RESEARCH COMMUNICATION

On the substrate specificity of α -crystallin as a molecular chaperone

Kali P. DAS* and Witold K. SUREWICZ†

Departments of Ophthalmology and Biochemistry, University of Missouri School of Medicine, Room EC-214, Columbia, MO 65212, U.S.A.

α -Crystallin, the major protein of the ocular lens, acts as a molecular chaperone by preventing the thermal aggregation of proteins. However, in contrast with many other heat shock proteins, α -crystallin fails to protect proteins from aggregation during refolding reactions. Our results indicate that α -crystallin

has substrate specificity different from other chaperones and recognizes specific non-native intermediates formed on the denaturation pathway only, with no affinity for intermediates formed on the refolding pathway.

INTRODUCTION

α -Crystallin is the major protein of the vertebrate ocular lens. It consists of two 20 kDa subunits, α A and α B [1–3]. The A and B chains show a high degree of sequence identity and have a similar, predominantly β -sheet secondary structure [4,5]. These subunits self-associate to form a large macromolecular complex with molecular mass of approx. 800 kDa. While the tertiary and quaternary structure of α -crystallin has not yet been determined, a host of structural models have been proposed, including layered and micellar structures and a rhombic dodecahedron model [6–8]. However, none of these models is fully consistent with all experimental findings.

Believed to be strictly a lens-specific protein until recently, α -crystallin has now been found in many non-lenticular tissues [9–11]. Furthermore, the presence of α B-crystallin has been associated with a number of neurodegenerative disorders [9,10]. Another important recent development is the finding that α -crystallin shares extensive structural and functional similarities with small heat-shock proteins (sHSPs) and is inducible by heat and other stress conditions [3,11–13]. Members of the family of sHSPs are abundant in the cytosol and in organelles of various eukaryotes. Similar proteins can also be found in prokaryotic organisms [14]. However, no obvious function has been associated with the ubiquitous sHSPs. A new light on the potential physiological role of sHSPs in general, and α -crystallin in particular, has been shed by a recent discovery that α -crystallin acts *in vitro* as a chaperone by preventing the aggregation of proteins denatured by heat or other insults. The chaperone activity of α -crystallin was first reported by Horwitz [15] and quickly confirmed by others [16–20]. This observation has been extended subsequently to other sHSPs [13,21]. The above findings have led to speculation [14,15] that α -crystallin plays a dual role in the eye: (i) as a structural protein which contributes to the refractive properties of the lens and (ii) as a molecular chaperone which protects other damaged lens proteins from aggregation. The chaperone function of α -crystallin and related sHSPs is also likely to be important in other tissues.

Chaperone activity constitutes a general property of different

classes of HSPs [14,22–24]. Probably the best characterized of those is HSP60 from *Escherichia coli*, known also as the chaperonin GroEL. In common with other chaperones, α -crystallin appears to bind non-native proteins. However, the molecular mechanisms of the interaction between α -crystallin and its substrates remain unknown. In the present study, we have addressed the question of the specificity of α -crystallin in recognizing different protein folding intermediates. Our results indicate that α -crystallin is significantly different from other known chaperones in that it does not recognize the folding intermediates formed during protein refolding reactions *in vitro*. The substrate specificity of α -crystallin appears to be limited to specific non-native intermediates that occur on the denaturation pathway only.

MATERIALS AND METHODS

Materials

The α -, low-molecular-mass β - (β_L) and γ -crystallin fractions were isolated from young bovine lenses. Briefly, the lenses obtained from a local slaughterhouse were separated into nucleus and cortex. The cortical portion was homogenized in 10 mM Tris/0.02% NaN₃, pH 7.8, and centrifuged for 15 min at 27000 g. The water-soluble material was fractionated by gel filtration on a Sephadex G-200 column (5 × 75 cm) using 10 mM Tris, pH 7.8, as an elution buffer. Distinct peaks corresponding to α -, β_L - and γ -fractions were collected separately. The pooled α -crystallin fraction was purified further by Agarose A-5m chromatography to remove the minor high-molecular mass component. The purified crystallin fractions were concentrated and stored at –70 °C. GroEL (in 50% glycerol) was purchased from Epicentre Technologies (Madison, WI, U.S.A.). Immediately before use, GroEL was dialysed against 30 mM Tris/100 mM KCl/2 mM dithiothreitol (DTT) buffer (pH 7.2) to remove glycerol. Rhodanese (type II) and α_{S1} -casein were obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.) and used without further purification. All other chemicals were of analytical grade.

