Enzyme activity after resealing within ghost erythrocyte cells, and protection by α -crystallin against fructose-induced inactivation

Barry K. DERHAM and John J. HARDING¹

Nuffield Laboratory of Ophthalmology, University of Oxford, Walton Street, Oxford OX2 6AW, U.K.

The role of α -crystallin as a molecular chaperone has been shown in many *in vitro* studies. In the present paper, we report on the chaperone function of α -crystallin within resealed erythrocyte ghosts. Eight enzymes were individually resealed within erythrocyte ghosts and assayed at zero time and at 24 h. The ghost cell suspension was separated into soluble and membrane fractions. Five of the enzymes had significantly greater enzyme activity after 24 h than the control within the soluble fractions. Fructation

caused a decrease in enzyme activity (relative to the control). Resealing of α -crystallin within the ghost cell alongside the enzymes protected against inactivation by fructose within the soluble fraction.

Key words: α -crystallin, enzyme inactivation, fructation, ghost cell, membrane.

INTRODUCTION

The catalytic activity of an enzyme is usually investigated in dilute aqueous solutions. However, the compact nature of living cells differs greatly from experimental medium in cuvettes. To recreate a more physiological environment in which to study enzyme reactions, the enzyme and reactants should be contained within functional cells. Previous reports showed that incorporation of fructose dehydrogenase into liposomes has a profound effect on enzyme activity compared with the enzyme in the free state. Enzyme activation was greater by orders of magnitude than that observed in the presence of Triton, indicating that this higher activation was due to lipid–protein interactions [1]. Furthermore, attachment of glycolytic enzymes to the erythrocyte membrane has been studied. Most enzymes can be released from the membranes by hypo-osmotic haemolysis and have been classified as enzymes ‘loosely bound’ to cell membranes [2]. In contrast, fructose-bisphosphate aldolase and glyceraldehyde-phosphate dehydrogenase have been described as ‘firmly bound’ enzymes.

Previous experiments in our laboratory have demonstrated inactivation of enzymes by small molecules. *In vitro* glycation of proteins causes conformational changes to the proteins, exposing the hydrophobic core and allowing the oxidation of newly exposed thiols into disulphides, which act as cross-links [3]. Fructation caused a decrease in enzyme activity of all the enzymes under investigation *in vitro* [4–6]. Ghost cells, erythrocytes devoid of intracellular components, offer the ability to carry out previous experiments, but within the physiological confines of cells that include membrane channels, intrinsic and extrinsic proteins.

Enzyme inactivation *in vitro* was prevented by the presence of α -crystallin, a molecular chaperone. α -Crystallin, a lens structural protein and molecular chaperone (comprising of α A and α B subunits), has been shown to protect many enzymes from inactivation and heat-induced aggregation [7]. Horwitz [8] first characterized α -crystallin as a molecular chaperone *in vitro*, based on its ability to prevent heat-induced aggregation of lens proteins and enzymes. These protective capabilities have been demonstrated with other *in vitro* systems, including the pre-

vention of aggregation of insulin B chains, following the reduction of disulphide bonds [9] by refolding of guanidine-hydrochloride- or urea-denatured proteins [8,10] and prevention of inactivation of enzymes by small molecules [4,5]. Characterization of α -crystallin using these assays has indicated similar mechanisms of protection. However, the molecular mechanism of the interaction between α -crystallin and substrates remains enigmatic.

In the present paper, we have resealed various enzymes within ghost cells followed by fructation to investigate the activity of resealed enzymes and the chaperone function of α -crystallin in a more physiological environment. The enzymes catalase, glutathione reductase, aldehyde dehydrogenase and malate dehydrogenase displayed huge increases in activity.

The increase in activity of some enzymes within ghost cells suggests that the lipid bilayer and proteins inherent within them stabilize an active conformation of the enzymes. This may be due to decreasing the entropy, the activation energy, by either a decrease in ordering or orientation of the substrate molecules or the catalytic side chains in the enzyme active site. This could also be due to a crowding effect of the lipid/protein environment in the ghost cell.

MATERIALS AND METHODS

Chemicals

All chemicals and enzymes were obtained from Sigma (Poole, Dorset, U.K.). The enzymes used were malate dehydrogenase from porcine heart (EC 1.1.1.37), glyceraldehyde 3-phosphate dehydrogenase from rabbit muscle (EC 1.2.1.12), fumarase from porcine heart (EC 4.2.1.2), catalase from bovine liver (EC 1.11.1.6), glutathione reductase from bakers yeast (EC 1.6.4.2), superoxide dismutase from bovine liver (EC 1.15.1.1), xanthine oxidase from buttermilk (EC 1.1.3.22), aldehyde dehydrogenase from bakers yeast (EC 1.2.1.5), lactate dehydrogenase from rabbit muscle (EC 1.1.1.27), phosphoglyceric phosphokinase (EC 2.7.2.3) and creatine kinase type 1 from rabbit muscle

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

Figure 1 Elution profile of a haemolysed erythrocyte suspension loaded onto a Sepharose 2B size-exclusion column (5 cm $\times$ 28 cm) pre-equilibrated with hypo-osmotic Pipes buffer, pH 6, that was maintained at 0 °C

The void volume at approx. 70 ml contains the ghosts. Haemoglobin was eluted at 200 ml.

