Published in final edited form as:

FEBS Lett. 2007 May 15; 581(10): 1939–1943.

Specificity of α A-crystallin binding to destabilized mutants of β B1-crystallin.

Hassane S. Mchaourab^{*}, M. Satish Kumar, and Hanane A. Koteiche

Department of Molecular Physiology and Biophysics, Vanderbilt University, Nashville, TN 37232, USA

Abstract

To elucidate the structural and energetic basis of attractive protein interactions in the aging lens, we investigated the binding of destabilized mutants of β B1-crystallin to the lens chaperones, α -crystallins. We show that the mutations enhance the binding affinity to α A- but not α B-crystallin at physiological temperatures. Complex formation disrupts the dimer interface of β B1-crystallin consistent with the binding of a monomer. Binding isotherms obtained at increasing concentrations of β B1-crystallin deviate from a classic binding equilibrium and display cooperative-like behavior. In the context of β B1-crystallin unfolding equilibrium, these characteristics are reflective of the concentration-dependent change in the population of a dimeric intermediate that has low affinity to α A-crystallin. In the lens, where α -crystallin binding sites are not regenerated, this may represent an added mechanism to maintain lens transparency.

Keywords

α -crystallin; β -crystallin; chaperone; small heat-shock protein; cataract

1. Introduction

Lens aging leads to changes in its optical properties that partly reflect the evolution of protein-protein interactions in the fiber cells [1,2]. These quiescent, organelle-free cells contain high concentrations of three families of water-soluble proteins, the crystallins. Age-long accumulation of protein damage, truncations and side-chain modifications [3-6] in a low protein turn-over environment change the solubility and stability of the crystallins which can lead to formation of protein aggregates.

The major protein component, α -crystallin, is an oligomer of two subunits that are related to the small heat-shock protein (sHSP) superfamily. α -crystallin plays a central role in delaying the loss of refractivity and transparency through binding of aggregation-prone proteins [7]. The interaction of α -crystallin with its substrates, referred to as chaperone activity, is energetically driven by thermodynamic destabilization of the substrate [8]. Thus, this activity towards modified β - and γ -crystallin is a major contributor to protein associations in the aging lens. Consistent with this model, old and cataractous lenses have co-aggregates of unfolded and cross-linked crystallins [9].

^{*}Corresponding Author. Fax: (1)-615-322 3307, Email:hassane.mchaourab@vanderbilt.edu

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

In contrast to the extensive studies of α -crystallin chaperone activity using model substrates [10-12], relatively little is known concerning the structural and energetic aspects of β - and γ -crystallins binding to α -crystallin. Previous work predominantly utilized suppression of crystallins thermal aggregation at temperatures that exceed the melting temperature of the chaperone and the substrate [13,14]. We have developed a steady state assay for quantitative assessment of sHSP binding to substrates at physiological temperatures [8]. Using this approach, we demonstrated that a phosphorylation mimic of α B-crystallin “senses” β B2-crystallin thermodynamic stability binding the more destabilized mutants to a higher level [15]. Analysis of the interaction of α -crystallin with β - or γ -crystallin is not only relevant to the molecular understanding of lens transparency but has implications for the etiology of cataracts. In a mouse cataract model associated with a mutation in γ -crystallin, α -crystallin forms co-aggregates with this protein at a temperature well below its unfolding temperature [16].

In the lens, seven β -crystallins are found as polydisperse homo- and hetero-oligomers although recombinant β B1- and β B2-crystallin form dimers in vitro [2]. One of the building blocks is β B1-crystallin, a protein that undergoes extensive truncation as well as deamidation during lens aging [5]. Contrary to expectation, β B1- and β B2- crystallin have different dimerization motifs despite extensive sequence similarity and essentially identical secondary structure topology [17].

In this paper, we show that the multi-state unfolding equilibrium of β B1-crystallin [18] leads to anomalous concentration dependence and cooperative-like shape of the binding isotherms to α A-crystallin. We propose that an unfolding intermediate, which has marginal affinity to α -crystallin, buffers the changes in stability by reducing the population of the unfolded state compared to a two-state equilibrium. In the lens, this may be significant in delaying the formation of stable complexes with α -crystallin.

2. Materials and methods

2.1. Protein expression and purification

β B1-crystallin mutants were expressed, purified and labeled as previously described [18]. α A- and α B-crystallin were expressed and purified as previously described [8,11]. Bimane-labeled mutants of β B1-crystallin are referred to by the suffix B₂.

