α B-crystallin-assisted reactivation of glucose-6-phosphate dehydrogenase upon refolding

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α B-crystallin, a small heat-shock protein has been shown to prevent the aggregation of other proteins under various stress conditions. We have investigated the role of α B-crystallin in the reactivation of denaturant [GdmCl (guanidinium chloride)]-inactivated G6PD (glucose-6-phosphate dehydrogenase). Studies indicate that unfolding and inactivation of G6PD by GdmCl proceeds via formation of a molten globule-like state at low concentrations of GdmCl, which was characterized by having maximum surface hydrophobicity and no catalytic activity. At high concentrations of GdmCl, G6PD was completely unfolded, which upon dilution-induced refolding yielding 35 % of original activity. In contrast, no activity was recovered when G6PD was refolded from a molten globule-like state. Interestingly, refolding of completely unfolded G6PD in the presence of α B-crystallin resulted in 70 % gain of the original activity, indicating that α B-crystallin assisted in enhanced refolding of G6PD. Intriguingly, α B-

crystallin was unable to reactivate G6PD from a molten globule-like state. Size-exclusion chromatography data indicate that α B-crystallin-assisted reactivation of completely unfolded G6PD is concomitant with the restoration of the native structure of G6PD. Nonetheless, α B-crystallin failed to reactivate G6PD from preformed aggregates. Moreover, methylglyoxal-modified α -crystallin, which occurs in aged and diabetic cataract lenses, was less efficient in the reactivation of denaturant inactivated G6PD. Diminished chaperone-like activity of α -crystallin due to post-translational modifications may thus result in the accumulation of aggregated/inactivated proteins.

Key words: α B-crystallin, chaperone-like activity, glucose-6-phosphate dehydrogenase (G6PD), guanidinium chloride, refolding, small heat-shock protein.

INTRODUCTION

Small heat-shock proteins (sHSPs), distributed across prokaryotes and higher eukaryotes, are a special class of molecular chaperones involved in preventing the denaturation and aggregation of proteins, thus giving thermoresistance and aid in maintaining cellular protein homeostasis [1,2]. In the process of their action, sHSPs form soluble complexes with non-native proteins and prevents the formation of irreversible aggregates. These sHSP-bound substrates are further refolded to their native state by ATP-dependent chaperones, such as Hsp70–DnaK [3,4]. Almost all sHSPs exist as oligomeric complexes of 12–40 subunits, with monomeric sizes ranging from 14 to 26 kDa [1]. sHSPs are characterized by the presence of a conserved ‘ α -crystallin domain’ at their C-terminal region, spanning approx. 80–110 amino acids [5]. Several studies have demonstrated the critical role of ‘ α -crystallin domain’ in the structure and chaperone function of sHSP [6,7].

α -Crystallin, a prominent member of the sHSP family, constitutes the major portion of eye lens cytoplasm, reaching up to 50 % of the total soluble protein and contains two polypeptides, α A and α B [8–10]. However, in non-lenticular tissues, α -crystallin is mainly present either as α A- or α B-homopolymers [10]. α B-crystallin shows a wider extralenticular distribution and appears to have a physiological function in tissues like heart, kidney, brain and muscle [10]. Like other sHSPs, α -crystallin has been shown to suppress the aggregation of heat, UV-irradiated and chemically denatured proteins [8–11]. In this direction, α -crystallin has been

shown to facilitate proper refolding upon denaturation and also to protect several enzymes against inactivation [12–19]. However, it is still debated whether α -crystallin is capable of protecting enzymes from inactivation [9,11], more so the mechanism of enzyme reactivation by α -crystallin remains incomplete.

G6PD (glucose-6-phosphate dehydrogenase), a rate-limiting enzyme of the HMP (hexose monophosphate) pathway, is a dimeric protein of 104 kDa. A major function of the HMP pathway is to supply reducing equivalents (NADPH) required for various biosynthetic reactions as well as for the maintenance of GSH. NADPH and GSH along with glutathione reductase constitute the glutathione redox cycle and protect thiol groups of lens proteins from various insults [20]. Previously, we have shown that exposure of rat lens to UV radiation *in vitro* leads to inactivation of many enzymes including G6PD [21]. Later, we showed that α A- and α B-crystallins protect G6PD from UVB irradiation-induced inactivation [18]. In addition, it was also demonstrated that glycation-induced inactivation of G6PD was prevented in the presence of α -crystallin [22]. In the context of the demonstrated ability of α -crystallin to prevent inactivation of G6PD under different stress/denaturing conditions, it was of great interest to understand the ability of α B-crystallin to assist the refolding and reactivation of G6PD against GdmCl (guanidinium chloride)-induced inactivation, and to elucidate the possible mechanism of action. Therefore, in the present study, we investigated the effects of α B-crystallin during refolding and reactivation of G6PD against GdmCl-induced inactivation.