(EC 2.7.3.2). Sepharose 2B was from Pharmacia Ltd. (Milton Keynes, Bucks., U.K.). α -Crystallin was isolated from bovine lenses [11].

Preparation of ghost cells

Freshly drawn human blood (30 ml) was collected from volunteers and stored at 4 °C with heparin for a maximum of 3 days. Erythrocyte ghosts, free of haemoglobin, were prepared by gel filtration chromatography [12]. Blood (5–6 ml) was centrifuged (1000 *g*) for 10 min, the plasma and white cells aspirated and the erythrocytes resuspended at 0 °C with iso-osmotic Hepes buffer, pH 7.4 (20 mM Hepes and 146 mM NaCl). This procedure was repeated four times. After the final wash, the supernatant was aspirated and the packed erythrocytes (approx. 3 ml) were lysed with hypo-osmotic buffer (approx. 50 mOsm), pH 6 (15 mM Pipes and 0.1 mM EDTA) to give a 10% haematocrit. The suspension was shaken gently and cooled in an ice bath for 5 min before loading onto a Sepharose 2B size-exclusion column (5 cm $\times$ 28 cm), pre-equilibrated with the hypo-osmotic Pipes buffer and maintained at 0 °C by a cooling jacket with circulating antifreeze. The column was eluted at a constant flow rate of 30 ml/h Hepes buffer and fractions were collected in tubes in an ice bath to prevent resealing. The ghost cells were eluted in the void volume (70 ml) whereas the main haemoglobin band was eluted approx. 130 ml later (see Figure 1). The lysed cells were white and therefore practically haemoglobin-free. The lysed cells were collected by centrifugation (11000 *g*, 10 mins, 0 °C), the supernatant was aspirated and then the pellet was re-suspended in iso-osmotic Hepes buffer at 0 °C to prevent resealing. This washing procedure was repeated four times to remove the hypo-osmotic buffer, and also to keep the ghost cells in an open form. After the final wash, the supernatant was aspirated and the packed ghost cells were suspended in 3 ml of Hepes buffer.

Re-sealing ghost cells

To four individual tubes, (i) 1 mg/ml enzyme, (ii) 1 mg/ml enzyme and 40 mM fructose, (iii) 1 mg/ml enzyme and 1 mg/ml α -crystallin, and (iv) 1 mg/ml enzyme, 50 mM fructose

and 1 mg/ml α -crystallin were added. For controls, 1 mg/ml BSA and 1 mg/ml lysozyme were used instead of α -crystallin. Then 5 ml of resealing buffer and 0.5 ml of ghost cell suspension was added to each tube at 0 °C. Resealing buffer, pH 7.4, contained 10 mM NaCl, 140 mM KCl, 10 mM Mops, 2 mM dithiothreitol, 0.1 mM EGTA, 1 mM potassium phosphate and 0.15 mM MgCl₂. Also, 2 mM potassium ATP, 5 mM sodium phosphocreatine and 5 units/ μ l creatine kinase were added as an ATP-regenerating system to maintain ion channel and membrane integrity [13].

The tubes were kept on ice for 10 min, shaken, then incubated in a water bath at 37 °C for 30 min to reseal the ghost cells. The haematocrit of ghost cells was the same for all samples. After the resealing process, the individual tubes were centrifuged at 10000 *g*, the supernatant was aspirated and the ghost cells washed three times in Hepes buffer, pH 7 (20 mM Hepes and 146 mM NaCl). The ghost cell pellets within the individual tubes were then resuspended with 400 μ l of Hepes buffer, gently shaken and then incubated at 37 °C within a shaking water bath.

Sampling and lysing incubated ghost cells

At zero time and 24 h, 200 μ l of the individual ghost cell suspension was removed from the centrifugation tube to an Eppendorf tube for enzyme assays. The ghost cells, with enzyme released inside, were lysed by the addition of 200 μ l of Pipes buffer, pH 6 (15 mM Pipes and 0.1 mM EDTA) then pelleted at 12000 *g* for 5 min. Pelleted ghost cells were removed for SDS/PAGE analysis (see below). For enzyme assays (see below) 100 μ l aliquots of supernatant were used and assays were carried out in triplicate. The activity in 100 μ l of the membrane fraction (which was not solubilized for the assay) was also determined. Enzyme activity was expressed as specific activity after a minimum of five experiments. The data were analysed using Student's paired *t* test, with $P < 0.05$, $P < 0.01$ and $P < 0.001$ considered statistically significant.

Sealing test

To check that ghosts were sealed tightly in our procedure, in one experiment [¹⁴C]sucrose was sealed in with the ghosts, which were washed three times before both washed and unwashed samples were analysed by scintillation counting.

Enzyme assays

Samples (100 μ l) of the supernatant of lysed ghosts and the membrane fraction were taken for each of the following assays.

Catalase activity

Catalase activity was assayed by monitoring the decrease in absorbance at 240 nm resulting from the decomposition of hydrogen peroxide for 1 min at 37 °C [14]. The assay mixture consisted of 100 μ l of enzyme solution and 2.9 ml of H₂O₂ stock solution [0.1 ml of 30% (v/v) H₂O₂ diluted in 50 ml of 50 mM sodium phosphate buffer, pH 7].

