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Research Article

Alpha-crystallin: an ATP-independent complete molecular chaperone toward sorbitol dehydrogenase

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Abstract. α-Crystallin, the major component of the vertebrate lens, is known to interact with proteins undergoing denaturation and to protect them from aggregation phenomena. Bovine lens sorbitol dehydrogenase (SDH) was previously shown to be completely protected by α-crystallin from thermally induced aggregation and inactivation. Here we report that α-crystallin, in the presence of the SDH pyridine cofactor NAD(H), can exert a remarkable chaperone action by favoring the recovery of the enzyme activity from chemically denaturated SDH up to 77%. Indeed, even in the absence of the cofactor, α-crystallin present at a ratio with SDH of 20:1 (w:w) allows a recovery of 35% of the enzyme activity. The effect of ATP in enhancing α-crystallin-promoted SDH renaturation appears to be both nonspecific and to not involve hydrolysis phenomena, thus confirming that the chaperone action of α-crystallin is not dependent on ATP as energy donor.

Key words. α-Crystallin; sorbitol dehydrogenase; molecular chaperone; enzyme renaturation; protein refolding; small heat shock protein.

Alpha-crystallin, the major protein of the mammalians lens, is a multimer of about 800 kDA formed by two subunits, αA and αB, each of approximately 20 kDA and with a sequence homology of approximately 55% [1]. Initially believed to be present only in the lens, as a relevant factor responsible for transparency, refractive power and structural stability, α-crystallin is in fact widely distributed in different tissues [2–4]. Alpha-crystallin subunits belong to the small heat shock protein (sHsp) family, a class of molecular chaperones of low monomer size (12–40 kDA) that can be induced by heat and other stress conditions [5]. Alpha-crystallin appears to be up regulated in a number of neurodegenerative diseases [6–10] and involved in negative regulation of apoptosis [11, 12]. Moreover, mutation or lack of the αA gene induces cataract in mice and humans [13], while mutation or lack of the αB gene causes a desmin-related myopathy [14] or hyperproliferation and genomic instability, respectively [15, 16].

A common feature of sHsps is a conserved C-terminal region of about 90 amino acids called the 'α-crystallin domain,' responsible for their molecular chaperone action. Alpha-crystallin has been shown to exert its chaperone action by preventing protein aggregation [17, 18], preserving enzyme activity [19–21], refolding the target protein into a functional conformation [22–24] or conferring cell thermotolerance [25]. However, factors and conditions modulating the chaperone action of α-crystallin still need to be defined. In this respect, phosphorylation of α-crystallin has been indicated as a potential regulatory mechanism. Phosphorylation of α-crystallin and other sHsps often leads to a decrease in the oligomer size; the experimental results in terms of activity modulation are controversial, reporting both a decrease [26] or an increase [27, 28] of the chaperone function. Phosphorylation of α-crystallin was also observed by exposure of cell

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systems to different stress conditions [28] and it was shown to be involved in the actin polymerization/depolymerization process [29]. Despite such lines of evidence, the physiological relevance of α-crystallin phosphorylation is still debated [26, 30].

To assess whether α-crystallin acts in an ATP-dependent or -independent manner is very important for understanding the molecular mechanism of this chaperone. ATP can form complexes with α-crystallin, as demonstrated by different techniques, including intrinsic tryptophan fluorescence spectroscopy, ^{31}P NMR and isotope exchange measurements [31–35]. However, modulation of the functional behavior of α-crystallin through the ATP binding or hydrolysis is still controversial. Lines of evidence indicate that α-crystallin chaperone activity increases in the presence of ATP; both human [35] and bovine [36] αB-crystallin, in the presence of ATP, reduce the aggregation of thermal-stressed citrate synthase and enhance reactivation of the enzyme after chemical denaturation. Moreover, ATP, added to the complex of denatured xylanase together with α-crystallin was shown to facilitate the release of the active enzyme [24]. On the other hand, other lines of evidence showed that α-crystallin acts as an ATP-independent chaperone toward thermal aggregation of citrate synthase, α-glucosidase and rhodanese [37, 38]; similarly, reactivation of chemically denatured xylose reductase by α-crystallin was shown to be independent of ATP hydrolysis [23]. Recently, ATP binding, but not its hydrolysis was shown to induce, through the exposure of additional hydrophobic sites on α-crystallin, a reversible association between α-crystallin and its protein substrate [39].