Abbreviations used: ANS, 8-anilino-1-naphthalene-sulphonic acid; CS, citrate synthase; G6PD, glucose-6-phosphate dehydrogenase; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GdmCl, guanidinium chloride; HMP, hexose monophosphate; MGO, methylglyoxal; SEC, size-exclusion chromatography; sHSP, small heat-shock protein; XR, xylose reductase.

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MATERIALS AND METHODS

Materials

ATP, ANS (8-anilino-1-naphthalene-sulphonic acid), dithiothreitol, glucose 6-phosphate, G6PD (from *Leuconostoc mesenteroides*), GdmCl, NADP (sodium salt), lysozyme and MGO (methylglyoxal) were obtained from Sigma. α B-crystallin clone was a gift from Professor J. M. Petrash (Washington University, St. Louis, MO, U.S.A.). Unless otherwise stated, all other chemicals were of highest purity available and procured from local companies.

Purification of recombinant human α B-crystallin

Human recombinant α B-crystallin was purified according to the methods described earlier [23]. Purity of α B-crystallin was found to be 99% as analysed by SDS/PAGE. Concentration of α B-crystallin was calculated based on the absorbance at 280 nm, using molar absorption coefficient $\epsilon_{280} = 19\,000\text{ M}^{-1}\cdot\text{cm}^{-1}$ [24]. Recombinant α B-crystallin molecular mass is taken as 650 kDa.

Purification of α -crystallin

α -Crystallin was purified from calf lenses as described earlier [25]. The purity of α -crystallin was assessed by SDS/PAGE and protein concentration determined by modified Lowry method [37]. Molecular mass of calf α -crystallin is taken as 800 kDa.

Denaturant-induced unfolding of G6PD

Stock solutions of G6PD made by reconstituting the lyophilized enzyme in 50 mM Tris/Cl buffer (pH 7.4) were centrifuged at 10 000 *g* to remove insoluble particles, if any. G6PD homogeneity as assessed by SDS/PAGE was 99%. Unfolding of G6PD was performed by mixing the stock solutions of G6PD and GdmCl (8 M) with 50 mM Tris/Cl buffer (pH 7.4) to give the desired concentration of protein (0.5 μ M) and denaturant (0–6 M). Solutions were incubated at room temperature (25°C) overnight, and intrinsic tryptophan fluorescence (both intensity and emission maximum) of the protein was measured. Molecular mass of the G6PD dimer is taken as 104 kDa.

CD studies

CD spectra were recorded at room temperature using a Jasco J-810 spectropolarimeter. All spectra are an average of six accumulations. Far- and near-UV CD spectra of G6PD equilibrated at different concentrations of GdmCl in 10 mM Tris/Cl buffer (pH 7.4) were recorded at room temperature using cells of 0.2 and 0.5 cm path length respectively. All spectra were corrected with respective blanks. Protein concentrations used for far- and near-UV were 0.1 and 0.5 mg/ml respectively. The CD data are expressed as molar ellipticity ($\text{deg}\cdot\text{cm}^2\cdot\text{dmol}^{-1}$).

Refolding/reactivation of G6PD

G6PD (15 μ M in 50 mM Tris/Cl buffer, pH 7.4) was denatured by rapidly mixing with an equal volume of 2.4 and 8 M GdmCl solution in polypropylene tubes and allowed to stand for 3 h at room temperature. Dilution-induced (1:30 and 1:50 for 1.2 and 4 M incubated G6PD respectively) refolding and renaturation was studied at room temperature in renaturation buffer (50 mM Tris/Cl buffer, pH 7.4) in the presence and absence of α B-crystallin at the concentrations indicated in the Figure legends. ATP, KCl and MgCl_2 were included in the renaturation buffer at 5, 10 and 2.5 mM respectively for reactivation studies in the presence of ATP.

Heat-induced inactivation of G6PD

G6PD (0.25 μ M) in 100 mM Tris buffer (pH 7.4) was incubated in the absence or presence of 0.125 μ M α B-crystallin at 42°C. Activity of G6PD kept in ice was taken as 100%.

Enzyme assay

G6PD activity was assayed by a spectrophotometric method as described earlier [21], with the exception that 100 mM glycylglycine buffer was replaced with 100 mM Tris/Cl buffer (pH 7.4). Increase in absorbance at 340 nm due to NADP reduction was monitored in a Cary 100 UV/Vis Bio spectrophotometer. The percentage protection was calculated using the formula: % protection = $((A_0 - A)/(A_0)) \times 100$, where A_0 and A represent the activity of G6PD in the absence and presence of α B-crystallin or ATP respectively.