Malate dehydrogenase activity

Malate dehydrogenase activity was determined by monitoring the decrease in absorbance of NADH at 340 nm during oxidation by oxaloacetate, at 37 °C [4]. All solutions were prepared in 0.05 M potassium phosphate buffer, pH 7.5. The assay mixture consisted of 0.5 mM oxaloacetate, 0.2 mM NADH and 100 μ l of enzyme solution.

Glutathione reductase activity

Glutathione reductase activity was determined by measuring the decrease in absorption at 339 nm as NADPH was used to reduce oxidized glutathione (GSSG). The assay was based on that of Goldberg and Spooner [15], with slight modifications. The assay mixture consisted of 15 mM EDTA, 63.5 mM GSSG and 100 μ l of enzyme, made up to 3 ml with 0.12 M potassium phosphate buffer, pH 7.2. The solution was incubated at 37 °C for 4.5 min in a quartz cuvette in a spectrophotometer and 50 μ l of 9.6 mM NADPH [in 1% (w/v) NaHCO₃] was added, stirred and absorbance was recorded for 1 min.

Aldehyde dehydrogenase activity

Aldehyde dehydrogenase activity was determined by monitoring the initial rate of NADH production at 340 nm with 0.2 mM NAD⁺ and 1 mM acetaldehyde in 0.05 M sodium pyrophosphate buffer, pH 8.4, at 37 °C [16]. The reaction was initiated by the addition of 100 μ l of enzyme solution.

Lactate dehydrogenase activity

Lactate dehydrogenase activity was determined by monitoring the decrease in absorbance of NADH at 340 nm as it is oxidized to NAD⁺, at 37 °C [17]. All solutions were prepared in 0.1 M potassium phosphate buffer, pH 7. The assay mixture consisted of 23 mM sodium pyruvate, 12 mM NADH and 100 μ l of enzyme solution made up to 3 ml.

Fumarase activity

Fumarase activity was determined by monitoring the increase in absorbance at 240 nm as fumarate was formed from malate, at 37 °C. The assay was adapted from that used by Kelley and Price [18]. All solutions were prepared in 0.1 M potassium phosphate buffer, pH 7.6. The assay mixture consisted of 50 mM malate and 100 μ l of enzyme solution.

Glyceraldehyde 3-phosphate dehydrogenase activity

Glyceraldehyde 3-phosphate dehydrogenase activity was assayed by monitoring the decreased absorbance at 340 nm resulting from the oxidation of NADH for 1 min [19] at 25 °C, in a coupled system with 3-phosphoglyceric phosphokinase. The assay mixture consisted of 5 mM D(-)-phosphoglyceric acid tricyclohexylammonium; 1.1 mM sodium ATP; 0.9 mM EDTA; 2 mM magnesium sulphate; 0.2 mM NADH, sodium salt, 2 μ l of 3-phosphoglyceric phosphokinase (7 units/ml) and 100 μ l of enzyme solution in 100 mM triethanolamine buffer, pH 7.6, made up to a final assay volume of 3 ml.

Superoxide dismutase activity

Superoxide dismutase activity was determined by monitoring the change in absorbance at 550 nm, at 37 °C, in a coupled assay. The assay was based on that of McCord and Fridovich [20]. All solutions were prepared in 0.12 M potassium phosphate buffer, pH 7.8, at 25 °C, plus 0.1 mM EDTA. Cytochrome *c* (10 μ M) is reduced in the presence of xanthine (50 μ M) and 50 μ l of xanthine oxidase (at 1.08 mg/100 μ l). Superoxide dismutase (100 μ l) inhibits this reduction over 1 min at 550 nm. The solution was made up to 3 ml with the above buffer.

SDS/PAGE

SDS/PAGE (12.5 % gel) was performed as described by Laemmli [21] under reducing conditions with a Bio-Rad system. Ghost cells (20 μ l) were dissolved in sample buffer [21] containing 5% (v/v) 2-mercaptoethanol. Coomassie Brilliant Blue G was used to detect the polypeptide bands. The relative abundance of sample band was determined by quantitative analysis of digital photographs of gels on computer (Labworks, UVP Products, Upland, CA, U.S.A.).

RESULTS

The ghost cells were eluted from the Sepharose 2B size-exclusion column in the void volume, whereas haemoglobin and cellular enzymes were eluted later (Figure 1). The preparation is comparable to a previously published gel filtration method of ghost cell preparation [12]. The collected eluted ghost cells are in their open form allowing various proteins to be incorporated into them before resealing.

It was shown that added enzymes and α -crystallin (approx. 800 kDa) were incorporated inside the ghost cell (Figure 2). Catalase alone (lane 2) and α -crystallin alone (lane 3) showed clear bands at their expected subunit masses of 80 kDa and approx. 20 kDa respectively. The ghosts themselves (lane 4) gave the usual pattern for erythrocyte membrane proteins. Ghosts that were resealed with catalase inside showed the extra band at 80 kDa (lane 5). Ghosts that were resealed in the presence of α -crystallin showed the extra band at 20 kDa (lane 6). Ghosts that were resealed with both proteins present showed extra bands at both 20 kDa and 80 kDa, indicating the efficient sealing of these proteins within the ghost cells. Similar results were seen when other enzymes were resealed, albeit with different subunit masses. The amounts of enzyme and α -crystallin were determined by densitometry of the SDS/PAGE gel. The ratio of catalase to α -crystallin was 1:1 by mass, a ratio previously observed between target enzymes and α -crystallin when α -crystallin protected the enzyme activity [14]. At these ratios, BSA gave no protection.