An indication of the involvement of α-crystallin in a multichaperone network comes from a clear stimulatory effect of ATP on the reactivation and release of folding-competent polypeptides bound to α-crystallin, observed in the presence of reticulocyte lysate [40] or Hsp60 and Hsp70 [40, 41].

Alpha-crystallin was previously shown to protect thermally stressed sorbitol dehydrogenase (L-iditol: NAD^+ 2-oxidoreductase, E.C. 1.1.1.14) (SDH) from both aggregation and inactivation [21]. Here we report that, beside the above protective action exerted on the native SDH, α-crystallin is able to elicit the refolding of the chemically denatured enzyme, affecting both the rate and the extent of recovery of active SDH. The results indicate that the recovery of SDH activity induced by α-crystallin is strongly enhanced by the enzyme pyridine cofactor. The observed stimulatory effect of ATP on SDH refolding could not be associated with a specific ATP-dependent mechanism.

Materials and methods

Materials

Calf eyes were obtained from a local abattoir soon after slaughtering; the lenses were removed and frozen at –20 °C until used.

Molecular-weight markers for SDS-PAGE and gel filtration, dithiothreitol (DTT), NAD^+, β-D(–) fructose, D-sorbitol, ATP, ADP, Ado, Guo, nicotinamide mononucleotide (NMN), β-γ-methylene adenosine 5′-triphosphate (AMP-PCP) and lysozyme were from Sigma-Aldrich. NADH was supplied by Boehringer, EDTA by Serva Feinbiochemica, guanidine hydrochloride (GdnCl) by Fluka and YM30 ultrafiltration membrane and Matrex Green-A by Amicon. The electrophoretic equipment was from Bio-Rad. Sepharose 6B was from Amersham Biosciences. All other chemicals were of reagent grade.

Enzyme purification and assay

SDH was purified to electrophoretic homogeneity as previously described [42]. For determination of the molar concentration of SDH, a molecular-weight value of 39.6 kDa for the single subunit of the tetrameric enzyme was used. The final enzyme preparation (approximately 2 mg/ml), with a specific activity of 21 U/mg, was stored at 4 °C in 10 mM sodium phosphate pH 7 (S-buffer), supplemented with 2 mM DTT and 0.1 mM NADH. The purified enzyme was dialyzed against S-buffer just before use.

The assay of enzyme activity was performed at 37 °C as previously described [42] by following the decrease in absorbance at 340 nm in a reaction mixture (0.5 ml final volume) containing 0.24 mM NADH and 0.4 M D-fructose in 100 mM Tris/HCl buffer pH 7.4. The rate of NADH oxidation measured in a parallel assay in which the substrate was omitted was subtracted as a blank. One unit of enzyme activity is the amount of SDH that catalyzes the oxidation of 1 μmol/min of NADH.

Alpha-crystallin isolation

All procedures were carried out at 4 °C, unless otherwise stated. Frozen lenses were suspended (0.15 g/ml) in S-buffer and homogenized in a Potter-Elvehjem homogenizer. The suspension was then centrifuged at 25,000 × g for 30 min. An aliquot of the resulting supernatant was applied to a Sepharose 6B column (1.5 × 65 cm), and the elution was performed with S-buffer at a flow rate of 3 ml/h. The eluted fractions (1 ml) containing low-molecular-weight α-crystallin were analyzed by SDS-PAGE, pooled, concentrated on Amicon YM30 membrane to a final protein concentration of approximately 2 mg/ml and then stored at –20 °C until use. For the determination of the molar concentration of α-crystallin, a molecular-weight value of 800 kDa was used.

Preparation of chemically denatured SDH and recovery of enzyme activity

SDH (final concentration 1 mg/ml) was denatured by incubation for 2 h at 25 °C in Tris/HCl 100 mM pH 7.4 in the presence of 6 M GdnCl, 2 mM DTT and 100 μM EDTA.

The recovery of active SDH after the GdnCl treatment was performed by a 100-fold dilution of the denatured enzyme preparation in Tris/HCl 100 mM pH 7.4 and incubation at 25 °C, for the appropriate time in the presence of α-crystallin and/or different compounds, as specified. At different times, aliquots were withdrawn and SDH activity measured. The extent of recovery of enzyme activity from denatured SDH is given as the percent of the activity of a control sample of native SDH treated in the same conditions.