Fluorescence measurements

Tryptophan fluorescence emission spectra (300–400 nm) of 0.1 mg/ml G6PD as a function of GdmCl (0–6 M) were recorded by excitation at 295 nm. Fluorescence of ANS in the presence of native- and GdmCl-denatured G6PD was measured by excitation at 390 nm and following the emission between 450 and 550 nm. G6PD (0.1 mg/ml) was incubated with 50 μ M ANS for 30 min at room temperature, and fluorescence of the protein-bound dye was measured. All measurements were performed in 50 mM Tris/Cl buffer (pH 7.4). Background fluorescence due to buffer, ANS and GdmCl were corrected with appropriate protein and buffer blanks. All the measurements were taken at 25°C on a Jasco FP-6500 spectrofluorometer using 3 nm spectral bandwidths for both excitation and emission.

Gel filtration chromatography

The interaction of α B-crystallin with G6PD during its refolding was studied by gel filtration chromatography. After allowing to refold, the samples were centrifuged at 10 000 *g* for 10 min at 4°C to remove insoluble protein, if any, then filtered using a 0.22 μ m filter and applied on to a 600 mm $\times$ 7.5 mm TSK-G4000 SW column (Tosoh, Tokyo, Japan) connected to a Shimadzu HPLC system. The column was equilibrated and proteins were eluted with 100 mM sodium phosphate buffer (pH 7.2) containing 100 mM sodium sulphate and 0.05% sodium azide with a flow rate of 1 ml/min. After refolding, the molar ratio of G6PD and α B-crystallin was 1:1 (0.75 μ M each).

Incubation of α L-crystallin with MGO

A stock of 1 M MGO solution was prepared in 100 mM sodium phosphate buffer (pH 7.5) and the pH was adjusted to neutral if required. α -Crystallin was incubated with 10 mM MGO in 100 mM sodium phosphate buffer (pH 7.5) at 37°C in the dark, under sterile conditions [16]. After 72 h, the unbound MGO was removed by dialysis against 100 mM sodium phosphate buffer (pH 7.5) and protein concentration was determined by modified Lowry method.

RESULTS AND DISCUSSION

The biologically active three-dimensional structure of a protein is obtained through a series of folding events. The folding process for oligomeric proteins is more complex as the acquisition of the quaternary structure entails both intramolecular refolding of the individual polypeptide chains and simultaneous intermolecular interactions between the various subunits. While some

Figure 1 Unfolding of G6PD with GdmCl followed by intrinsic tryptophan fluorescence

G6PD (0.1 mg/ml) was incubated in 0–6 M GdmCl overnight at room temperature before fluorescence measurements were made. (A) Relative fluorescence emission intensity at 340 nm and (B) emission wavelength maximum (λ_{max}). a.u., arbitrary units.

proteins can fold/refold to their active form spontaneously, some need involvement of a group of molecules called chaperones. Refolding studies of several enzymes *in vitro* provided important clues regarding the function of these chaperones. However, a central question in sHSP-assisted refolding is the restoration of enzyme activity. We have investigated the role of α B-crystallin on refolding-induced reactivation of G6PD to gain greater insights into the chaperone-like function of α B-crystallin.

GdmCl-induced unfolding of G6PD

GdmCl-induced unfolding of G6PD was monitored by tryptophan fluorescence. Figure 1 shows the changes in the intrinsic tryptophan fluorescence of the protein as a function of GdmCl concentration. Initially, at low concentrations of GdmCl (0.4 M), G6PD exhibits increase in tryptophan fluorescence with a blue shift in emission. The emission maximum gradually shifted towards the red end of the spectrum and a decrease in fluorescence intensity was observed with increasing concentrations of GdmCl. These results indicate complete exposure of the tryptophan residues to polar environment. Further, the results also suggest that the unfolding pathway of G6PD consists of more than one intermediate and this is expected, as G6PD is a multimeric protein. In order to obtain further insights into G6PD unfolding, we have studied the changes in secondary and tertiary structure of G6PD at various concentrations of GdmCl by CD spectroscopy. Figure 2(A) shows the far-UV CD spectra of G6PD at different concentrations of GdmCl. Unlike tryptophan fluorescence, negligible changes were noticed in CD spectra up to 1.2 M GdmCl. Even at 2.0 M GdmCl concentration, G6PD retained substantial amount of secondary structure. G6PD lost its secondary structural elements completely above 3 M GdmCl. In contrast with changes observed in secondary structure, G6PD lost its tertiary structure at 1.2 M GdmCl as the protein exhibited an altered signal in the

Figure 2 Unfolding of G6PD with GdmCl followed by CD spectroscopy

(A) Far-UV CD spectra of G6PD in different concentrations of GdmCl. Curves 1–6 represent the spectra of the protein in 0, 0.9, 1.2, 2.0, 3.0 and 4.0 M GdmCl respectively. (B) Near-UV CD spectra of G6PD in different concentrations of GdmCl. Curves 1–3 represent the spectra of the protein in 0, 1.2 and 3.0 M GdmCl respectively.