2.2. Binding isotherms of β B1-crystallin to α -crystallin

Binding isotherms were constructed at fixed concentrations of bimane-labeled β B1-crystallin. For each isotherm, samples containing a fixed concentration of β B1-crystallin and varying amounts of α A-crystallin were incubated for two hours at 37°C. The fluorescence emission of the samples was obtained using a PTI L-format spectrofluorometer equipped with an RTC2000 temperature controller. The bimane probe was excited at 380 nm and the fluorescence emission recorded in the 420 nm – 500 nm range. The bimane intensity at 470 nm was plotted versus the molar ratio of α A- to β B1-crystallin to construct a binding isotherm. Analysis of the binding isotherms was carried out using non-linear least squares as described previously [8].

3. Results

3.1. General methodology

The goal of this paper is to define the energetic threshold for the formation of a stable complex between α - and β -crystallins. Our approach uses site-directed mutagenesis to reduce the substrate free energy of unfolding and then spectroscopically monitor the partitioning of the substrate between folded in solution and stably bound to α -crystallin. Binding is driven by the

dynamic population of unfolded states and its level depends on the energetic balance between binding to the chaperone and refolding [8].

Two destabilized mutants of β B1-crystallin that target the domain packing interface are used in this study, L206A and E208L. To detect binding, a bimane label was introduced either at the native C79 or at site K117C in a background where C79 was replaced with a valine. Analysis of the unfolding of these mutants and the effects of the introduction of the probe has been reported [18]. Binding isotherms are constructed at a fixed β B1-crystallin concentration by monitoring the change in bimane emission intensity in the presence of α -crystallin. The binding levels can be manipulated by changing the fixed concentration of β B1-crystallin in a range defined by the dissociation constant.

3.2. Destabilized β B1-crystallin mutants bind α A-crystallin and the phosphorylation mimic of α B-crystallin

Binding curves in Figure 1A and 1B show that at physiological pH and temperature, α A-crystallin binds destabilized mutants of β B1-crystallin. Addition of α A-crystallin leads to a reduction in the emission intensity of a bimane label attached at site 79 of β B1-crystallin and analysis by size exclusion chromatography confirms the formation of a stable complex (data not shown). Binding does not substantially shift the wavelength of maximum emission suggesting subtle changes in the polarity of the bimane environment. As expected, at similar concentrations the most destabilized mutant L206A binds extensively while the labeled WT (C79B₂) shows little change in the bimane intensity (Figure 1A). Increasing the fixed concentration of β B1-crystallin leads to incipient biphasic shape suggestive of two-mode binding. At low molar ratios of α A-to β B1-crystallin the fluorescence of the bimane increases while at high molar ratio it decreases (Figure 1A). Previous studies using the model substrate T4L have demonstrated that highly destabilized mutants activate a low affinity but high capacity binding mode [8].

At concentrations where significant binding of C79B₂ is observed, both L206A and E208L display evidence of biphasic behavior (Figure 1B). The limited contribution of low-affinity binding hinders the use of non-linear least squares to fit the L206A isotherms. Therefore, the analysis was carried out for selected curves that appear to be predominantly monophasic. For C79B₂, the parameters obtained are consistent with the predominance of high-affinity binding previously correlated with the binding of four α -crystallin subunit to one substrate molecule [8].

Binding to WT α B-crystallin is marginal at these concentrations (data not shown). However, Figure 1C shows that the bimane-labeled β B1-crystallins bind the phosphorylation mimic of α B-crystallin, α B-D3, where three serines (S19, S45, S59) were replaced with aspartates. This is consistent with previous analysis demonstrating that phosphorylation of α B-crystallin is required to activate substrate binding [8,15]. Binding leads to blue shift in the wavelength of maximum emission consistent with a transition of the bimane to a more hydrophobic environment (data not shown).

3.3. Structural features of bound β B1-crystallin

To probe the structure of bound β B1-crystallin, we monitored the dimer interface through the attachment of a bimane probe at site K117. In the crystal structure, this residue is in contact with tryptophan 174 from the symmetry-related subunit. Tryptophans quench bimane emission in a distance-dependent manner [19]. Unfolding relieves the quenching of the bimane at site K117 through the separation of the two monomers [18]. Thus, the trp/bimane pair provides a fingerprint of the dimer interface.