Other methods

Protein concentration was determined by the Coomassie-Blue-binding assay [43], using bovine serum albumin as a standard. SDH and α-crystallin localization in the elution profiles of chromatographic analyses was carried out by SDS-PAGE according to Laemmli [44], using 12% acrylamide slab gels, 0.75 mm thick. The following standards were used for apparent molecular weight calibration: bovine serum albumin (M_r 66,000), ovalbumin (M_r 45,000), glyceraldehyde-3-phosphate dehydrogenase (M_r 36,000), human erythrocyte carbonic anhydrase (M_r 29,000), trypsinogen (M_r 24,000) and trypsin inhibitor (M_r 20,000). Gels were stained by the silver stain technique [45].

Results

Recovery of enzyme activity from GdnCl-denatured SDH

Inactive SDH, after dilution in the recovery buffer (see Materials and methods), slowly reactivates with time, reaching in 2 h a recovery of approximately 10% (fig. 1). Alpha-crystallin, present in the incubation mixture, increased the rate and the extent of the recovery of enzyme activity in a concentration-dependent manner, being able, at an α-crystallin:SDH ratio of 20:1 (w:w), to induce in 2 h a recovery of approximately 35%. Further incubation up to 24 h gave an increase in the recovery to 45% (data not shown). In these conditions, the recovered activity remained stable for at least 72 h. No changes in the recovery were observed when the incubation was performed by reducing GdnCl from 60 mM (standard conditions) to 15 mM (data not shown). Lysozyme (a thermally stable protein displaying a rather low ability to interact with other proteins) present instead of α-crystallin in the incubation mixture at a lysozyme:SDH ratio of 20:1 (w:w) was ineffective in favoring the recovery of SDH activity. Temperature appeared to be a relevant factor in the refolding of denatured SDH; a decrease in the temperature from 25 to 4 °C after 30 min of incubation of denatured SDH undergoing renaturation determined a block in the refolding process (data not shown).

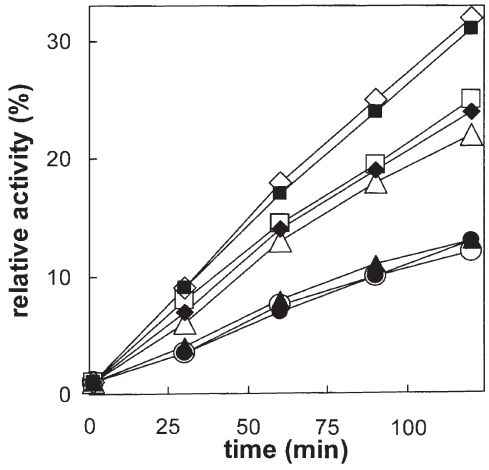

Figure 1. Renaturation of SDH induced by α-crystallin. Chemically denatured SDH (10 μg/ml) was incubated alone (●) or in the presence of the following α-crystallin:SDH (w:w) ratios: 1:1 (○), 5:1 (△), 10:1 (□), 20:1 (◇), 30:1 (■) and 50:1 (◆). Closed triangles (▲) refer to the incubation of chemically denatured SDH in the presence of lysozyme at a ratio lysozyme:SDH of 20:1 (w:w). See Materials and methods for details.

Evidence of interactions between α-crystallin and SDH undergoing refolding was obtained by an affinity chromatography approach (fig. 2). The elution profile of a mixture of α-crystallin and native SDH (20:1; w:w) applied on a Matrex Green A column (fig. 2A), showed two distinct peaks, each containing a single molecular species. In fact, SDS-PAGE analysis revealed (fig. 2A, inset) that the first peak contained only α-crystallin and the second, obtained after elution with NADH, contained exclusively SDH. The elution profile of a mixture of denaturated SDH undergoing renaturation in the presence of α-crystallin is shown in figure 2B. In this case, SDS-PAGE analysis (fig. 2B, inset) revealed that, while the first peak contained exclusively α-crystallin, the second peak contained both SDH and α-crystallin. The interaction between α-crystallin and renaturing SDH was confirmed by gel filtration chromatography on Superdex200 HR10/30 in conditions previously described [18]. In fact, coelution of α-crystallin and denatured SDH was observed (data not shown). With such an approach, we could verify the release of the recovered active enzyme from the α-crystallin:SDH complex after 24 h renaturation. In fact, in this case, no coelution of α-crystallin and active SDH was observed (data not shown).