Figure 3 ANS-associated fluorescence of G6PD as a function of GdmCl concentration

a.u., arbitrary units.

near-UV region (Figure 2B), suggesting that the intermediate(s) exhibit substantially loosened side-chain packing. At 3 M GdmCl, G6PD was in a completely unfolded state as indicated by CD and fluorescence spectroscopy. Binding of the fluorescent probe, ANS, to the hydrophobic surfaces on the protein results in an increase in its emission intensity, while the emission maximum shifts to shorter wavelengths. ANS can be used to detect partially unfolded/refolded intermediate states with exposed hydrophobic surfaces as in the case of a molten globule-like state. Therefore, the nature of the intermediates during the unfolding of G6PD was further characterized by ANS binding. As shown in Figure 3, a maximum increase in the fluorescence intensity of ANS binding to G6PD was observed at 1.2 M GdmCl, indicating maximum exposure of hydrophobic surfaces. G6PD showed complete collapse in its surface hydrophobicity at and above 3 M GdmCl, representing the formation of a completely unfolded state. The

Figure 4 Enzyme activity of G6PD at different concentrations of GdmCl

G6PD (0.5 μ M) was incubated in 0–6 M GdmCl for 4 h at room temperature before enzyme activity was assayed. Results shown are average for three independent assays.

unfolding nature of G6PD in the present study was consistent with a previous study, where it was demonstrated that the enzyme undergoes a conformational change, exposing tryptophan residues to the solvent, with some loss of secondary structure and a complete loss of enzyme activity, without dissociation of dimer at the first transition state [26]. Therefore we have further investigated the inactivation of G6PD as a function of GdmCl concentration. Figure 4 shows the activity of enzyme at different concentrations of denaturant. There was a marginal decrease in the enzyme activity up to 0.9 M GdmCl but a sharp decrease and complete loss of activity thereafter. However, it should be noted that the molten globule-like state (1.2 M GdmCl) was associated with the loss of enzyme activity. Hence, subsequent studies on α B-crystallin-assisted refolding of G6PD were carried out using 1.2 and 4.0 M GdmCl that represent the molten globule-like and completely unfolded states of G6PD respectively.

α B-crystallin-assisted reactivation of G6PD

As shown in Figure 5, dilution-induced spontaneous refolding of G6PD from molten globule-like state at 25 °C showed negligible enzyme activity, whereas that from completely unfolded state yielded approx. 35% of the original activity (Figure 5A). Low yields of reactivation could be mainly due to off pathway aggregation as high-molecular mass-inactive aggregates were observed (as insoluble precipitate upon centrifugation) in the process of refolding, more so with refolding of molten globule-like state. Perhaps exposure of sticky hydrophobic surfaces at 1.2 M GdmCl, as shown by ANS binding (Figure 3), may be responsible for the complete aggregation of partially unfolded intermediates and loss of activity. Remarkably, the presence of α B-crystallin enhanced the extent of reactivation of completely unfolded G6PD, yielding 70–80% of the original activity within 3 h (Figure 5A). Furthermore, the extent of α B-crystallin-assisted renaturation of completely unfolded G6PD increased in a concentration-dependent manner, in that as low as 0.1 μ M α B-crystallin could recover 50% of original activity by 3 h of refolding (Figure 5B). Increase in the yield of active G6PD in the presence of α B-crystallin could be due to the partitioning of G6PD into a fraction that is committed to reactivate. To confirm that the increase in recovery of G6PD activity was specifically associated with the presence of α B-crystallin, renaturation of G6PD was initiated in the presence of lysozyme under similar conditions. As shown in Figure 5(A), unlike α B-crystallin, lysozyme failed to recover G6PD activity. These results show that α B-crystallin specifically assisted the proper refolding of the GdmCl-denatured G6PD and consequently its reactivation. Since G6PD exists as

Figure 5 Reactivation of G6PD in the absence and presence of α B-crystallin

(A) Reactivation of G6PD followed enzyme denaturation in 1.2 (molten globule-like) and 4.0 M GdmCl (completely unfolded). G6PD was refolded from the completely unfolded state in the absence ($\circ$) and in the presence of either 0.5 μ M α B-crystallin ($\bullet$) or 1 μ M lysozyme ($\blacktriangle$). Similarly, G6PD was refolded from molten globule-like state in the absence ($\square$) and in the presence of 0.5 μ M α B-crystallin ($\blacksquare$). (B) Reactivation of G6PD with various concentrations of α B-crystallin. G6PD was refolded from the completely unfolded state in the absence ($\circ$) and in the presence of 0.1 ($\blacktriangledown$), 0.5 ($\bullet$), 1.0 ($\blacksquare$) and 10 μ M α B-crystallin ($\blacklozenge$). Results shown are the average for three independent experiments.

a molten globule-like state at 1.2 M GdmCl, it might bind to α B-crystallin with higher affinity and hence greater reactivation. Intriguingly, α B-crystallin was unable to reactivate G6PD from a molten globule-like state (Figure 5A). Furthermore, we also investigated whether the presence of α B-crystallin along with G6PD during its unfolding to molten globule-like state will aid in reactivation upon refolding. However, presence of α B-crystallin during unfolding did not assist the reactivation of G6PD from molten globule-like state (results not shown).