The amount of enzyme and α -crystallin within the four individual tubes was the same throughout the experiment from zero time to 24 h. The pH of the incubations was determined

Figure 2 SDS/PAGE of catalase and α -crystallin before and after resealing into erythrocyte ghosts

Lanes 1 and 8, molecular-mass markers; lane 2, catalase; lane 3, α -crystallin; lane 4, ghost cells alone; lane 5, ghost cells re-sealed with catalase present; lane 6, ghost cells resealed with α -crystallin present; lane 7, ghost cells resealed with both α -crystallin and catalase present.

Figure 3 Effects of fructose and α -crystallin on the enzyme activity of catalase in soluble (SOL) and membrane (MEMB) fractions at zero time and 24 h after resealing into erythrocyte ghosts

Statistically significant differences are indicated: *, $P < 0.05$; **, $P < 0.01$ and ***, $P < 0.001$.

Figure 4 Effects of fructose and α -crystallin on the enzyme activity of malate dehydrogenase in soluble (SOL) and membrane (MEMB) fractions at zero time and 24 h after resealing into erythrocyte ghosts

Statistically significant differences are indicated, as for Figure 3.

at zero time and 24 h and there was no major change in pH (results not shown).

To confirm that the lysing and resealing procedure produced effectively sealed ghost cells, [14 C]sucrose was resealed to check for leakiness. After three washes and leaving the ghost cells for 2 h, the counts in the supernatant were similar to those in the supernatant of unlabelled cells. The ghost cells were then centrifuged, lysed and counts of [14 C]sucrose measured. Approx. 70 % of the counts were recovered from the inside of the ghost cells (results not shown). This indicates that the [14 C]sucrose did not leak out of the ghosts and that they were resealed efficiently.

Controls without fructose

The total enzyme activity in the soluble and insoluble fractions respectively at zero time and after 24 h following resealing into erythrocyte ghosts is shown in Figures 3–10. The activity of catalase increased 5-fold in the soluble fraction over 24 h, whereas the activity of the membrane fraction remained at control levels (Figure 3). The activity of malate dehydrogenase in the soluble fraction increased over 5-fold, whereas the membrane fraction remained at control levels which was double that of the soluble fraction (Figure 4). The activity of glutathione reductase in-

Figure 5 Effects of fructose and α -crystallin on the enzyme activity of glutathione reductase in soluble (SOL) and membrane (MEMB) fractions at zero time and 24 h after resealing into erythrocyte ghosts

Statistically significant differences are indicated, as for Figure 3.

Figure 6 Effects of fructose and α -crystallin on the enzyme activity of aldehyde dehydrogenase in soluble (SOL) and membrane (MEMB) fractions at zero time and 24 h after resealing into erythrocyte ghosts

Statistically significant differences are indicated, as for Figure 3.

creased almost 4-fold in the soluble fraction, whereas the membrane fraction increased 5-fold from a lower control level (Figure 5). However, the majority of the total enzyme activity was found in the soluble fraction both at zero time and after 24 h. The activity of aldehyde dehydrogenase increased almost 6-fold in the soluble fraction whereas that in the membrane fraction doubled (Figure 6). The activities of lactic dehydrogenase (Figure 7) and fumarase (Figure 8) changed less; both enzymes increased slightly after 24 h in the soluble fraction, but the activity in the membrane fraction did not significantly change from zero time. In contrast, glyceraldehyde 3-phosphate dehydrogenase (Figure 9) and superoxide dismutase (Figure 10) lost activity during incubation in both soluble and membrane fractions. The soluble

fraction decreased marginally, whereas the membrane fractions lost approx. half of the activity after 24 h.

Fructose

The effect of fructation upon total enzyme activity in the soluble and insoluble fractions in the 24 h following the resealing into erythrocyte ghosts is also shown in Figures 3–10. Although the activity of catalase increased 2-fold after 24 h incubation with fructose, there was a 60 % decrease compared with the control activity (without fructose) (Figure 3). There was no change in activity in the membrane fraction, which remained at control levels. There was a marginal increase in activity of malate

Figure 7 Effects of fructose and α -crystallin on the enzyme activity of lactate dehydrogenase in soluble (SOL) and membrane (MEMB) fractions at zero time and 24 h after resealing into erythrocyte ghosts

Statistically significant differences are indicated, as for Figure 3.