ATP and NAD/H effect on SDH reactivation

The effect of ATP in eliciting the chaperone action of α-crystallin on SDH reactivation was investigated. The

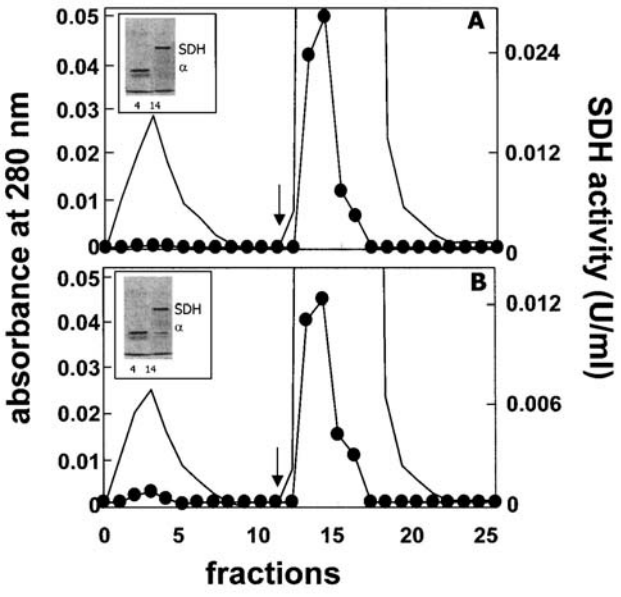

Figure 2. Chromatographic separation of α-crystallin from native SDH and denatured SDH undergoing reactivation. (*A*) Native SDH (0.024 mg/ml) supplemented with α-crystallin 1:20 (w:w) in S-buffer containing 60 mM GdnCl was applied (0.8 ml) to a Matrex Green A column (0.8 × 2.0 cm) and eluted with S-buffer at a flow rate of 0.8 ml/min. Fractions of 0.8 ml were collected. SDH was eluted supplementing the S-buffer with 0.1 mM NADH (arrow). SDH activity (●) and absorbance at 280 nm (–) were measured. The inset refers to the SDS-PAGE analysis of the indicated fractions. (*B*) SDH (2.4 mg/ml) denaturated by treatment for 2 h at 25 °C with 6 M GdnCl was incubated after 1:100 dilution in the presence of an α-crystallin:SDH ratio of 20:1 (w:w) for 60 min and subjected to the Matrex Green A chromatography in the conditions described above. The inset refers to SDS-PAGE analysis of the indicated fractions.

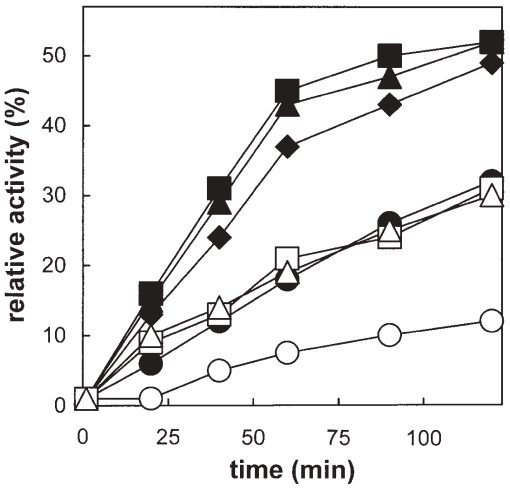

Figure 3. Influence of ATP and related compounds on SDH renaturation. Chemically denatured SDH (10 μg/ml) was incubated alone (○) or in the presence of 3.5 mM of either ATP (□) or ADP (△). Closed symbols refer to incubations of SDH with α-crystallin 20:1 (w:w) alone (●) or supplemented with 3.5 mM of the following: ATP (■), ADP (◆), and AMP-PCP (▲). See Materials and methods for details.