Findings of the present study are not in agreement with the studies reported by Rawat and Rao [17], where α -crystallin could assist in the reactivation of GdmCl-denatured XR (xylose reductase) from its molten globule. Whereas Ganea and Harding [15] reported that the extent of reactivation of 0.3 M GdmCl-treated GAPDH (glyceraldehyde-3-phosphate dehydrogenase) in the absence or presence of α -crystallin was the same. In contrast, the reactivation was much greater in the presence of α -crystallin when GAPDH was inactivated with higher concentration (4 M) of GdmCl. This discrepancy may be attributed to the existence of a non-native-like state of GAPDH at higher but not lower concentrations of GdmCl. Strikingly, based on our own and other observations [26], G6PD assumes a non-native (molten globule-like) state at lower concentrations but gets completely unfolded at higher concentrations of GdmCl.

Therefore, to understand the basis of α B-crystallin-assisted renaturation of the completely unfolded, but not the molten globule-like state of G6PD, we have investigated the interaction of these two states of G6PD with α B-crystallin by gel filtration.

Figure 6 SEC of G6PD upon refolding in the absence and presence of α B-crystallin

(A) Elution profile of G6PD and α B-crystallin under native conditions. (B) Elution profiles of G6PD refolded in the absence of α B-crystallin from molten globule-like state (solid line) and completely unfolded state (dotted line). (C) Elution profiles of G6PD refolded in the presence of α B-crystallin from molten globule-like state (solid line) and completely unfolded state (dotted line).

Gel filtration analysis of G6PD reactivation

As shown in Figure 6(A), α B-crystallin and G6PD when fractionated together under native conditions eluted corresponding to their respective oligomeric sizes on an SEC (size-exclusion chromatography) column (i.e. 650 and 104 kDa), thus suggesting no interaction between these proteins under native conditions. Refolding of G6PD from its molten globule-like state showed no

peak corresponding to native G6PD, whereas from the completely unfolded state showed a partially recovered native G6PD peak on SEC (Figure 6B). Furthermore, spontaneous refolding of both molten globule-like and unfolded states of G6PD showed peaks indicating formation of soluble high-molecular mass species (> 200 kDa) that could have resulted from misfolding (Figure 6B), in addition to the insoluble aggregates. However, there was no measurable enzyme activity associated with the soluble aggregates (Table 1). Hence, the absence of a peak corresponding to native G6PD on SEC upon spontaneous refolding of molten globule-like state explains the complete loss of activity. On the other hand, partial recovery of a peak corresponding to native G6PD upon refolding of the unfolded state justifies 35 % enzyme reactivation.

Interestingly, when the unfolded state of G6PD was allowed to refold in the presence of α B-crystallin, a remarkable increase in native G6PD peak was observed, concomitant with the disappearance of the soluble aggregates (Figure 6C). Increased reactivation of G6PD in the presence of crystallin could be due to the formation of native G6PD and/or the complex of G6PD with α B-crystallin. However, only native G6PD exhibited enzyme activity but not the complex (Table 1). Thus it can be argued that α B-crystallin restored the activity of G6PD largely by preventing the formation of both soluble and insoluble aggregates and hence, shifted the refolding equilibrium to the formation of native G6PD. α B-crystallin, although prevented the formation of soluble aggregates upon refolding of the molten globule-like state, could not restore the native G6PD peak (Figure 6C).

These results suggest that α B-crystallin may form complexes with different species of G6PD during the course of refolding, but reactivation appears to be species-specific, depending on their conformational state. Perceptible differences were observed in recognition of various intermediates in α B-crystallin-mediated reactivation of enzymes. For instance, α B-crystallin could reactivate GdmCl-denatured XR from its molten globule-like state, but not from a completely unfolded state [17]. In case of CS (citrate synthase), it was reported that α -crystallin transiently interacts with the early unfolding intermediates but forms a stable complex with the late unfolding intermediates during heat-induced denaturation [14]. However, the stable complex of CS and α -crystallin exhibited no enzyme activity. On the other hand, few other studies reported occurrence of enzyme activity even when α -crystallin formed complexes with GAPDH and sorbitol dehydrogenase [15,27]. Moreover, studies also implicate that stability of the substrate (or its intermediate) could influence its interaction with α -crystallin [28]. Thus it appears that α -crystallin-assisted reactivation of enzymes upon refolding/renaturation involves various mechanisms and largely depends on the substrate and its various conformations in solution.

