α-Crystallin assists the renaturation of glyceraldehyde-3-phosphate dehydrogenase

Elena GANEA* and John J. HARDING†[1]

*Institute of Biochemistry, Splaiul Independentei 296, sect 6, 77700 Bucharest, Romania, and †Nuffield Laboratory of Ophthalmology, University of Oxford, Walton Street, Oxford OX2 6AW, U.K.

α-Crystallin, a major lens protein, has many of the properties of a molecular chaperone, but its ability to assist refolding of proteins has been less certain. In the present work it was shown that α-crystallin specifically increased the reactivation of guanidine-denatured glyceraldehyde-3-phosphate dehydrogenase with most of the activity being recovered. In the incubation mixture the recovered enzyme activity was partly free but mostly it appeared in a protective complex with α-crystallin. The aggregation of the denatured enzyme on dilution from the guanidine solution was prevented. Thus α-crystallin not only protects against aggregation and inactivation of enzymes during denaturation, but can also prevent aggregation and assist recovery of the native structure during renaturation.

Key words: complex formation, guanidine hydrochloride, molecular chaperones, protein folding.

INTRODUCTION

In vivo, the correct folding of some proteins is assisted by molecular chaperones, a group of proteins which maintain protein integrity under physylogical and stress conditions. Some of these chaperones are also known as heat-shock proteins (Hsps), as their expression increases when cells are exposed to high temperatures. A large number of Hsps has been described in various compartments of the cell, and the major classes Hsp70, Hsp60 and Hsp90 co-operate in their chaperoning function [1].

A special class of molecular chaperones are the small heat-shock proteins (sHsps), with polypeptides of low monomeric size (12-40 kDa), which exist in cells as large oligomers (300–800 kDa) and have chaperone activity.They share a sequence of about 100 amino acids in a conserved region of the C-terminal half, called the α-crystallin domain, homologous to the lens α-crystallin, a member of this group [2]. Unlike the large Hsps, some of the sHsps refold proteins *in vitro* in an ATP-independent manner [2].

There are many data on sHsp from bacteria, plants and yeast [3,4], but less is known about mammalian sHsps. Recently, a newly described member of this class of mammalian proteins, Hsp20, abundant in skeletal muscle and heart has been characterized as a less active chaperone [5]. Unlike Hsp20, α-crystallin, a major lens crystallin and also a non-lenticular protein, showed chaperone-like activity, in that it prevented heat-induced aggregation of proteins *in vitro* [6] and protected enzymes against inactivation induced by sugars [7,8], cyanate [9] or steroids [10]. α-Crystallin has structural and functional similarities with sHsps [2]; however, its mechanism of action is still poorly understood. It was thought to be incapable of protecting enzyme activity against thermal inactivation [6] but such protection was provided for catalase [11]. There are contradictory results for the role of α-crystallin in protein folding; it was unable to re-fold rhodanese denatured in 6 M guanidine hydrochloride (GuHCl) [12], but it appeared to assist the reactivation of GuHCl-denaturated xylose reductase [13] and heat-inactivated citrate synthase [14,15]. Various stress factors such as heat, pH changes and chaotropic agents can cause structural changes to proteins and make them non-functional. Different factors can induce different alterations to the native structure (e.g. heat and GuHCl), chaotropic agents such as GuHCl or urea being more effective in disrupting the non-covalent interactions essential for the native conformation. These changes could affect different parts of the molecule [16].

Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (E.C.1.2.1.12) is an important enzyme in glycolysis, and oxidizes and subsequently phosphorylates glyceraldehyde 3-phosphate in the glycolytic direction. Recent studies reported other biological properties of the GAPDH protein. As a membrane protein, GAPDH functions in endocytosis; in the cytoplasm, it is involved in translational regulation; in the nucleus it has a function in nuclear tRNA export, DNA replication and DNA repair [17]. The enzyme is involved in some diseases; increased GAPDH levels have been reported in the brain of patients with Down's syndrome [18], and a decrease of the enzyme activity has been reported in human lens with age and in cataract [19]. GAPDH has been extensively studied for structural changes during denaturation. It has been demonstrated that glycation modifies the enzyme conformation and the resistance to inactivation by GuHCl [20], and it has been shown that inactivation precedes allosteric and conformational changes during denaturation by GuHCl [21]. GADPH renatures spontaneously to a small extent, although there is extensive aggregation, as assessed by turbidity measurements [22]. The chaperone protein, GroEL, with or without GroES, decreased the aggregation but impaired the reactivation. Stable chaperone complexes were identified by SDS/PAGE [22]. The enzyme was inactivated *in vitro* by fructose 6-phosphate and prednisolone-21-hemisuccinate, and α-crystallin was not able to protect GAPDH against the inactivation [23].