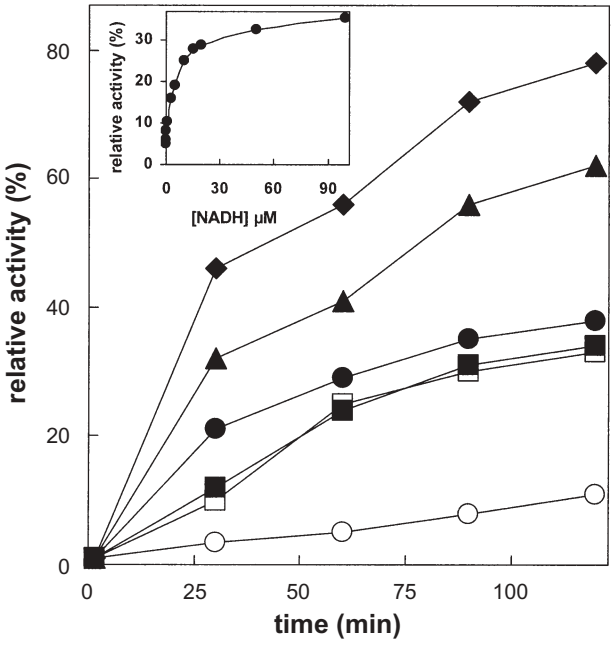

Figure 4. Influence of NAD(H) on SDH renaturation. Chemically denatured SDH (10 μg/ml) was incubated in the presence of 0.1 mM NADH either alone (□) or in the presence of the following: α-crystallin 20:1 (w:w) (◆), lysozyme 20:1 (■), α-crystallin 20:1 (w:w) plus 3.5 mM ATP (▲).Circles refer to the incubation performed with SDH alone (○) or in the presence of α-crystallin 20:1 (w:w) (●). The inset refers to the recovery of enzyme activity from GdnCl-denatured SDH incubated as above in the presence of the indicated concentrations of NADH. No differences were observed when NADH was substituted by NAD^+. See Materials and methods for details.

presence during reactivation of SDH of ATP ranging from 0.1 to 3.5 mM accounted for a recovery of the enzyme activity of 30 %. When α-crystallin was simultaneously present (α-crystallin:SDH, 20:1; w:w), the recovered activity increased up to 50% (fig. 3). Further addition of 3.5 mM $MgCl_2$ and 10 mM KCl, a condition shown to favor the action of ATP-dependent molecular chaperones [40, 41], gave essentially the same results (data not shown). The effect on the SDH reactivation exerted by ADP and by the nonhydrolyzable ATP analogue AMP-PCP is also reported in figure 3. Both molecules gave essentially the same results observed for ATP. Moreover, no significant differences with respect to ATP were observed in the presence of other nucleosides and nucleotides, namely Ado, AMP, Guo, IMP and NMN (data not shown).

A remarkable effect on SDH renaturation was offered by the SDH cofactor. Both NAD^+ and NADH induced at 0.1 mM, even in the absence of α-crystallin, a recovery of the enzyme activity up to 35% (fig. 4). The effectiveness of NAD results from the low levels required to induce SDH renaturation (fig. 4, inset). When α-crystallin and NAD(H) were both present in the refolding mixture, the recovery of SDH activity was raised to 78% (fig. 4). The

reactivation of SDH in the presence of AMP and NMN together in the mixture (0.1 mM each) was essentially the same as that obtained with the single species (data not shown). When in addition to α-crystallin and NADH, ATP was present, the recovery of the SDH activity was lower than that observed in the absence of the nucleotide (fig. 4, compare diamonds with triangles).

Discussion

The relevance of α-crystallin in favoring the refolding of denatured proteins is clearly evident in the remarkable effect of this molecular chaperone on the recovery of a native functional structure of chemically denatured bovine lens SDH. An increase in the ratio α-crystallin:SDH up to 20:1 allows a maximal recovery of 35% of the expected enzyme activity (fig. 1). SDH was previously shown, under thermal stress conditions, to be an excellent target for the α-crystallin protective action, both in terms of antiaggregation and enzyme activity preservation [21]. Both effects were explained on the basis of the formation of two different kinds of adduct. While the antiaggregation action of α-crystallin was associated with the generation of a quite stable complex between inactive SDH and multimeric 800-kDA α-crystallin, preservation of the active enzyme was associated with the formation of a transient complex between SDH and low-molecular-weight oligomeric α-crystallin. In the present study, the ratio 20:1 (w:w) α-crystallin:SDH, optimal for enzyme renaturation, is suggestive of a possible 1:1 α-crystallin multimer:SDH subunit interaction. However, the complexity of the process(es) leading to the recovery of a quaternary functional structure of SDH from the denatured enzyme renders difficult at this point any proposals for a functional mechanism of the chaperone action of α-crystallin.