α B-crystallin does not solubilize the preformed aggregates

Furthermore, we have examined if α B-crystallin can influence the solubilization of the preformed aggregates and subsequent

Table 1 G6PD activity of the HPLC fractions corresponding to chaperone-substrate complex and native G6PD

G6PD was refolded in the absence (–) and presence (+) of α B-crystallin from molten globule-like state and completely unfolded state. Activity of native G6PD that was refolded from completely unfolded state in the absence of α B-crystallin was considered as 100 %. N.A., no activity.

HPLC peak	Refolded from molten globule-like state		Refolded from completely unfolded state	
	(–) α B-crystallin	(+) α B-crystallin	(–) α B-crystallin	(+) α B-crystallin
Chaperone-substrate complex	–	N.A.	–	N.A.
Native G6PD	N.A.	N.A.	100 %	187 %

Figure 7 Ability of α B-crystallin to dissolve preformed aggregates of G6PD upon refolding

(A) Completely unfolded G6PD was refolded in the absence (○) and presence of α B-crystallin added at 0 h (●), 1 h (■) and 2 h (▲) after initiation of refolding (indicated by arrows). (B) Elution profiles of G6PD refolded in the presence of α B-crystallin, added at 0 h (dotted line) and 2 h (solid line) after initiation of refolding following denaturation by 4 M GdmCl.

reactivation. For this, refolding of completely unfolded G6PD was initiated in a renaturation buffer lacking α B-crystallin, thus producing a kinetic competition between folding and aggregation pathways of G6PD, and α B-crystallin was added at later time points. As shown in Figure 7(A), increasing lag-time before the addition of α B-crystallin was associated with a dramatic decrease in the ability of α B-crystallin to restore G6PD activity. Furthermore, to confirm the inability of α B-crystallin to dissolve preformed G6PD aggregates, we analysed the protein mixtures in time-lag experiments by gel filtration. The results suggest that the presence of α B-crystallin at later time points does not restore native G6PD peak (Figure 7B). These observations together prove that preformed aggregates are not probably dissolved by α B-crystallin.

Effect of ATP on G6PD refolding

In the presence of ATP, α B-crystallin has been shown to be more effective in terms of its chaperone activity and also in protecting the chaperone against proteolytic digestion [25,29,30]. Although it has been shown that ATP binds to α -crystallin [25,31], the mechanism of ATP-enhanced protection is not known. However, ATP binding was shown to release target proteins bound to α -crystallin, which might be further refolded with the help of co-chaperones that require ATP [32]. In the present study, we have investigated whether presence of ATP aids in the refolding of G6PD. α B-crystallin-assisted reactivation of G6PD was enhanced marginally by 15% in the presence of ATP, reaching up to 90% of the original activity (Figure 8). Nevertheless, ATP alone has improved spontaneous refolding of G6PD by 6–8% (Figure 8). Although, ATP seems to enhance α -crystallin-assisted

Figure 8 Role of ATP in α B-crystallin-mediated protection of G6PD

G6PD was refolded from the completely unfolded state in the absence (○) and in the presence of either 5 mM ATP (■) or 0.5 μ M α B-crystallin (●) or 0.5 μ M α B-crystallin plus 5 mM ATP (▲). Results shown are the average for three independent experiments.

Figure 9 Reactivation of G6PD from the completely unfolded state by native and MGO-modified α -crystallin

Bar 1, G6PD refolded alone; bars 2 and 3, G6PD refolded in the presence of native and 10 mM MGO-modified α -crystallin respectively. G6PD incubated under similar conditions in the absence of GdmCl was considered as control (100%) to calculate percentage activity. Results shown are the average ($\pm$ S.E.M.) for three independent experiments.

reactivation of G6PD, ATP did not improve the renaturation efficiency of α -crystallin remarkably.

Effect of post-translational modification on α -crystallin ability to refold G6PD

Various post-translational modifications, including non-enzymatic glycation, which are implicated in the formation of cataract development, have been shown to affect α -crystallin chaperone-like activity [16,33–36]. Previously, we have reported that upon MGO modification, bovine α -crystallin showed diminished protection to enzyme inactivation against UV light and heat [16]. Hence, we have studied the ability of MGO-modified α -crystallin to reactivate GdmCl-denatured G6PD. As shown in Figure 9, MGO-modified α -crystallin was unable to restore the activity of completely unfolded G6PD upon refolding. Our present observations, together with the previous data [16], merit attention in terms of significance of post-translational modifications that affect α -crystallin chaperone-like activity.