Chaperone activity assays

The ability of GroEL and α -crystallin to suppress the aggregation of substrate proteins during refolding from guanidinium chloride was measured by light-scattering assay [25,26]. The substrate proteins (rhodanese, β_1 - and γ -crystallins) were denatured by 4 h incubation at room temperature in 30 mM Tris/100 mM KCl/1 mM DTT buffer containing 6 M guanidinium chloride. The unfolded proteins were diluted 100-fold (0.12–0.20 μ M final concentration) by injection at 25 °C into 1.5 ml of 30 mM Tris/100 mM KCl buffer containing different amounts of α -crystallin or GroEL. Light scattering was followed using the SLM 8100 spectrofluorimeter with excitation and emission set at 400 nm. During the dilution and turbidity measurements, the solutions were continuously stirred with a magnetic bar.

Suppression of thermally induced aggregation of rhodanese was measured in a Cary 1 spectrophotometer. The enzyme (final concentration of 4.5 μ M) was added to 1 ml of 30 mM Tris/100 mM KCl buffer (pH 7.2) with or without α -crystallin. The mixture was heated at 47 °C and the apparent absorption due to light scattering at 400 nm was followed as a function of time.

Isothermal reaction calorimetry

Titration calorimetry experiments were performed at 25 °C using a Hart Scientific Microtitration Calorimeter. A 1 ml volume of α -crystallin solution (6.7 mg/ml) in 50 mM Tris/100 mM KCl buffer was placed in the reaction cell and, after thermal equilibration was reached, the titration was performed by adding 8 μ l aliquots of α -casein solution (5 mg/ml) in the same buffer.

RESULTS

In order to compare the substrate specificity of α -crystallin with that of the extensively studied chaperonin GroEL, we used rhodanese as a model substrate protein. The folding intermediates of rhodanese and their interaction with GroEL are relatively well characterized. In particular, it has been shown that GroEL is active in preventing the aggregation of rhodanese both during refolding from chemical denaturants [25] as well as during inactivation by heat [27].

The ability of α -crystallin to recognize non-native intermediates of rhodanese on the denaturation pathway was probed by measuring the effect of α -crystallin on the aggregation of the enzyme during thermal denaturation at 47 °C. Figure 1 shows the aggregation kinetics of rhodanese as indicated by light scattering at 400 nm and the suppression of aggregation in the presence of various amounts of the chaperone. Complete prevention of aggregation occurred at the molar ratio of α -crystallin monomer to rhodanese of 1.2:1 or higher (which corresponds to approx. 33 molecules of the enzyme per molecule of 800 kDa α -crystallin oligomer). Lower concentrations of α -crystallin resulted in partial protection. The results obtained with rhodanese are very similar to those reported previously for other heat-treated proteins [2,15–17]. They confirm the notion that α -crystallin is very effective in recognizing and binding non-native intermediates formed during thermal denaturation of proteins.

Drastically different results were obtained when the effect of α -crystallin on the aggregation of rhodanese was probed under refolding conditions. In accord with previous studies [25], dilution of rhodanese from 6 M guanidinium chloride into the buffer results in extensive protein aggregation. As shown in Figure 2, the presence of α -crystallin in the buffer failed to suppress this aggregation. Notably, no protective effect could be detected even when the concentration of oligomeric α -crystallin exceeded three

Figure 1 Thermal aggregation of rhodanese at 47 °C (A) and its suppression by 0.045 μ M α -crystallin (1.8 μ M on the monomer basis) (B) and 0.135 μ M α -crystallin (5.4 μ M on the monomer basis) (C)

The concentration of rhodanese was 4.5 μ M. Molecular masses of the oligomeric and monomeric α -crystallin were taken as 800 and 20 kDa respectively.

Figure 2 Aggregation of rhodanese during refolding from 6 M guanidinium chloride in the absence (a) and presence of α -crystallin at a concentration (oligomer) of 0.21 μ M (b), 0.42 μ M (c) and 0.71 μ M (d)

A 20 μ M solution of rhodanese (15 μ l) in 6 M guanidinium chloride was injected at 100 s into 1.5 ml of a dilution buffer, and protein aggregation was followed by light scattering.

times the molar concentration of rhodanese. The apparent lack of protection in these experiments is rather surprising. Therefore, to verify the correctness of our experimental procedure, the measurements were also performed using the chaperonin GroEL instead of α -crystallin. In agreement with the previous report [25], complete suppression of rhodanese aggregation was observed at an equimolar concentration of GroEL and the enzyme (Figure 3).

Refolding experiments from guanidinium chloride similar to

Figure 3 Aggregation of rhodanese during refolding from 6 M guanidinium chloride in the absence (A) and presence (B) of GroEL at a concentration (oligomer) of 0.24 μ M

Experimental details are as given in the legend to Figure 2.