Figure 8 Effects of fructose and α -crystallin on the enzyme activity of fumarase in soluble (SOL) and membrane (MEMB) fractions at zero time and 24 h after resealing into erythrocyte ghosts

Statistically significant differences are indicated, as for Figure 3.

dehydrogenase in the soluble fraction over 24 h incubation in ghosts with fructose; however, there was a 65% decrease compared with the control (without fructose) activity (Figure 4). The enzyme activity in the membrane fraction decreased by approx. 30%. During fructation of glutathione reductase there was a slight increase in activity over 24 h, but compared with the control (without fructose) there was a 70% decrease in activity in the soluble fraction (Figure 5). There was no change in the membrane fraction enzyme activity; however, there was a 90% decrease in activity compared with the control activity (without fructose). During fructation of aldehyde reductase there was an increase in basal level activity in both fractions compared with

the control activity at zero time (Figure 6). However, after 24 h, the soluble fraction had lost approx. 80% activity and the membrane fraction approx. 50% activity. Similarly, fructation of lactate dehydrogenase caused an increase in basal level activity (not significant) compared with the control (without fructose) at zero time (Figure 7), but, after 24 h, the activity in the soluble fraction had decreased by approx. 40%. Activity in the membrane fraction decreased from an already low basal activity level. Fumarase activity in the soluble fraction decreased by approx. 80% after 24 h (Figure 8), whereas activity in the membrane fraction decreased marginally (not significant). Fructation of glyceraldehyde 3-phosphate dehydrogenase caused

Figure 9 Effects of fructose and α -crystallin on the enzyme activity of glyceraldehyde 3-phosphate dehydrogenase in soluble (SOL) and membrane (MEMB) fractions at zero time and 24 h after resealing into erythrocyte ghosts

Statistically significant differences are indicated, as for Figure 3.

Figure 10 Effects of fructose and α -crystallin on the enzyme activity of superoxide dismutase in soluble (SOL) and membrane (MEMB) fractions at zero time and 24 h after resealing into erythrocyte ghosts

Statistically significant differences are indicated, as for Figure 3.

a decrease in activity of approx. 60% after 24 h, which was greater than the decrease in the control (without fructose) (Figure 9). The membrane fraction decreased less than the control (without fructose) after 24 h. Fructation caused an approx. 65% decrease in superoxide dismutase activity in the soluble fraction (Figure 10), and over 50% decrease in the membrane fraction.

α -Crystallin

The effect on activity of resealing α -crystallin, together with the enzyme, within erythrocyte ghosts was determined (Figures 3–10). The activity of catalase increased 5-fold (same as control activity) in the soluble fraction after 24 h, whereas the

activity of the membrane fraction remained at control levels (Figure 3). Similarly, the activity of malate dehydrogenase doubled in the soluble fraction after 24 h, whereas the membrane fraction remained at control levels set at a greater basal level activity than for the soluble fraction (Figure 4). Glutathione reductase activity increased approx. 3-fold in the soluble fraction after 24 h (Figure 5), although the membrane fraction remained at a low basal level. The activity of aldehyde dehydrogenase in both soluble and membrane fractions did not significantly change after 24 h (Figure 6), with both having similar activities, unlike the control (without α -crystallin), where activity was markedly enhanced. The activity of lactate dehydrogenase decreased slightly over 24 h in both soluble and membrane fractions;

however, the basal levels at zero time were very similar to the control activities (without α -crystallin) (Figure 7). The activity of fumarase in the soluble fraction increased slightly over 24 h from a lower basal level than the control activity (without α -crystallin) at zero time (Figure 8). The membrane fraction approx. doubled in activity. The activity of glyceraldehyde 3-phosphate dehydrogenase decreased in both the soluble fraction and the membrane fraction by a similar magnitude to that of incubation with control ghosts (without α -crystallin) (Figure 9). However, the activity of superoxide dismutase was decreased by about 70 % over 24 h in the soluble fraction, whereas there was no change in the membrane fraction, which had a lower basal level (Figure 10).

α -Crystallin + fructose

The presence of α -crystallin during fructation protected all the resealed enzymes from inactivation by varying degrees (Figures 3–10). The inhibition of catalase by fructose was prevented by α -crystallin and the activity was maintained to approx. 70 % of the control within the soluble fraction after 24 h (Figure 3). When BSA or lysozyme were resealed instead of α -crystallin as controls, neither protein afforded any form of protection from fructation. This was seen with all enzymes studied (results not shown). The membrane fraction remained at control activity. Inhibition of malate dehydrogenase was prevented by α -crystallin and activity within the soluble fraction was restored to approx. 65 % of control activity after 24 h (Figure 4). The decrease in membrane activity following fructation was reversed and activity was restored to approx. 80 % of control activity. The presence of α -crystallin restored activity of glutathione reductase to approx. 60 % of control activity within the soluble fraction (Figure 5), although there was little recovery of activity within the membrane fraction. Aldehyde dehydrogenase activity was maintained by α -crystallin following fructation after 24 h (Figure 6). Although the soluble activity did not match that of the control (without fructose), the activity was 6-fold that of the activity with fructose. The membrane fraction was maintained at control levels. The inhibition of lactate dehydrogenase was recovered by the presence of α -crystallin and activity in the soluble fraction was maintained at control levels (Figure 7). However, the membrane fraction activity decreased slightly, after 24 h, from control levels. Fumarase activity following fructation and resealing of α -crystallin was maintained at zero time levels, which were higher than the level with fructose alone, although not as high as those of α -crystallin or control (Figure 8). The membrane fraction activity after 24 h with fructose and α -crystallin was approx. 3-fold greater than that with fructose alone. The inhibition of glyceraldehyde 3-phosphate dehydrogenase by fructose was prevented by α -crystallin and activity was restored to control levels within the soluble fraction (Figure 9). The membrane fraction had not been affected by fructose, and the addition of α -crystallin had no further effect. The inhibition of superoxide dismutase was recovered in the soluble fraction to control levels (Figure 10). The membrane fraction activity was raised 2-fold above the control when α -crystallin was present with fructose.