In summary, we show that α B-crystallin assists in the reactivation of G6PD and prevents misfolding/aggregation upon refolding. Although, α B-crystallin formed complexes with soluble aggregates and the molten globule-like state of G6PD, it could not channel them into native form. Hence, α B-crystallin appears to reactivate conformation-specific unfolding/refolding

Scheme 1 Representation of the possible mechanism of α B-crystallin-assisted reactivation of G6PD from denaturant-induced inactivation

N_2 , native dimeric G6PD; I_{MG} , molten globule-like intermediate; I_N , native-like intermediate; I_U , unfolding intermediate; U and A, completely unfolded and aggregated G6PD respectively.

intermediates, probably by providing the necessary energetics to these intermediates for crossing the kinetic barriers in achieving the native state. Based on these observations, a possible mechanism for α B-crystallin-assisted reactivation of G6PD is shown in Scheme 1. α B-crystallin may reversibly interact with the unfolding intermediates of G6PD, favouring the formation of active G6PD through a native-like intermediate. Thus chaperoning of denaturant-inactivated G6PD by α B-crystallin involves two distinct mechanisms: (i) formation of a complex with partially or completely unfolded substrates, thus preventing the aggregation of G6PD and (ii) favouring the refolding of specific intermediates (completely unfolded) to active G6PD.