In the present work we have studied the denaturation of GAPDH by GuHCl and the effect of α-crystallin on enzyme reactivation.

MATERIALS AND METHODS

Materials

Rabbit muscle D-glyceraldehyde-3-phosphate dehydrogenase, guanidinium chloride, D(−)-3-phosphoglyceric acid [tri(cyclohexylammonium) salt] βNADH (sodium salt), ATP (disodium

Abbreviations used: GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GuHCl, guanidine hydrochloride; Hsp, heat-shock protein; DTT, dithiothreitol.

[1] To whom correspondence should be addressed (e-mail john.harding@eye.ox.ac.uk).

salt), 3-phosphoglycerate kinase and all other chemicals were obtained from Sigma. TSK HW-55 gel was obtained from Anachem, Sephacryl S-300 was from Pharmacia and Econo-Pac DEAE Affi-Gel Blue cartridge from Bio-Rad.

Isolation of α-crystallin

α-Crystallin was isolated from bovine lenses by gel-permeation chromatography on TSK HW-55 gel, by the method of Beswick and Harding [24].

GAPDH activity assay

GAPDH activity was measured spectrophotometrically at 25 °C by monitoring the decrease in absorbance of NADH for 1 min at 340 nm [25]. The reaction mixture (3.03 ml) contained 2.5 ml of 0.1 M potassium phosphate buffer, pH 7.6, 6 mM glycerate 3-phosphate, 1.1 mM ATP, 0.9 mM EDTA, 1.7 mM $MgSO_4$, 0.02 mM NADH, 14.8 units/ml phosphoglycerate kinase (E. C.2.7.2.3), as an auxiliary enzyme used in a coupled assay, and 0.01–0.02 ml of enzyme solution.

GAPDH inactivation by GuHCl and renaturation experiments

GAPDH (10 mg/ml) in denaturation buffer [0.1 M phosphate buffer, pH 7.6, 1 mM dithiothreitol (DTT)] was incubated at 0–4 °C for various times with 0.3 M or 4 M GuHCl, and the activity was assayed. The renaturation experiments were performed on the enzyme (10 mg/ml) incubated at 0–4 °C for 2 h in denaturation buffer with or without 0.3 M or 4 M GuHCl. At the end of this period, each sample was immediately diluted 1:100 in renaturation buffer (0.1 M phosphate buffer, pH 7.6, 5 mM NADH and 5 mM DTT) [26]. Aliquots (40 μl) of the incubation mixture (40 μg enzyme, 2.8 units) were withdrawn at various times, and the enzymic activity was measured. The renaturation was estimated as a percentage of the control (enzyme incubated under the same conditions). In some experiments, α-crystallin, BSA or lysozyme, each at a concentration of 1 mg/ml was added to the incubation mixture. Triplicate samples were taken for each assay and each experiment was done at least three times.

Affinity chromatography of the refolded GAPDH

GAPDH refolded by 100-fold dilution in the renaturation buffer, in the presence or absence of α-crystallin, was separated on DEAE Affi-Gel Blue prepacked cartridge.The protein was eluted from the column with 50 mM phosphate buffer, pH 7.1, in a continuous gradient of 0–1 M NaCl, and fractions of 2 ml were collected. The enzymic activity and protein concentration were assayed.

Gel-filtration chromatography of the refolded GAPDH

GAPDH refolded in the presence or absence of α-crystallin was separated by gel filtration on a Sephacryl S-300 column (64 cm × 1 cm). The protein was eluted from the column with 50 mM phosphate buffer, pH 6.7, at a flow rate of 0.5 ml/min, and 1.5 ml fractions were collected. The absorbance at 280 nm was measured in each fraction, the protein peaks were collected, dialysed against distilled water and the enzyme activity assayed.

RESULTS AND DISCUSSION

GAPDH inactivation by GuHCl

It has been shown previously that incubation of GAPDH with 0.3 M GuHCl for 24 h at room temperature inactivated the enzyme completely, but it still bound NADH with negative co-operativity, higher GuHCl concentrations being necessary to produce protein unfolding [21].