Figure 4 Aggregation of β_L -crystallin (final concentration of 7.5 μ g/ml or 0.125 μ M, assuming an average molecular mass of 60 kDa) during refolding from 6 M guanidinium chloride in the absence (a) and presence (b) of α -crystallin at a concentration (oligomer) of 0.47 μ M

A 0.75 mg/ml solution of β_L -crystallin (15 μ l) in 6 M guanidinium chloride was injected into 1.5 ml of the dilution buffer and protein aggregation was followed by light scattering.

those described for rhodanese were also performed using β_L - and γ -crystallins as substrate proteins. However, also in these cases α -crystallin did not offer any protection from aggregation (Figure 4). Since the surface hydrophobicity of α -crystallin and its ability to prevent aggregation of denatured proteins appear to increase with temperature [18,20], it was of interest to find out whether elevated temperature could also improve the ability of α -crystallin to prevent the aggregation of proteins under the refolding conditions. Dilution experiments from guanidinium chloride into the buffer containing α -crystallin preincubated at 50 $^{\circ}$ C failed to detect any protective effect of α -crystallin against the aggregation of β_L - or γ -crystallin (aggregation curves not shown).

To further probe the substrate specificity of α -crystallin, we

examined the association between α -crystallin and a model disordered protein, α -casein. It has been shown previously that α -casein competes with unfolded substrates for binding to GroEL [25]. The interaction between α -casein and GroEL can be measured easily by isothermal reaction calorimetry [29]. However, when α -crystallin instead of GroEL was used in calorimetric experiments, we failed to detect any measurable binding between the former protein and α -casein.

DISCUSSION

Molecular chaperones are defined as proteins that bind and stabilize an otherwise unstable conformer of another protein and by doing so facilitate the correct fate of this protein *in vivo* [14,22–24]. This general property is shared by a number of structurally unrelated heat-shock (or stress) proteins. The key activities measured *in vitro* that characterize the protein as a molecular chaperone include (i) the ability to suppress aggregation during protein refolding and (ii) the ability to protect from aggregation during protein denaturation under stress conditions.

α -Crystallin, the major component of the ocular lens, appears to fulfil the broad definition of a molecular chaperone by being able to recognize non-native conformations of protein substrates without interacting with the native states. Indeed, α -crystallin has been shown to prevent non-specific aggregation of proteins under a variety of denaturing conditions, including thermal inactivation [15–17], UV irradiation [18] or reduction of disulphide bonds [19,20]. However, much less information is available regarding the ability of this putative chaperone to interact with non-native states formed during protein-folding reactions. It has been reported previously that α -crystallin, when co-dissolved in 5–6 M guanidinium chloride with γ -crystallin, prevents the aggregation of the latter protein which occurs (in the absence of α -crystallin) upon removal of the chaotropic agent by dialysis [2,28]. While we could reproduce this observation, its interpretation remains ambiguous. Since α -crystallin was dissolved in concentrated guanidinium chloride, it is possible that the binding occurs to the denatured chaperone. To avoid this ambiguity, the refolding experiments described in the present study were performed under conditions which preserve the native structure of α -crystallin. Our data, obtained for three substrates, one model enzyme and two lens proteins, clearly show that α -crystallin in its native conformation is unable to bind folding intermediates formed *in vitro* during the renaturation process.

The present data point to a significantly different substrate specificity of α -crystallin as compared with other chaperone proteins. Thus, the majority of known chaperones can recognize and bind non-native structures formed *in vitro* during protein-folding reactions [25,26,31,32]. In the case of GroEL, the chaperone activity against the aggregation of thermally inactivated enzymes has also been reported [27,30]. In contrast, the specificity of α -crystallin appears to be limited solely to specific conformational intermediates that occur on the denaturation pathway, with no affinity for the intermediates formed during refolding reactions *in vitro*. Furthermore, unlike GroEL, α -crystallin has no affinity for α -casein, a model protein which is largely disordered and has no well-defined secondary structure.

The apparently different substrate specificity of α -crystallin as compared with other chaperones probably reflects distinct physiological functions of the various members of the chaperone family. Thus, proteins such as GroEL, HSP70, HSP90 or the SecB/SecA system are believed to constitute an integral part of protein folding and/or protein translocation machinery *in vivo* [14,22–24]. Their mechanism of action involves trapping of early

folding intermediates and subsequent release of the substrates in an ATP-dependent fashion. This may be contrasted with α -crystallin, which appears to have no ATPase activity and is unlikely to be part of a general folding machinery. The role of the latter protein may be limited to binding (probably in an irreversible manner) of aggregation-prone non-native structures that are formed in response to various physiological stress conditions. Although restricted in substrate specificity, the chaperone function of α -crystallin (and presumably that of other sHSPs) is likely to be of considerable physiological significance, both in the lens as well as in non-lenticular tissues. In particular, with its high capacity to prevent the aggregation of denaturing proteins, α -crystallin appears to be uniquely suited to assist in maintaining the transparency of the ocular lens.