The protective effect was not due to a preferential fructation of α -crystallin over the enzyme. The protective effect is specific as incorporation of BSA or lysozyme into the ghost cells did not protect the enzymes from inactivation and therefore displayed no chaperone protective ability (results not shown).

The ghost cell still maintains some enzymes within the membrane, although most are released after hypo-osmotic haemolysis and extensive washing. The fact that there are differences between enzyme activities within the membrane fraction may indicate

that those enzymes may not have been washed away from the membrane. We tested this possibility; when the enzymes were assayed using ghost cells, the background activities were very low, which indicated that the unusually high enzyme activity in the soluble and membrane fractions are those from the particular resealed enzyme observed (results not shown). Membrane-bound enzyme would not explain an observed increase in enzyme activity over 24 h.

DISCUSSION

The process of ghost cell preparation from erythrocytes, by lysis followed by size-exclusion chromatography, produced very pure intact membranes with fully operational channels and cytoskeleton. Production of erythrocyte ghosts by hypo-osmotic lysis results in the formation of a large number of pores in the erythrocyte membrane, estimated to be 300 Å in diameter [22]. Molecules that have been resealed include albumin (70 kDa) [23], ferritin (474 kDa; diameter, 80 Å) and gold particles (10–15 nm) [22].

Eight enzymes from glycolysis, the citric acid cycle and detoxifying systems were individually resealed within the 'open' ghost cells and the enzyme activity determined after 24 h. Hypo-osmotic lysing, followed by a short centrifugation, separated the soluble and membrane fractions. Whereas the activity of enzymes *in vitro* usually decreases over 24 h, the enzymes resealed within the ghosts maintained or increased activity by several-fold (with the exception of glyceraldehyde 3-phosphate dehydrogenase and superoxide dismutase, which decreased slightly). This finding was quite unexpected. Catalase, malate dehydrogenase, glutathione reductase and aldehyde dehydrogenase exhibited a 5-fold or greater increase in activity over 24 h within the soluble fraction. The activity within the control membrane fraction varied considerably. Of the eight enzymes, catalase, aldehyde dehydrogenase and glyceraldehyde 3-phosphate dehydrogenase had the same activity at zero time as the soluble fraction. The enzymes glutathione reductase, lactate dehydrogenase and superoxide dismutase had lower basal activity levels than the soluble fraction, whereas malate dehydrogenase had a higher level of enzyme activity in the membrane fraction.

This difference between malate dehydrogenase activity within the membrane fraction was shown not to be due to insufficient washing of the membranes, and residual enzymes could not explain an observed increase in enzyme activity over 24 h. The low basal activity and decrease in enzyme activity within the membrane fraction (with the exception of malate dehydrogenase, glutathione reductase and lactate dehydrogenase) could be due to the enzyme moving so far into the membrane that it is difficult to release. Such enzymes would show a resistance to fructation since lysine residues may be hidden within the membrane; however, there is no correlation between low basal activity or decrease in enzyme activity and degree of enzyme inhibition by fructose.

All enzyme soluble fractions showed a decrease in activity from fructation, compared with the control, that varied between enzymes, although this was not always observed in the membrane fraction. This may be due to a low amount of enzyme in the membrane fraction and a shielding effect of the membrane upon the enzyme during fructation. Glycation has been shown to inactivate many enzymes *in vitro* such as glutathione reductase [24], malate dehydrogenase [4,11] and catalase [25]. Incubation of fructose with α -crystallin did not cause any decrease in chaperone function [26]. Similar decreases in enzyme activity and protection from α -crystallin in the cell system correlate well with *in vitro* experiments.

The effect of α -crystallin alone upon the enzyme activity within the ghost cell varied, but it always protected against fructose-induced inactivation. Catalase, malate dehydrogenase, glutathione reductase and fumarase all increased in activity in the presence of α -crystallin, although this activity was not of the same magnitude as the control activity after 24 h. α -Crystallin does not interact with native or stable unfolded proteins [27,28] and cannot refold enzymes such as catalase [14], carbonic anhydrase [29] or rhodanase [27], although it could refold glyceraldehyde 3-phosphate dehydrogenase [30]. This suggests that α -crystallin may be sterically hindering enzyme activity. Glyceraldehyde 3-phosphate dehydrogenase activity decreased significantly in the presence of α -crystallin. This is quite an unstable enzyme, which could have acted as a 'seed' for α -crystallin (or other enzyme molecules) to form larger aggregates. Large aggregates are seen in the lens as cataracts and in the insoluble, disease-causing protein aggregates seen in the brain in neurodegenerative diseases such as Alzheimer's disease. Unfortunately, the efficiency of α -crystallin decreases with age [11].