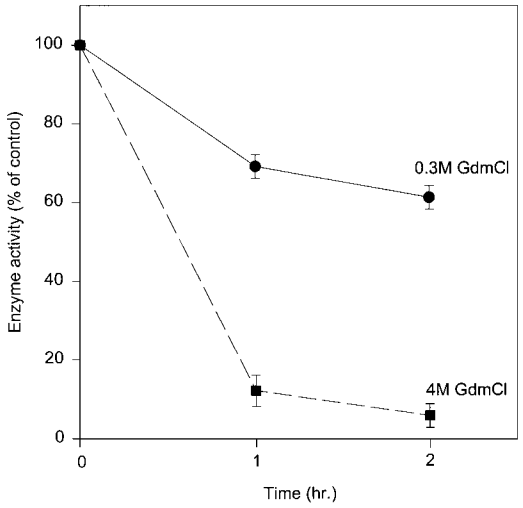

Figure 1 GAPDH inactivation by GuHCl

GAPDH (10 mg/ml) was incubated with 0.3 M or 4 M GuHCl (GdmCl) in 0.1 M phosphate buffer, pH 7.6, containing 1 mM NADH for 0, 1 or 2 h at 0–4 °C.A control sample was incubated under the same conditions. The use of continuous and broken lines is not pertinent.

In the present experiment GAPDH was incubated with two GuHCl concentrations, 0.3 M or 4 M, for various time intervals.

GAPDH was inactivated by GuHCl in a time- and concentration-dependent manner. After incubation with 0.3 M GuHCl for 2 h, the activity decreased to 61 % and with 4 M GuHCl to 5.5 % of the control (Figure 1). Longer incubation (5 h) induced greater inactivation, 84.6 % by 0.3 M and total inactivation by 4 M GuHCl (results not shown).

GAPDH renaturation in the absence or presence of α-crystallin

After 2 h of inactivation, aliquots of incubation mixture were diluted 1:100 in the renaturation buffer with or without α-crystallin (1 mg/ml) and incubated, in parallel, at room temperature and at 0–4 °C. A better reactivation of GAPDH was found by incubation at room temperature (Figure 2). The enzyme activity measured immediately after dilution was taken to be the activity at zero time for the renaturation assay.

The enzyme was reactivated by dilution in the absence and in the presence of α-crystallin; the reactivation was much greater in the presence of α-crystallin when the GAPDH was inactivated by 4 M GuHCl (Figure 2). After 8 h of renaturation, the activity of GAPDH inactivated by 4 M GuHCl recovered to 25 % without, and to 71 % with, α-crystallin (Figure 2, curves 4 M G and 4 M G+C). The extent of reactivation of the 0.3 M GuHCl-inactivated enzyme in the absence and in the presence of α-crystallin was essentially the same. Compared with the activity present at zero time of the renaturation, this represents an increase of 14 % in the absence and 17 % in the presence of α-crystallin for the 0.3 M GuHCl inactivated enzyme, whereas the 4 M inactivated enzyme recovered 17.4 % activity without, and 63 % with α-crystallin. The results indicate that the enzyme inactivated by the lower concentration of GuHCl reactivates spontaneously and similarly to the enzyme inactivated by 4 M GuHCl, whereas in the presence of α-crystallin the extent of

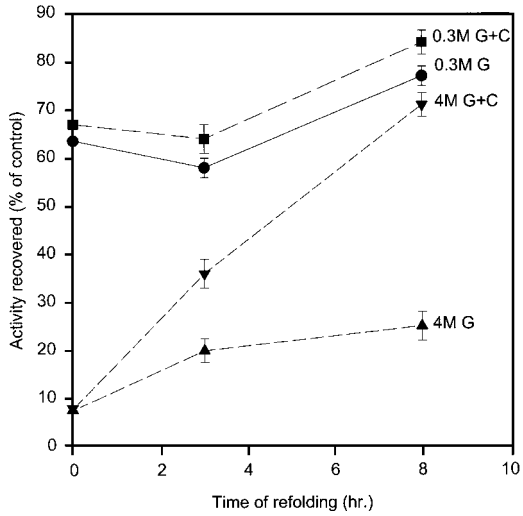

Figure 2 Reactivation of GuHCl denatured GAPDH

The enzyme inactivated by 0.3 M GuHCl (0.3 M G) or 4 M GuHCl (4 M G) was renatured by dilution in the renaturation buffer in the absence (0.3 M G or 4 M G) or presence of 1 mg/ml α-crystallin (0.3 M G + C or 4 M G + C). The activity recovered was calculated as a percentage of the control (GAPDH and GAPDH + α-crystallin activities respectively, under the same conditions). The use of continuous, dashed and broken lines is not pertinent.