At position K117, complex formation with α A-crystallin or α B-D3 induces a bimane signal change of opposite sign compared to Figure 1. The increase in the emission intensity reflects the separation of the trp and bimane upon binding (Figure 2). This result indicates extensive structural rearrangements at the dimer interface and is consistent with dissociation of the dimer and binding of β B1-crystallin in a monomeric form.

3.4. Cooperative-like binding isotherms and differential binding by α A-crystallin and α B-D3

The shape of the binding curves for α A-crystallin deviates from simple monophasic behavior for all mutants in the K117B₂ background. At low molar ratio of α A to β B1-L206A (Figure 2A), the curve is flat suggesting lack of binding or alternatively a small contribution to the signal from the bound species. Similarly, increasing the fixed concentration of β B1-E208L (Figure 3A) leads to anomalous changes in the shape of the binding curves. The curves are expected to be left shifted at concentrations of the binding partners comparable to the dissociation constant. Figure 3 shows that increasing the fixed concentration of the β B1-crystallin does not significantly change the signal intensity at low molar ratio and the shape of the binding curve is similar to that of a classic cooperative transition. Efficient binding requires a critical ratio of α A-to β B1-crystallin. The critical molar ratio appears correlated to the overall stability; it has a smaller value for K117B₂-L206A than K117B₂. The cooperative-like shape is not observed in the binding of α B-D3 and the curves could be fit assuming a single mode of binding (Figure 2B). Furthermore, the curves obtained at higher fixed concentrations of β B1-crystallin display the expected left shift.

There are two possible origins of the shape changes in the binding isotherms of the destabilized mutants. First, binding may occur in two modes, high- and low-affinity, with opposite fluorescence signal changes that cancel each other. In this case, extensive binding is expected for K117B₂ in the flat region. Analysis of mixtures of α A and mutant β B1-crystallin by size exclusion chromatography shows a substantial fraction of unbound β B1-crystallin (data not shown). Furthermore, the curves could not be fit using a two-mode binding model [8].

Because the flat region depends on the fixed concentration of β B1-crystallin, a more likely origin is the reported concentration-dependent reduction in the fractional population of the unfolded state [18]. The unfolding curves of β B1-crystallin show a change in the midpoint of the transition over the concentration range where the anomalous binding curves are observed. This suggests that increasing the fixed concentration of β B1-crystallin reduces the population of the species that bind α -crystallin and that the intermediate has low binding affinity to α A-crystallin.

4. Discussion

Scheme 1

The results presented in this paper can be interpreted using the thermodynamic model of chaperone activity previously proposed for sHSP [20]. Three coupled equilibria model the dependence of binding on the substrate free energy of unfolding (equation 1) and on the activation state of the chaperone (equation 2). Equation 1 was modified to reflect the multi-state equilibrium of β B1-crystallin [18]: D is the native dimer and I₂ is a dimeric intermediate.

The binding-induced increase in the emission intensity of the bimane at site K117C suggests that bound β B1-crystallin is monomeric. For β B1-crystallin, loss of dimeric structure is coupled to unfolding; therefore we assume that the stably bound state is extensively unfolded (equation 3). A systematic study of the bound structure of T4L monitoring multiple trp/bimane pairs reveals that the bound conformation is extensively unfolded (Claxton D.P., and Mchaourab, H.S., unpublished results).

α A-crystallin affinity and capacity to β B1-crystallin follows the trends observed for T4L. Levels of binding correlate with effective ΔG_{unf} of the mutants with two-mode binding observed for the most destabilized mutant L206A. Nevertheless, there are substantial differences in the shape and concentration dependence of the binding isotherms between T4L and β B1-crystallin. The origin of these specific characteristics can be rationalized using eqs. 1-3. The coupling of unfolding and dissociation implies that the fractional population of the unfolded state (f_u) is inversely related to the total protein concentration (P_t) while the fractional population of the intermediate (f_I) increases with higher P_t . The equilibrium constant that describes the transition from the intermediate (I_2) to the unfolded state is given by $K_{\text{eq}} = [P_t] f_u^2 / f_I$ [21]. Consequently, the decrease in the level of binding at higher β B1-crystallin concentration with binding of β B1 in an unfolded conformation and suggests that the intermediate (I) has no measurable affinity to α A-crystallin.