α -Crystallin protected resealed enzymes in the soluble fraction from fructation to varying degrees. α -Crystallin protects enzymes from inactivation by small molecules in dilute solution [4–6,24,25]. Glyceraldehyde 3-phosphate dehydrogenase was not protected from inactivation by α -crystallin in dilute solution [31], but is protected in this more physiological environment. The membrane fraction proved more resistant and maintained control levels. The protection is not a result of α -crystallin reacting with free inactivators. α -Crystallin does not simply compete for fructose, as incorporation of radiolabelled fructose with proteins such as lysozyme, which has a similar lysine content, and bovine serum albumin, which has a greater lysine content, all bind fructose at a similar rate and displayed no chaperone protective ability [4]. Protection may be via complex formation or by a more dynamic mechanism that would allow enzymes to retain their functional state. Previous experiments have demonstrated that increased incorporation of radiolabelled fructose mirrored a decline in activity of glucose 6-phosphate dehydrogenase [5].

A previous study showed that incorporation of fructose dehydrogenase into bilayers of small unilamellar vesicles made of several phospholipids resulted in an 8-fold higher activation compared with that of the enzyme in the free state [1]. It was suggested that the effect of phospholipids might be attributed to a hydrophobic interaction with the enzyme that stabilizes an active conformation of the enzyme. This could explain how the enzyme activity increases while the concentration of enzyme protein stays the same over the 24 h within the ghost cell. The enzyme becomes more energetically stable.

Enzyme catalytic power is usually determined in dilute solutions in buffer, far removed from the environment in the cell. Most theories that try to explain enzyme catalysis pertain to some aspect of entropy. Pauling [32] first suggested that an enzyme might stabilize the transition state of a reaction and thereby speed up the reaction by binding the transition state and lowering the energy of activation along the reaction co-ordinate. So what factors preside in the ghost cell membrane that lowers the free energy of the transition state?

The ghost cell environment can be seen as an organic solvent that can be utilized to stabilize proteins by the ability to form multiple hydrogen bonds and act like the solvent water, thereby lowering the energy of activation. Strong hydrogen bonds can form and since the active site of an enzyme is no longer aqueous once it has closed around a substrate, the properties of hydrogen bonds in organic solvents are highly pertinent to enzymic catalysis [33].

The chaperone function of α -crystallin provides a very attractive mechanism for the important role of cell protection, possibly by a similar mechanism to that by which α -crystallin maintains precarious enzyme activities and protein conformations within the lens. Overexpression of α A-crystallin in HeLa and NIH3T3 cells increased cellular resistance; also overexpression of α B-crystallin in *Escherichia coli* conferred thermotolerance [34,35]. The α B-crystallin gene promoter is subject to stress-mediated transcriptional control and is expressed outside the lens, so it is very likely that α -crystallin plays an important role, not just within the lens, but also throughout the body, in chaperoning proteins against inactivation and aggregation. Cellular stress causes a decrease of α B-crystallin levels from the soluble fraction, which gradually recover to the control level and above. Cytoplasmic α B-crystallin is translocated upon stress to the insoluble fraction, perinuclear and membrane regions a process which is reversible upon removal of stress providing protein synthesis is not inhibited [36].

The present results have shown that the *in vitro* chaperone assays can be re-enacted within a more physiological environment. The ghost cell assay is more physiological than previous chaperone assays, which have been criticized for operating at non-physiological temperatures. The mechanism of protection of enzymes by α -crystallin remains enigmatic. The increase in enzyme activity after incubation inside ghost cells can be attributed to an increase in stability offered by the lipid bilayer.

We are grateful to the Knoop Junior Research Fellowship and the Wellcome Trust for their support.

REFERENCES

- 1 Kheirloom, A., Miyazato, K., Katoh, S. and Sada, E. (1991) Incorporation and activation of a membrane-bound enzyme in bilayers of liposomes. *Appl. Microbiol. Biotechnol.* **35**, 521–524.
- 2 Duchon, G. and Collier, H. B. (1971) Enzyme activities of human erythrocyte ghosts: effects of various treatments. *J. Membr. Biol.* **6**, 138–157.
- 3 Beswick, H. T. and Harding, J. J. (1987) Conformational changes induced in lens α - and γ -crystallins by modification with glucose 6-phosphate. Implications for cataract. *Biochem. J.* **246**, 761–769.
- 4 Heath, M. M., Rixon, K. C. and Harding, J. J. (1996) Glycation-induced inactivation of malate dehydrogenase protection by aspirin and a lens molecular chaperone, α -crystallin. *Biochim. Biophys. Acta* **1315**, 176–184.
- 5 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.
- 6 Hook, D. W. and Harding, J. J. (1998) Protection of enzymes by α -crystallin acting as a molecular chaperone. *Int. J. Biol. Macromol.* **22**, 295–306.
- 7 Derham, B. K. and Harding, J. J. (1999) α -Crystallin as a molecular chaperone. *Prog. Retin. Eye Res.* **18**, 463–509.
- 8 Horwitz, J. (1992) α -Crystallin can function as a molecular chaperone. *Proc. Natl. Acad. Sci. U.S.A.* **89**, 10449–10453.
- 9 Farahbakhsh, Z. T., Huang, Q. L., Ding, L. L., Altenbach, C., Steinhoff, H. J., Horwitz, J. and Hubbell, W. L. (1995) Interaction of α -crystallin with spin-labelled peptides. *Biochemistry* **34**, 509–516.
- 10 Raman, B., Ramakrishna, T. and Rao, C. M. (1995) Rapid refolding studies on the chaperone-like α -crystallin. Effect of α -crystallin on refolding of β - and γ -crystallins. *J. Biol. Chem.* **270**, 19888–19892.
- 11 Derham, B. K. and Harding, J. J. (1997) The effects of ageing on the chaperone-like function of rabbit α -crystallin, comparing three methods of assay. *Biochim. Biophys. Acta* **1336**, 187–194.
- 12 Wood, P. G. and Passow, H. (1981) Techniques for the modification of the intracellular composition of red blood cells. In *Techniques in Cellular Physiology* (Baker, P. F., ed.), vol. P1, part I(12), pp. 1–43, Elsevier/North-Holland, Shannon, Ireland.
- 13 Glynn, I. M. and Hoffman, J. F. (1971) Nucleotide requirements for sodium–sodium exchange catalysed by the sodium pump in human erythrocytes. *J. Physiol.* **218**, 239–256.