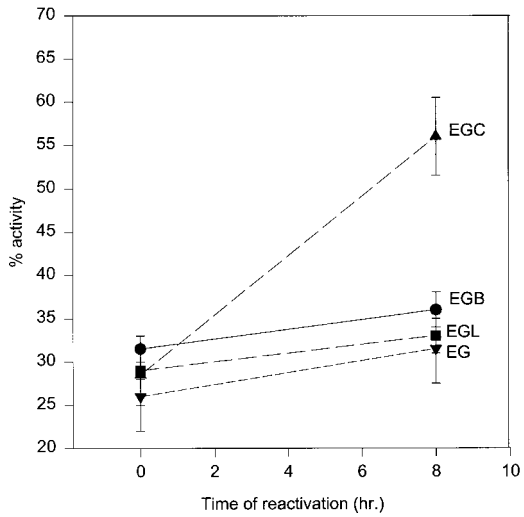

Figure 3 Specific reactivation of GAPDH by α-crystallin

The 4 M GuHCl-inactivated GAPDH was incubated for 8 h in the renaturation buffer in the absence (EG) or presence of α-crystallin (EGC), BSA (EGB) or lysozyme (EGL) (1 mg/ml of each).The recovered activity was calculated as a percentage of the controls (GAPDH, GAPDH + α-crystallin, GAPDH + BSA or GAPDH + lysozyme respectively, under the same conditions). The use of continuous, dashed and broken lines is not pertinent.

reactivation is higher for the enzyme inactivated by 4 M than by 0.3 M GuHCl.

It has been shown that lower concentrations of GuHCl do not induce conformational changes [20]. Intrinsic and ANS (8-anilino-1-naphthalenesulphonic acid) fluorescence spectra suggested a partial unfolding of the enzyme with 4 M GuHCl [21].This could explain the greater effect of α-crystallin on the reactivation of 4 M GuHCl-inactivated GAPDH, as it has been suggested that α-crystallin shows a substrate specificity and interacts with non-native intermediates formed in the denaturation pathway [13,27].

The stoichiometry of GAPDH reactivation (recovery of 63% activity) by α-crystallin shows that two molecules of enzyme were protected by multimeric α-crystallin assembly (800000 Da). This is consistent with the presence of a cavity within the structure of α-crystallin [28]. If protection was provided on the outside of the aggregate, more enzyme molecules could be protected per α-crystallin aggregate.

Specificity of α-crystallin in GAPDH renaturation

The specificity of α-crystallin activity was examined by incubating 4 M GuHCl-inactivated GAPDH in renaturation buffer in the presence or absence of BSA, lysozyme or α-crystallin, at the same concentration (1 mg/ml) and under the same conditions. Enzymic activity was measured immediately after dilution of the inactivated enzyme in buffer with or without BSA, lysozyme or α-crystallin, and after various time intervals (Figure 3). There was no significant reactivation by BSA or lysozyme (4.5% and 4% reactivation respectively, at 8 h compared with zero time of reactivation), whereas reactivation by α-crystallin (28%) was significant. The results show that α-crystallin specifically assisted the correct refolding of the GuHCl-denaturated GAPDH and consequently its reactivation.

Under similar conditions another molecular chaperone, GroEL, completely suppressed the reactivation of GAPDH, and the enzyme was renatured only when MgATP or MgATP and GroES were added [26].

Affinity chromatography of renatured GAPDH

For a better understanding of the mechanism of refolding of GuHCl-denatured GAPDH and the contribution of α-crystallin

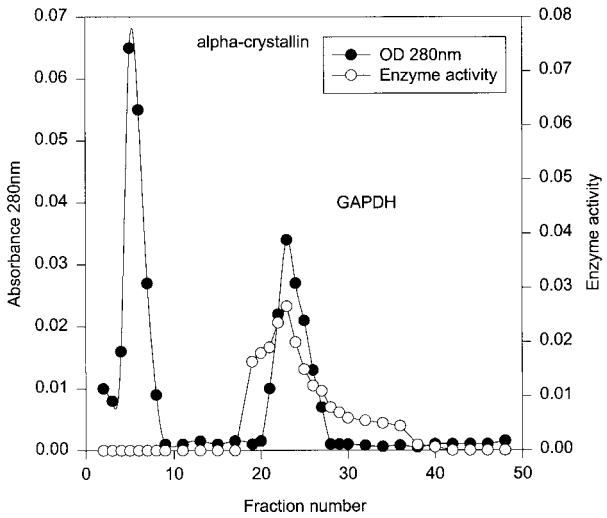

Figure 4 Separation of native GAPDH and α-crystallin by affinity chromatography

GAPDH (2 mg protein) and α-crystallin (1.5 mg protein) were dissolved in 50 mM phosphate buffer, pH 7.1, and separated by affinity chromatography on DEAE Affi-Gel Blue (bed volume 5 ml). The proteins were eluted from the column with a 0–1 M NaCl gradient in 50 mM phosphate buffer, pH 7.1, at a flow rate of 1 ml/min, and 2 ml fractions were collected. ●, protein measured as absorbance (OD) at 280 nm.