Conversely, selective binding to the unfolded state implies that an increase in concentration of the α A-crystallin (the x-axis in Figure 3A) leads to an effective decrease in the apparent stability of the intermediate similar to the one induced by lower protein concentration. The model predicts, therefore, that the shape of the binding curve will reflect the cooperative nature of the dissociation of the dimeric intermediate.

The lack of the anomalous concentration dependence in the C79B₂ background can be explained by its higher stability relative to the K117B₂ background [18]. In the latter, the concentration range over which the dimer intermediate is stabilized corresponds to the range used for binding isotherms. For C79B₂, the effect of the intermediate is implicit and is manifested by the overall moderate affinity to α A-crystallin (Figure 1A).

Destabilized mutants of β B1- and β B2-crystallin [15] marginally bind α B-crystallin. This may suggest a limited role of α B-crystallin in preventing the aggregation of β B-crystallins. Binding reported by previous studies occurred under strongly denaturing conditions such as high temperature where the favorable free energy of equation 1 drives the coupled equilibrium thereby overriding the unfavorable activation state of α B-crystallin. The lack of a cooperative-like shape for the binding isotherms to α B-D3 may reflect either its higher affinity to β B1-crystallin or a fundamental difference at the mechanistic level including binding to the dimeric intermediate. Analysis using a larger set of mutants will be necessary to distinguish between these two possibilities.

There are two consequences of the exceedingly high protein concentration in the lens. First, even with dissociation constants in the micromolar range, our coupled model predicts substantial binding for relatively stable proteins. Second, crowding may act to enhance the dissociation constants effectively pulling the coupled equations to the right [22]. However, the findings in this paper suggest that for β B1-crystallin, these effects are counteracted by the concentration dependence of the intermediate states stability which serves to reduce the overall affinity to α -crystallin.

Acknowledgments.

This work was supported by grant R01-12018 from the National Eye Institute, NIH. The authors thank Derek P. Claxton for critical reading of the manuscript.

Abbreviations

sHSP, small heat-shock proteins; T4L, T4 lysozyme; α B-D3, α B-crystallin S19D/S45D/S59D; WT, wild type.

References

1. Bloemendal H, de Jong W, Jaenicke R, Lubsen NH, Slingsby C, Tardieu A. Ageing and vision: structure, stability and function of lens crystallins. *Progress in Biophysics & Molecular Biology* 2004;86:407–85. [PubMed: 15302206]
2. Jaenicke R, Slingsby C. Lens Crystallins and their Microbial Homologs: Structure, Stability and Function. *Critical Reviews in Biochemistry and Molecular Biology* 2001;36:435–499. [PubMed: 11724156]
3. Ajaz MS, Ma Z, Smith DL, Smith JB. Size of human lens beta-crystallin aggregates are distinguished by N-terminal truncation of betaB1. *Journal of Biological Chemistry* 1997;272:11250–5. [PubMed: 9111027]
4. Lampi KJ, et al. Age-related changes in human lens crystallins identified by two-dimensional electrophoresis and mass spectrometry. *Experimental Eye Research* 1998;67:31–43. [PubMed: 9702176]
5. Lampi KJ, Oxford JT, Bachinger HP, Shearer TR, David LL, Kapfer DM. Deamidation of human β B1 alters the elongated structure of the dimer. *Experimental Eye Research* 2001;72:279–88. [PubMed: 11180977]
6. Ma Z, Hanson SR, Lampi KJ, David LL, Smith DL, Smith JB. Age-related changes in human lens crystallins identified by HPLC and mass spectrometry. *Experimental Eye Research* 1998;67:21–30. [PubMed: 9702175]
7. Horwitz J. The function of α -crystallin in vision. *Seminars in Cell and Developmental Biology* 2000;11:53–60. [PubMed: 10736264]
8. Sathish HA, Stein RA, Yang G, Mchaourab HS. Mechanism of chaperone function in small heat-shock proteins. Fluorescence studies of the conformations of T4 lysozyme bound to alphaB-crystallin. *J. Biol. Chem* 2003;278:44214–44221. [PubMed: 12928430]
9. Truscott RJW. Age-related nuclear cataract-oxidation is the key. *Experimental Eye Research* 2005;80:709–25. [PubMed: 15862178]
10. Carver JA, Lindner RA, Lyon C, Canet D, Hernandez H, Dobson CM, Redfield C. The interaction of the molecular chaperone alpha-crystallin with unfolding alpha-lactalbumin: a structural and kinetic spectroscopic study. *Journal of Molecular Biology* 2002;318:815–27. [PubMed: 12054825]
11. Mchaourab HS, Dodson EK, Koteiche HA. Mechanism of Chaperone Function in Small Heat-Shock Proteins. Two-Mode Binding of the Excited States of T4 Lysozyme Mutants by α A-Crystallin. *Journal of Biological Chemistry* 2002;277:40557–40566. [PubMed: 12189146]
12. Rao PV, Horwitz J, Zigler JS Jr. Alpha-crystallin, a molecular chaperone, forms a stable complex with carbonic anhydrase upon heat denaturation. *Biochemical & Biophysical Research Communications* 1993;190:786–93. [PubMed: 8094957]
13. Horwitz J. α -crystallin can function as a molecular chaperone. *Proceedings of the National Academy of Sciences of the United States of America* 1992;89:10449–53. [PubMed: 1438232]
14. Weinreb O, van Rijk AF, Dovrat A, Bloemendal H. In vitro filament-like formation upon interaction between lens alpha-crystallin and betaL-crystallin promoted by stress. *Investigative Ophthalmology & Visual Science* 2000;41:3893–7. [PubMed: 11053291]
15. Sathish HA, Koteiche HA, Mchaourab HS. Binding of destabilized betaB2-crystallin mutants to alpha-crystallin: the role of a folding intermediate. *Journal of Biological Chemistry* 2004;279:16425–32. [PubMed: 14761939]
16. Liu H, et al. Crystallin {gamma}B-I4F mutant protein binds to {alpha}-crystallin and affects lens transparency. *Journal of Biological Chemistry* 2005;280:25071–8. [PubMed: 15878859]
17. Van Montfort RLM, Bateman OA, Lubsen NH, Slingsby C. Crystal structure of truncated human betaB1-crystallin. *Protein Science* 2003;12:2606–12. [PubMed: 14573871]