This work was supported in part by the grant from Research to Prevent Blindness, Inc., U.S.A.

REFERENCES

- Hoenders, H. J. and Bloemendal, H. (1981) in *Molecular and Cellular Biology of the Eye Lens* (Bloemendal, H., ed.), pp. 279–326, John Wiley and Sons, New York
- Horwitz, J. (1993) *Invest. Ophthalmol. Vis. Sci.* **34**, 10–21
- Groenen, P. J. T. A., Merck, K. B., de Jong, W. W. and Bloemendal, H. (1994) *Eur. J. Biochem.* **225**, 1–19
- Thomson, J. A. and Augusteyn, R. C. (1989) *Biochim. Biophys. Acta* **994**, 246–252
- Surewicz, W. K. and Olesen, P. R. (1995) *Biochemistry* **34**, 9655–9660
- Tardieu, A., Laporte, D., Licinio, P., Krop, B. and Delaye, M. (1986) *J. Mol. Biol.* **192**, 711–724
- Augusteyn, R. C. and Koretz, J. F. (1987) *FEBS Lett.* **222**, 1–5
- Wistow, G. (1993) *Exp. Eye Res.* **56**, 729–732
- Iwaki, T., Kume-Iwaki, A., Liem, R. K. H. and Goldman, J. E. (1989) *Cell* **57**, 71–78
- Lowe, J., McDermott, H., Pike, I., Spendlove, I., Landon, M. and Mayer, R. J. (1992) *J. Pathol.* **166**, 61–68
- Sax, C. M. and Piatigorsky, J. (1994) *Adv. Enzymol.* **69**, 155–201
- Klemenz, R., Frohli, E., Steiger, R. H., Schafer, R. and Aoyama, A. (1991) *Proc. Natl. Acad. Sci. U.S.A.* **88**, 3652–3656
- Merck, K. B., Groenen, P. J. T. A., Voorter, C. E. M. et al. (1993) *J. Biol. Chem.* **268**, 1046–1052
- Jakob, U. and Buchner, J. (1994) *Trends Biochem. Sci.* **19**, 205–211
- Horwitz, J. (1992) *Proc. Natl. Acad. Sci. U.S.A.* **89**, 10449–10453
- Rao, P. V., Horwitz, J. and Zigler, J. S. (1993) *Biochem. Biophys. Res. Commun.* **190**, 786–793
- Wang, K. and Spector, A. (1994) *J. Biol. Chem.* **269**, 13601–13608
- Raman, B. and Rao, C. M. (1994) *J. Biol. Chem.* **269**, 27264–27268
- Farahbakhsh, Z. T., Huang, Q.-L., Ding, L.-L. et al. (1995) *Biochemistry* **34**, 509–516
- Das, K. P. and Surewicz, W. K. (1995) *FEBS Lett.* **369**, 321–325
- Jakob, U., Gaestel, M., Engel, K. and Buchner, J. (1993) *J. Biol. Chem.* **268**, 1517–1520
- The Biology of Heat Shock Proteins and Molecular Chaperones* (1994) (Morimoto, R. I., Tissieres, A. and Georgopoulos, C., eds.), Cold Spring Harbor Laboratory Press, New York
- Hendrick, J. P. and Hartl, F.-U. (1993) *Annu. Rev. Biochem.* **62**, 349–384
- Becker, J. and Craig, E. A. (1994) *Eur. J. Biochem.* **219**, 11–23
- Martin, J., Langer, T., Boteva, R., Schramel, A., Horwich, A. L. and Hartl, F.-U. (1991) *Nature (London)* **352**, 36–42
- Wiech, H., Buchner, J., Zimmermann, R. and Jakob, U. (1992) *Nature (London)* **358**, 169–170
- Mendoza, J. A., Lorimer, G. H. and Horowitz, P. M. (1992) *J. Biol. Chem.* **267**, 17631–17634
- Boyle, D., Gapalakrishnan, S. and Takemoto, L. (1993) *Biochem. Biophys. Res. Commun.* **192**, 1147–1154
- Lin, Z., Schwarz, F. P. and Eisenstein, E. (1995) *J. Biol. Chem.* **270**, 1011–1014
- Holl-Neugebauer, B., Rudolph, R., Schmidt, M. and Buchner, J. (1991) *Biochemistry* **30**, 11609–11614
- Langer, T., Lu, C., Echols, H., Flanagan, J., Hayer, M. K. and Hartl, F. U. (1992) *Nature (London)* **356**, 683–689
- Randall, L. L., Topping, T. B. and Hardy, S. J. S. (1990) *Science* **248**, 860–863