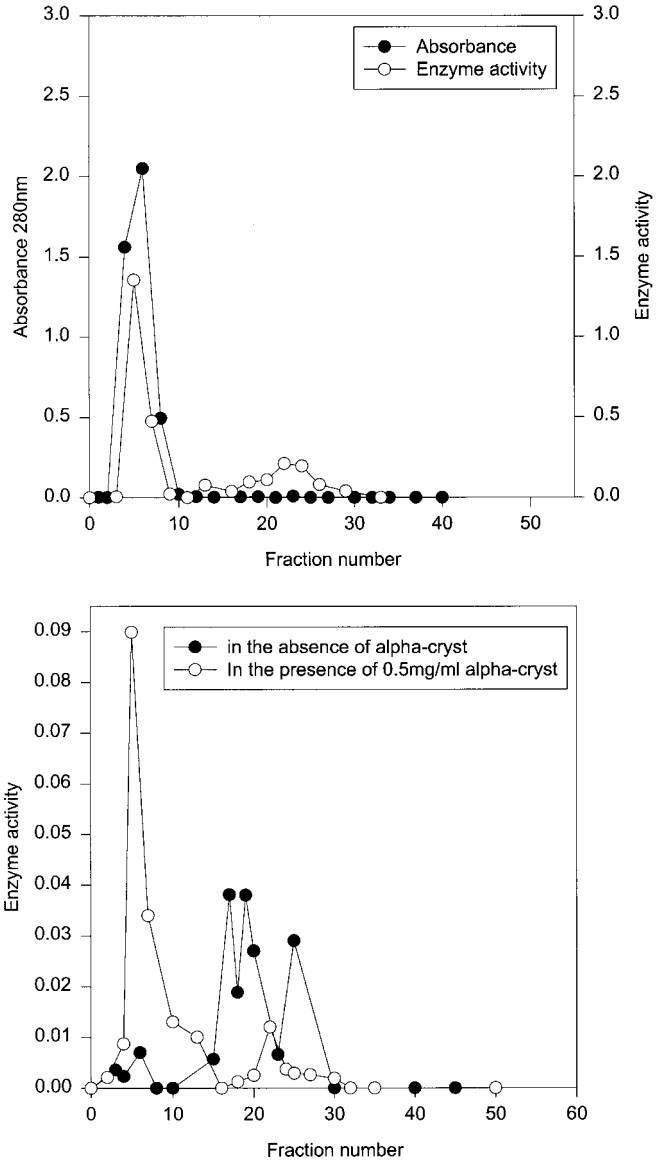

Figure 5 Affinity chromatography on Affi-Gel Blue of native GAPDH and renatured GAPDH in the renaturation buffer

Elution conditions were as described in the legend to Figure 4. Upper panel: elution profile of the native GAPDH (600 μg protein) dissolved in 1 ml renaturation buffer (5 mM NADH, 1 mM DTT in 0.1 M potassium phosphate buffer pH 7.1). ●, protein measured as absorbance at 280 nm. Lower panel: elution profile of GAPDH, renatured by 100-fold dilution in the renaturation buffer in the absence (●) or presence (○) of 0.5 mg/ml α-crystallin (alpha-cryst).

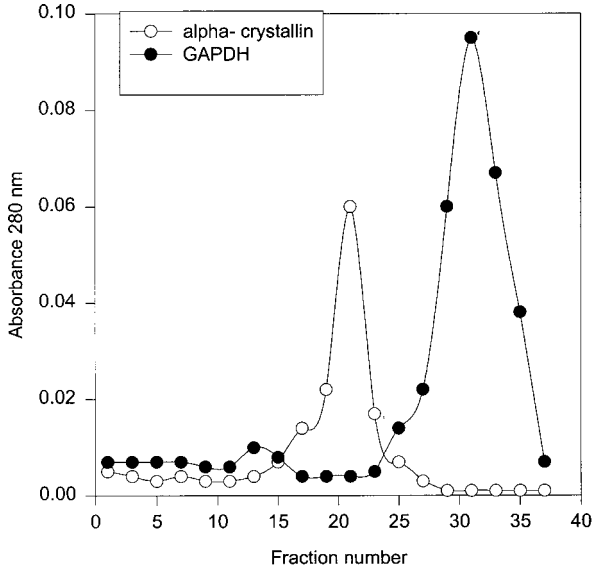

Figure 6 Gel-filtration analysis of native GAPDH and α-crystallin on Sephacryl S-300 HR