The occurrence of a rather strong interaction between α-crystallin and the enzyme undergoing renaturation was observed by both Matrex Green A affinity chromatography (fig. 2) and gel filtration chromatography (data not shown).

As shown in figure 2, the interaction of α-crystallin with the Matrex Green A resin occurs only in the presence of the denatured enzyme undergoing renaturation and is likely mediated by the enzyme itself. An NADH concentration of 0.1 mM, shown to be adequate for eluting the enzyme from the affinity chromatographic support, determines the release of α-crystallin too (fig. 2B). No interaction between α-crystallin and Matrex Green A was observed when α-crystallin alone (data not shown) or in mixtures with native SDH (fig. 2A) were analyzed. Worth noting is that the renatured enzyme does not appear to interact any more with α-crystallin, since α-crystallin and active SDH do not coelute from the gel filtration column (data not shown).

The pyridine cofactor NAD, previously shown to be a good stabilizer of the pure enzyme during storage [42] and also to exert partial protection of the enzyme against thermal inactivation [21], exerts a striking effect in favoring the reactivation of chemically denatured SDH. Thus, irrespective of its redox status, NAD favors a recovery of active enzyme from denatured SDH up to 35% (fig. 4), even in the absence of α-crystallin. When α-crystallin and NAD(H) were both present in the refolding mixture, the recovery of SDH activity was raised to 78%, indicating an additive effect of the two molecular species. Indeed, the remarkable effect of NAD in promoting SDH reactivation can be monitored at rather low concentrations (fig. 4, inset) suggesting that the denatured enzyme is actually prone to regaining an effective binding domain for the cofactor.

ATP, at 3.5 mM, induces a recovery of SDH activity up to 30%. The addition of α-crystallin enhances the recovery up to 50%. Such an action of ATP appears to be neither specific nor to involve hydrolysis phenomena. The same effect is in fact observed when ATP is substituted by a number of purine nucleotides and nucleosides, as well as NMN and the nonhydrolyzable ATP analogue AMP-PCP (fig. 3). Whether ATP exerts its action through its binding either to α-crystallin or, mimicking NAD, to the newly reconstituting cofactor site on the target protein, cannot be assessed at present. Indeed, ATP has the potential to interact with both protein molecules. An ATP-binding site has been characterized on α-crystallin [31] and a functional role for ATP binding was recently proposed [39]; on the other hand, ATP, can in the millimolar concentration range, inhibit SDH activity [I. Marini, unpublished observation]. Thus, the inhibitory action on the SDH reactivation process observed when ATP was added to the α-crystallin/NAD reactivating mixture (fig. 4), could derive either from a competition between ATP and NAD at the cofactor site of SDH or from a true modulation of the interaction efficiency of α-crystallin with the target protein, the latter possibly due to a more compact structure of α-crystallin when bound to ATP [39] from which the active enzyme will no longer be released.

Although the potential of α-crystallin to act as a molecular chaperone is supported by a wide mass of data, its protective action in several stress conditions and with several different target proteins is still defined as a 'chaperone-like' activity. This term for the α-crystallin action, taking into account the well-assessed antiaggregation and stabilization effect of α-crystallin on different targets, essentially comes from the lack of clear evidence for the ability of α-crystallin to favor the correct folding of denatured proteins to native functional structures. Another relevant aspect playing against complete acceptance of α-crystallin as a complete chaperone is the lack, for α-crystallin, of clear evidence for an energy requirement in terms of ATP consumption in order to accomplish its

functions. In fact, despite clear evidence of the ability of α-crystallin to interact with ATP [32–35, 39] and to be itself a target of phosphorylation [26-29], the role of ATP either as direct energy donor during the refolding process or as an indirect α-crystallin functional modulating agent, is not yet clarified.

The present data on the almost complete SDH renaturation contributes significantly to the growing body of data indicating that α-crystallin has indeed the ability to elicit protein refolding. This action of α-crystallin appears not to be dependent on ATP as energy donor, but essentially associated with intrinsic structural features of this chaperone molecule.

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