18. Koteiche HA, Kumar MS, Machaourab HS. Analysis of β B1-crystallin unfolding equilibrium by spin- and fluorescence labeling. Evidence of a dimeric intermediate. FEBS LETTERS. 2007Submitted
19. Mansoor SE, McHaourab HS, Farrens DL. Mapping proximity within proteins using fluorescence spectroscopy. A study of T4 lysozyme showing that tryptophan residues quench bimane fluorescence. Biochemistry 2002;41:2475–84. [PubMed: 11851393]
20. Koteiche HA, McHaourab HS. Mechanism of a Hereditary Cataract Phenotype: Mutations in α A-crystallin activate substrate binding. Journal of Biological Chemistry 2006;281:14273–9. [PubMed: 16531622]
21. Grimsley JK, Scholtz JM, Pace CN, Wild JR. Organophosphorus hydrolase is a remarkably stable enzyme that unfolds through a homodimeric intermediate. Biochemistry 1997;36:14366–74. [PubMed: 9398154]
22. Minton AP. The influence of macromolecular crowding and macromolecular confinement on biochemical reactions in physiological media. Journal of Biological Chemistry 2001;276:10577–80. [PubMed: 11279227]

Figure 1.

(A) Differential binding of bimane-labeled $\beta B1$ -WT and $\beta B1$ -L206A. At higher concentrations, L206A shows clear evidence of two-mode binding. (B) Binding of $\beta B1$ -crystallin and its mutants in the $C79B_2$ background to αA -crystallin at 37°C. The concentration of $\beta B1$ was 30 μM . (C) Binding of $\beta B1$ -crystallin and its mutants to αB -D3 at 37°C. The concentration of $\beta B1$ was 20 μM . The solid lines in panels A and C are a non-linear least-squares fit assuming a single mode of binding. n is the number of $\beta B1$ binding sites per αA -crystallin subunit. K_D is the dissociation constant.

Figure 2.
Binding of β B1-L206A in the C79V/K117B₂ background to (A) α A-crystallin and (B) α B-D3.
The solid line in panel B is a non-linear least squares fit assuming a single mode of binding.

Figure 3.
Cooperative-like binding isotherms of (A) β B1-K117B₂/E208L and (B) β B1-K117B₂ to α A-crystallin.