Native GAPDH (0.6 mg protein in 1 ml renaturation buffer) and α-crystallin (2 mg protein in 1 ml renaturation buffer) were separated on Sephacryl S-300 HR (64 cm × 1 cm), eluted at a flow rate of 0.5 ml/min with 50 mM phosphate buffer, pH 6.7. Fractions (1.5 ml) were collected and the absorbance at 280 nm was measured in each fraction.

to the recovery of enzyme activity, the behaviour of the renatured enzyme on an affinity column, which binds dehydrogenases, has been studied. The elution pattern of the native GAPDH and α-crystallin separation by affinity chromatography on DEAE Affi-Gel Blue is shown in Figure 4. α-Crystallin was eluted with the void volume, whereas the enzyme was bound to the column and eluted later, in fractions 20–30 (Figure 4).

When NADH was present, the native GAPDH elution pattern was modified by competition between the coenzyme and the affinity gel for the enzyme binding (Figure 5, upper panel). In the presence of NADH, the enzyme eluted together with its coenzyme in the void volume and the protein and activity peaks were superimposed. A small amount of activity was detected in fractions 20–30, corresponding to the enzyme bound to the column (Figure 5, lower panel). The GuHCl-denatured GAPDH, refolded in the absence or presence of α-crystallin, was separated by affinity chromatography and the enzyme activity measured in the eluted protein fractions (Figure 5, lower panel). In the presence of α-crystallin, GAPDH activity was observed in a similar position to that of the native enzyme in the presence of NADH. This indicates that α-crystallin assisted the renaturation of the enzyme to its native state. The possibility that α-crystallin protects GAPDH by forming a complex was investigated by size-exclusion chromatography (see below). GAPDH renatured in the absence of α-crystallin showed some activity, distributed over several fractions, but only a very small peak was eluted as the native enzyme (Figure 5, lower panel). Most of the activity was present in the enzyme species with more affinity for the column gel than the native enzyme, suggesting the presence of some active intermediates, which could be a mixture of active and inactive molecules. This suggestion is supported by the data reported by Ovadi et al. [29], which showed that, in solution, GAPDH exists as a tetramer–dimer equilibrium mixture and both forms are enzymically active. The dissociation of the tetramer into monomers and dimers by chaotropic agents, and renaturation of the enzyme by dilution in renaturation buffer could produce recombination of the subunits. The interaction observed between active and inactive GAPDH molecules [29] could explain the results of the present study. In addition, it has been suggested that the enzyme contains two different NAD^+ binding sites, i.e. sites which tightly bind 2 molecules of NAD^+, and sites which bind two molecules of NAD^+ relatively loosely [20]. The conformational changes of the enzyme during refolding