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REFERENCES

- Welsh, M. J. and Gaestel, M. (1998) Small heat-shock protein family: function in health and disease. *Ann. N.Y. Acad. Sci.* **851**, 28–35
- Haslbeck, M., Braun, N., Stromer, T., Richter, B., Model, N., Weinkauff, S. and Buchner, J. (2004) Hsp42 is the general small heat shock protein in the cytosol of *Saccharomyces cerevisiae*. *EMBO J.* **23**, 638–649
- Houry, W. A. (2001) Chaperone-assisted protein folding in the cell cytoplasm. *Curr. Protein Pept. Sci.* **2**, 227–244
- Young, J. C., Agashe, V. R., Siegers, K. and Hartl, F. U. (2004) Pathways of chaperone-mediated protein folding in the cytosol. *Nat. Rev. Mol. Cell. Biol.* **5**, 781–791
- de Jong, W. W., Caspers, G. J. and Leunissen, J. A. (1998) Genealogy of the alpha-crystallin–small heat-shock protein superfamily. *Int. J. Biol. Macromol.* **22**, 151–162
- Lentze, N., Studer, S. and Narberhaus, F. (2003) Structural and functional defects caused by point mutations in the alpha-crystallin domain of a bacterial alpha-heat shock protein. *J. Mol. Biol.* **328**, 927–937
- van Montfort, R. L., Basha, E., Friedrich, K. L., Slingsby, C. and Vierling, E. (2001) Crystal structure and assembly of a eukaryotic small heat shock protein. *Nat. Struct. Biol.* **8**, 1025–1030
- Bloemendal, H., de Jong, W. W., Jaenicke, R., Lubsen, N. H., Slingsby, C. and Tardieu, A. (2004) Ageing and vision: structure, stability and function of lens crystallins. *Prog. Biophys. Mol. Biol.* **86**, 407–485
- Horwitz, J. (2003) Alpha crystallin. *Exp. Eye Res.* **76**, 145–153
- Bhat, S. P. (2003) Crystallins, genes and cataract. *Prog. Drug Res.* **60**, 205–262
- Ganea, E. (2001) Chaperone-like activity of α -crystallin and other small heat shock proteins. *Curr. Protein Pept. Sci.* **2**, 205–225
- Das, K. P., Petrash, J. M. and Surewicz, W. K. (1996) Conformational properties of substrate proteins bound to a molecular chaperone alpha-crystallin. *J. Biol. Chem.* **271**, 10449–10452
- Raman, B., Ramakrishna, T. and Rao, C. M. (1997) Effect of the chaperone-like alpha-crystallin on the refolding of lysozyme and ribonuclease A. *FEBS Lett.* **416**, 369–372
- Rajaraman, K., Raman, B., Ramakrishna, T. and Rao, C. M. (2001) Interaction of human recombinant alphaA- and alphaB-crystallins with early and late unfolding intermediates of citrate synthase on its thermal denaturation. *FEBS Lett.* **497**, 118–123
- Ganea, E. and Harding, J. J. (2000) Alpha-crystallin assists the renaturation of glyceraldehyde-3-phosphate dehydrogenase. *Biochem. J.* **345**, 467–472
- Kumar, M. S., Reddy, P. Y., Kumar, P. A., Suroliya, I. and Reddy, G. B. (2004) Effect of dicarbonyl-induced browning on alpha-crystallin chaperone-like activity: physiological significance and caveats of *in vitro* aggregation assays. *Biochem. J.* **379**, 273–282
- Rawat, U. and Rao, M. (1998) Interactions of chaperone alpha-crystallin with the molten globule state of xylose reductase. Implications for reconstitution of the active enzyme. *J. Biol. Chem.* **273**, 9415–9423
- Reddy, G. B., Reddy, P. Y. and Suryanarayana, P. (2001) AlphaA- and alphaB-crystallins protect glucose-6-phosphate dehydrogenase against UVB irradiation-induced inactivation. *Biochem. Biophys. Res. Commun.* **282**, 712–716
- Hook, D. W. and Harding, J. J. (1998) Protection of enzymes by alpha-crystallin acting as a molecular chaperone. *Int. J. Biol. Macromol.* **22**, 295–306
- Reddy, V. N. and Giblin, F. J. (1984) Metabolism and function of glutathione in the lens. *Ciba Found. Symp.* **106**, 65–87
- Reddy, G. B. and Bhat, K. S. (1998) Synergistic effect of UVB radiation and age on HMPS enzymes in rat lens homogenate. *J. Photochem. Photobiol. B* **43**, 56–60
- Ganea, E. and Harding, J. J. (1995) Molecular chaperones protect against glycation-induced inactivation of glucose-6-phosphate dehydrogenase. *Eur. J. Biochem.* **231**, 181–185
- Reddy, G. B., Das, K. P., Petrash, J. M. and Surewicz, W. K. (2000) Temperature-dependent chaperone activity and structural properties of human alphaA- and alphaB-crystallins. *J. Biol. Chem.* **275**, 4565–4570
- Horwitz, J., Huang, Q. L., Ding, L. and Bova, M. P. (1998) Lens alpha-crystallin: chaperone-like properties. *Methods Enzymol.* **290**, 365–383
- Kumar, M. S., Mrudula, T., Mitra, N. and Reddy, G. B. (2004) Enhanced degradation and decreased stability of eye lens alpha-crystallin upon methylglyoxal modification. *Exp. Eye Res.* **79**, 577–583
- Plomer, J. J. and Gafni, A. (1992) Denaturation of glucose-6-phosphate dehydrogenase from *Leuconostoc mesenteroides* by guanidine hydrochloride; identification of inactive, partially unfolded, dimeric intermediates. *Biochim. Biophys. Acta* **1122**, 234–242
- Marini, I., Moschini, R., Del Corso, A. and Mura, U. (2000) Complete protection by alpha-crystallin of lens sorbitol dehydrogenase undergoing thermal stress. *J. Biol. Chem.* **275**, 32559–32565
- Mchaourab, H. S., Dodson, E. K. and Koteiche, H. A. (2002) Mechanism of chaperone function in small heat shock proteins. Two-mode binding of the excited states of T4 lysozyme mutants by alphaA-crystallin. *J. Biol. Chem.* **277**, 40557–40566
- Muchowski, P. J. and Clark, J. I. (1998) ATP-enhanced molecular chaperone functions of the small heat shock protein human alphaB crystallin. *Proc. Natl. Acad. Sci. U.S.A.* **95**, 1004–1009
- Muchowski, P. J., Hays, L. G., Yates, III, J. R. and Clark, J. I. (1999) ATP and the core 'alpha-crystallin' domain of the small heat-shock protein alphaB-crystallin. *J. Biol. Chem.* **274**, 30190–30195
- Palmisano, D. V., Groth-Vasselli, B., Farnsworth, P. N. and Reddy, M. C. (1995) Interaction of ATP and lens alpha crystallin characterized by equilibrium binding studies and intrinsic tryptophan fluorescence spectroscopy. *Biochim. Biophys. Acta* **1246**, 91–97
- Wang, K. and Spector, A. (2001) ATP causes small heat shock proteins to release denatured protein. *Eur. J. Biochem.* **268**, 6335–6345
- Cherian, M., Smith, J. B., Jiang, X. Y. and Abraham, E. C. (1997) Influence of protein-glutathione mixed disulfide on the chaperone-like function of alpha-crystallin. *J. Biol. Chem.* **272**, 29099–29103
- Thampi, P., Hassan, A., Smith, J. B. and Abraham, E. C. (2002) Enhanced C-terminal truncation of alphaA- and alphaB-crystallins in diabetic lenses. *Invest. Ophthalmol. Vis. Sci.* **43**, 3265–3272
- Derham, B. K. and Harding, J. J. (1997) The effects of ageing on the chaperone-like function of rabbit alpha-crystallin, comparing three methods of assay. *Biochim. Biophys. Acta* **1336**, 187–194
- Derham, B. K. and Harding, J. J. (2002) Effects of modifications of alpha-crystallin on its chaperone and other properties. *Biochem. J.* **364**, 711–717
- Lowry, O. H., Rosebrough, N. H., Farr, A. L. and Randall, R. H. (1951) Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* **193**, 265–275