular organization of HAA oligomers in solution as opposed to in a crystal lattice, or the difference between full-length HAA versus tr-BAA, or both. According to the X-ray structure work (21), all amino acid residues bound to Zn^{2+} are part of the α -crystallin domain (22). Thus, it is likely that our results using HAA may have a wider applicability to other proteins containing the α -crystallin domain.

The chaperone complexes in our study were produced using temperatures (50–55°C), significantly higher than physiological temperature, even though the human lens may occasionally approach 50°C *in vivo* (23). We note that under these conditions, the secondary structure of HAA is still maintained (14, 24), which we have confirmed using CD. Such experimental conditions are generally necessary to model reactions *in vitro* that occur over long periods of time in the lens *in vivo*, and whose products accumulate over a lifetime due to negligible protein turnover. However, we have examined complex formation at 40°C, closer to physiological temperature, and show that the rate of HAA-HGD complex

formation drops significantly (Table S1). As expected, the yield of the HAA-HGD complex increases with the increase in reaction time and temperature.

Finally, Cu^{2+} and Zn^{2+} have been shown to increase the chaperone activity of α -crystallin *in vitro* (10). Our data show that complexation of α - with β/γ -crystallins should (1) lower the chaperone activity in the lens, and (2) lead to an increase in the concentration of free Cu^{2+} . Other reports suggest a correlation between cataract and elevation of *total* copper in the lens (25). Thus, a combination of these two independent effects, i.e. lowered chaperone activity, and increase in free Cu^{2+} could lead to a precipitous degradation of lens proteins due to aging and cataract, assuming that the *in vitro* results are applicable to the lens *in vivo*.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

The work is supported by National Institute of Health Grant EY010535. Dr. Geo Rajan's help in preparing the TOC figure is gratefully acknowledged.

Abbreviations

HAA	Human α A-crystallin
BAA	Bovine α A-crystallin
HGD	human γ D-crystallin
HBB	human β B1-crystallin
CD	circular dichroism
PAR	4-(2-pyridylazo) resorcinol
SEC	size-exclusion chromatography

REFERENCES

- Horwitz J. Proc Natl Acad Sci U S A. 1992; 89:10449–10453. [PubMed: 1438232]
- Derham BK, Harding JJ. Prog Retin Eye Res. 1999; 18:463–509. [PubMed: 10217480]
- McHaourab HS, Godar JA, Stewart PL. Biochemistry. 2009; 48:3828–3837. [PubMed: 19323523]
- Sharma KK, Santhoshkumar P. Biochim Biophys Acta. 2009; 1790:1095–1108. [PubMed: 19463898]
- Acosta-Sampson L, King J. J Mol Biol. 2010; 401:134–152. [PubMed: 20621668]
- Peschek J, Braun N, Franzmann TM, Georgalis Y, Haslbeck M, Weinkauf S, Buchner J. Proc Natl Acad Sci U S A. 2009; 106:13272–13277. [PubMed: 19651604]
- Goishi K, Shimizu A, Najarro G, Watanabe S, Rogers R, Zon LI, Klagsbrun M. Development. 2006; 133:2585–2593. [PubMed: 16728471]
- Wang K, Spector A. J Biol Chem. 1994; 269:13601–13608. [PubMed: 7909809]
- Ahmad MF, Singh D, Taiyab A, Ramakrishna T, Raman B, Rao CM. J Mol Biol. 2008; 382:812–824. [PubMed: 18692065]
- Biswas A, Das KP. Biochemistry. 2008; 47:804–816. [PubMed: 18095658]
- Singh D, Tangirala R, Bakthisaran R, Chintalagiri MR. Mol Vis. 2009; 15:2050–2060. [PubMed: 19862354]
- Ganadu ML, Aru M, Mura GM, Coi A, Mlynarz P, Kozlowski H. J Inorg Biochem. 2004; 98:1103–1109. [PubMed: 15149821]

13. Hawse JR, Cumming JR, Oppermann B, Sheets NL, Reddy VN, Kantorow M. Invest Ophthalmol Vis Sci. 2003; 44:672–679. [PubMed: 12556398]
14. Srinivas P, Narahari A, Petrash JM, Swamy MJ, Reddy GB. IUBMB Life. 2010; 62:693–702. [PubMed: 20836128]
15. Beebe DC, Holekamp NM, Shui YB. Ophthalmic Res. 2010; 44:155–165. [PubMed: 20829639]
16. McCall KA, Fierke CA. Anal Biochem. 2000; 284:307–315. [PubMed: 10964414]
17. Rasia RM, Bertoni CW, Marsh D, Hoyer W, Cherny D, Zweckstetter M, Griesinger C, Jovin TM, Fernandez CO. Proc Natl Acad Sci U S A. 2005; 102:4294–4299. [PubMed: 15767574]
18. Takemoto L, Boyle D. Curr Eye Res. 1994; 13:35–44. [PubMed: 8156824]
19. Lorca RA, Coddou C, Gazitua MC, Bull P, Arredondo C, Huidobro-Toro JP. J Neurochem. 2005; 95:499–512. [PubMed: 16190872]
20. Martins EO, Drakenberg T. Inorganica Chimica Acta-Bioinorganic Chemistry. 1982; 67:71–74.
21. Laganowsky A, Benesch JL, Landau M, Ding L, Sawaya MR, Cascio D, Huang Q, Robinson CV, Horwitz J, Eisenberg D. Protein Sci. 2010; 19:1031–1043. [PubMed: 20440841]
22. Caspers GJ, Leunissen JA, de Jong WW. J Mol Evol. 1995; 40:238–248. [PubMed: 7723051]
23. Friedrich MG, Truscott RJ. Invest Ophthalmol Vis Sci. 51:5145–5152. [PubMed: 20435594]
24. Datta SA, Rao CM. J Biol Chem. 1999; 274:34773–34778. [PubMed: 10574947]
25. Rasi V, Costantini S, Moramarco A, Giordano R, Giustolisi R, Gabrieli CB. Annals of Ophthalmology. 1992; 24:459–464. [PubMed: 1485742]

Figure 1.

SEC plots of heated and unheated mixtures of HAA and HGD (2:1 wt/wt). HAA and HGD mixtures were heated at 50°C (for 2 and 16h) and 55°C (for 9 and 16h). All chromatograms are normalized with respect to the total area under the two peaks. Increasing proportion of HGD in the complex shifts the high molecular weight peak progressively to the left.

Figure 2.

Lowering of the bound Cu^{2+} concentration (left axis, black) and bound Zn^{2+} concentration (right axis) with increasing weight% of HGD in the HAA-HGD complexes. Total protein concentration, $30.5 \mu\text{M}$. Variation of ellipticity (mdeg) for Cu^{2+} complexes measured at 575 nm (left axis, gray). Protein concentration was $600 \mu\text{M}$ for CD studies. Ellipticity values are shown as gray symbols with error bars, indicating the level of noise in the spectrum. The lines are linear fits of the data: black solid ($[\text{Cu}^{2+}]$), black dotted ($[\text{Zn}^{2+}]$) and gray (ellipticity).

Figure 3.

Comparison of the amount of bound Cu^{2+} and Zn^{2+} in HAA-HGD complexes, with those in HAA which was pre-heated to the same temperatures and incubation times used to make the HAA-HGD complexes. Data has also been corrected for Cu^{2+} bound to HGD alone.