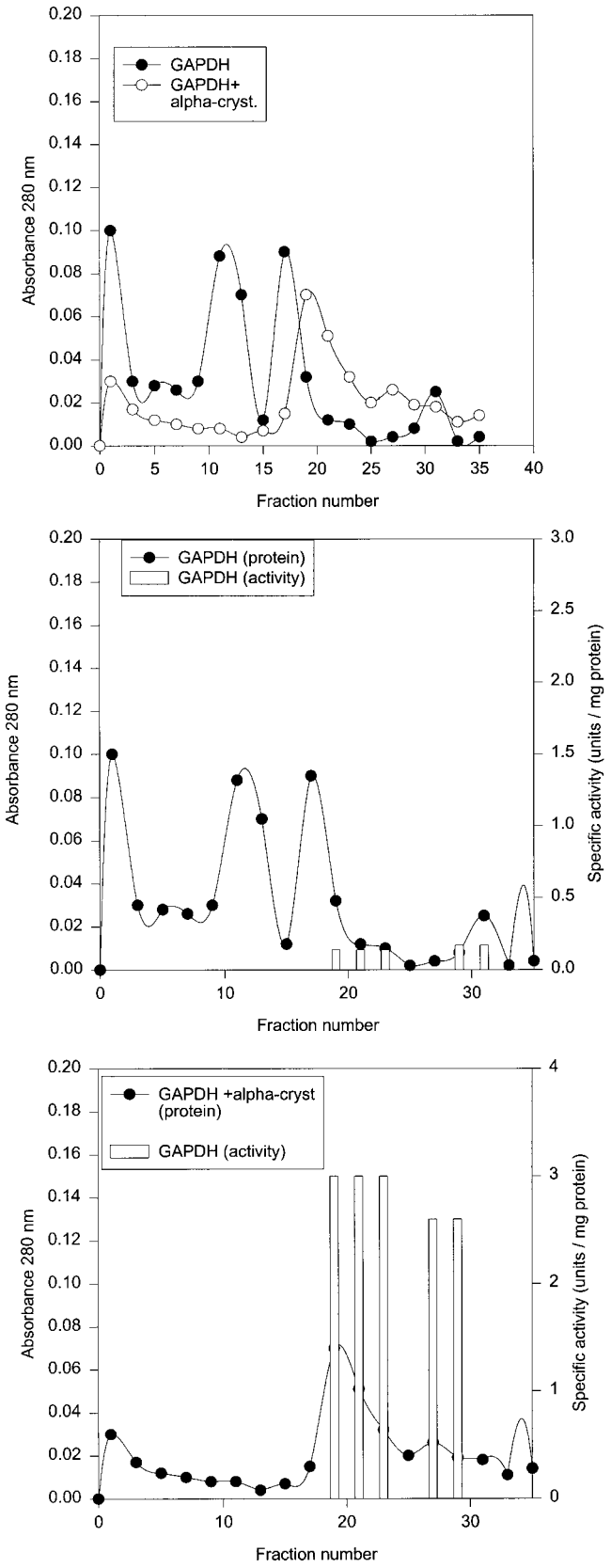

Figure 7 Gel-filtration on Sephacryl S-300 HR of GAPDH renatured with and without α-crystallin

Elution conditions were as described in the legend to Figure 6. Top panel; GAPDH (0.6 mg protein) was renatured by 100-fold dilution in renaturation buffer, in the absence or presence of 1 mg α-crystallin (alpha-cryst.). Middle panel: GAPDH refolded in the absence of α-crystallin. Bottom panel: GAPDH refolded in the presence of 1 mg/ml α-crystallin (alpha-cryst).

could expose one or other type of coenzyme binding site, thus influencing the enzyme's affinity for the column gel.

Gel-filtration chromatography of the refolded GAPDH

Most of the studies on the mechanism of protein unfolding by GuHCl are based on spectroscopic techniques. Recently, it has been shown that GuHCl unfolding of apoflavodoxin is a three-state mechanism in which a relatively stable intermediate is involved [30]. The CD data showed that the intermediate had significant secondary structure, whereas fluorescence spectroscopy indicated a lack of the characteristic tertiary structure of the native protein. Thus the intermediate had the characteristics of a molten globule state [31].

In order to characterize the intermediates of the GuHCl-unfolded GAPDH on the refolding pathway, we separated the components by gel-filtration on Sephacryl S-300. The elution profile of the native protein is shown in Figure 6. GAPDH was eluted in fraction 33 and α-crystallin in fraction 20 (Figure 6).

The GuHCl-unfolded GAPDH, renatured by dilution in the absence or presence of α-crystallin, was separated on a Sephacryl S-300 HR column (Figure 7, top panel). Most of the enzyme renatured in the absence of α-crystallin was eluted earlier than the native enzyme, indicating the presence of higher molecular mass material, presumably enzyme aggregates, and only a very small peak was eluted at the position of the native enzyme, indicating that a very small amount of the enzyme was renatured to its native state. The enzymic activity found in these peaks was very low: 0.17 unit/mg of protein in the peak corresponding to the native enzyme, and 0.14 units/mg of protein in the preceding peak, which was eluted as a slightly aggregated form (Figure 7, middle panel). There was no activity in the higher molecular mass peaks, suggesting larger aggregates, which had no GAPDH activity.

The enzyme renatured in the presence of α-crystallin (Figure 7, top panel) showed a main peak at the position of α-crystallin, but was eluted slightly earlier (maximum absorbance in fraction 19), suggesting that the enzyme eluted as a complex with α-crystallin; large aggregates were not observed. More than half of the enzyme activity was in the peak eluted as α-crystallin (3 units/mg of protein), and some activity was found close to the position of the native enzyme (2.5 units/mg of protein) (Figure 7, bottom panel). Therefore, the majority of the activity, restored by the presence of α-crystallin, is in a complex with α-crystallin, which could be in equilibrium with the free enzyme.

The heat-induced aggregation of β-crystallin preparations has been used widely to assess the chaperone activity of α-crystallin, but it has been shown [32] that much of the protein precipitated consisted of a group of heat-labile enzymes, including GAPDH. These authors showed that α-crystallin protected GAPDH against heat. In the present work, we have shown that, in addition to this protective effect, α-crystallin is able to assist in the restoration of activity of GAPDH previously denatured by GuHCl.

The results show that α-crystallin acted as a molecular chaperone by assisting the refolding of GAPDH, suppressing the aggregation occurring during the dilution, helping the enzyme to recover to its native state and thus to regain most of its activity.

We are grateful to Wellcome Trust for financial support.

REFERENCES

1 Hendrick, J. P. and Hartl, T. U. (1993) Annu. Rev. Biochem. **62**, 349–385
2 Merk, K., Groenen, P. J., Voorter, C. E., de Haard-Hoekman, W. A., Horwitz, J., Bloemendal, H. and de Jong, W. W. (1993) J. Biol. Chem. **268**, 1046–1052
3 Lee, G. J., Roseman, A. M., Saibil, H. R. and Vierling, E. (1997) EMBO J. **16**, 659–671
4 Kim, K. K., Kim, R. and Kim, S. H. (1998) Nature (London) **394**, 595–599
5 van de Klundert, F. A., Smulders, R. H., Gijsen, M. L., Lindner, R. A., Jaenicke, R., Carver, J. A. and de Jong, W. W. (1998) Eur. J. Biochem. **258**, 1014–1021
6 Horwitz, J. (1992) Proc. Natl. Acad. Sci. U.S.A. **89**, 10449–10453
7 Ganea, E. and Harding, J. J. (1995) Eur. J. Biochem. **231**, 9655–9660
8 Heath, M. M., Rixon, K. C. and Harding, J. J. (1996) Biochim. Biophys. Acta **1315**, 174–186
9 Ganea, E. and Harding, J. J. (1996) Biochem. Biophys. Res. Commun. **222**, 626–631
10 Hook, D. W. and Harding, J. J. (1996) FEBS Lett. **382**, 281–284
11 Hook, D. W. and Harding, J. J. (1997) Eur. J. Biochem. **247**, 380–385
12 Das, K. P. and Surewicz, W. K. (1995) Biochem. J. **311**, 367–370
13 Rawat, U. and Rao, M. (1998) J. Biol. Chem. **273**, 9415–9423
14 Jakob, U., Gaestel, M., Engel, K. and Buchner, J. (1993) J. Biol. Chem. **268**, 1517–1520
15 Muchowski, P. J. and Clark, J. I. (1998) Proc. Natl. Acad. Sci. U.S.A. **95**, 1004–1009
16 Matthews, C. R. (1993) Annu. Rev. Biochem. **62**, 653–683
17 Sirover, M. A. (1997) J. Cell Biochem. **66**, 133–140
18 Lubec, G., Labudova, O., Cairns, N. and Fountoulakis, M. (1999) Neurosci. Lett. **260**, 141–145
19 Jedziniak, J. A., Arredondo, L. M. and Meys, M. (1986) Curr. Eye Res. **5**, 119–126
20 He, R. Q., Li, Y. G., Wu, X. Q. and Li, L. (1995) Biochim. Biophys. Acta **1253**, 47–56
21 Jiang, R. F. and Tsou, C. L. (1994) Biochem. J. **303**, 241–245
22 Li, J. and Wang, C. C. (1999) J. Biol. Chem. **274**, 10790–10794
23 Hook, D. W. and Harding, J. J. (1997) Biochim. Biophys. Acta **1362**, 232–242
24 Beswick, H. T. and Harding, J. J. (1987) Biochem. J. **246**, 595–599
25 Bergmeyer, H. U. (1974) Methods in Enzyme Analysis, pp. 466–467, Verlagchemie GmbH, Weinheim
26 Li, X.-L., Le, X. D., Cai, H., Li, J., Yang, S. L., Wang, C. C., Wang, C. L. and Tsou, C. L. (1998) Biochem. J. **331**, 505–511
27 Lindner, R. A., Kaour, A. and Carver, J. A. (1995) J. Biol. Chem. **272**, 27722–27729
28 Haley, D. A., Horwitz, J. and Stewart, P. L. (1998) J. Mol. Biol. **277**, 27–35
29 Ovadi, J., Telegdi, M., Batke, J. and Keleti, T. (1971) Eur. J. Biochem. **22**, 430–432
30 van Mierlo, C. P., van Dongen, W. M., Vergeldt, F., van Berkel, W. J. and Steensma, E. (1998) Protein Sci. **11**, 2331–2344
31 Ptitsyn, O. B. (1995) Adv. Protein Chem. **47**, 83–229
32 Velasco, P. T., Lukas, T. J., Murthy, S. N. P., Douglas-Tabor, Y., Garland, D. L. and Lorand, L. (1997) Exp. Eye Res. **65**, 497–505

Received 20 July 1999/6 October 1999; accepted 8 November 1999