- 14 Hook, D. W. A. and Harding, J. J. (1997) Molecular chaperones protect catalase against thermal stress. *Eur. J. Biochem.* **247**, 380–385
- 15 Goldberg, D. M. and Spooner, R. J. (1987) Glutathione reductase. In *Methods of Enzymatic Analysis* (Bergmeyer, H.-U., ed.), vol. 3, 3rd edn, pp. 258–265, VCH, Weinheim, Germany
- 16 Ting, H.-H. and Crabbe, M. J. C. (1983) Bovine lens aldehyde dehydrogenase: kinetics and mechanism. *Biochem. J.* **215**, 361–368
- 17 Bergmeyer, H.-U., Bernt, E. and Hess, B. (1963) Lactic dehydrogenase. In *Methods of Enzymatic Analysis* (Bergmeyer, H.-U., ed.), pp. 736–743, VCH, Weinheim, Germany
- 18 Kelly, S. M. and Price, N. C. (1991) The unfolding and refolding of pig heart fumarase. *Biochem. J.* **275**, 745–749
- 19 Bergmeyer, H. U. (1973) Glyceraldehyde 3-phosphate dehydrogenase. In *Methods of Enzymatic Analysis*. (Bergmeyer, H. U., ed.), pp. 105–106, VCH, Weinheim, Germany
- 20 McCord, J. M. and Fridovich, I. (1969) Superoxide dismutase. An enzymic function for erythrocuprein (hemocuprein). *J. Biol. Chem.* **244**, 6049–6055
- 21 Laemmli, U. K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature (London)* **227**, 680–685
- 22 Seeman, P. (1967) Transient holes in the erythrocyte membrane during hypotonic hemolysis and stable holes in the membrane after lysis by saponin and lysolecithin. *J. Cell Biol.* **32**, 55–70
- 23 Colclasure, G. C. and Parker, J. C. (1991) Cytosolic protein concentration is the primary volume signal in dog red cells. *J. Gen. Physiol.* **98**, 881–892
- 24 Blakytyn, R. and Harding, J. J. (1995) Prevention of the inactivation of glutathione reductase by fructation using human α -crystallin. *Biochem. Soc. Trans.* **23**, 610S
- 25 Yan, H. and Harding, J. J. (1997) Glycation-induced inactivation and loss of antigenicity of catalase and superoxide dismutase. *Biochem. J.* **328**, 599–605
- 26 van Boekel, M. A., Hoogakker, S. E., Harding, J. J. and de Jong, W. W. (1996) The influence of some post-translational modifications on the chaperone-like activity of α -crystallin. *Ophthalmic Res.* **28** (Suppl. 1), 32–38
- 27 Das, K. P. and Surewicz, W. K. (1995) On the substrate specificity of α -crystallin as a molecular chaperone. *Biochem. J.* **311**, 367–370
- 28 Carver, J. A., Guerreiro, N., Nicholls, K. A. and Truscott, R. J. (1995) On the interaction of α -crystallin with unfolded proteins. *Biochim. Biophys. Acta* **1252**, 251–260
- 29 Rao, P. V., Horwitz, J. and Zigler, Jr, J. S. (1993) α -Crystallin, a molecular chaperone, forms a stable complex with carbonic anhydrase upon heat denaturation. *Biochem. Biophys. Res. Commun.* **190**, 786–793
- 30 Ganea, E. and Harding, J. J. (2000) α -Crystallin assists the renaturation of glyceraldehyde 3-phosphate dehydrogenase. *Biochem. J.* **345**, 467–472
- 31 Hook, D. W. and Harding, J. J. (1997) Inactivation of glyceraldehyde 3-phosphate dehydrogenase by sugars, prednisolone-21-hemisuccinate, cyanate and other small molecules. *Biochim. Biophys. Acta* **1362**, 232–242
- 32 Pauling, L. (1946) Molecular architecture and biological reactions. *Chem. Eng. News* **24**, 1375–1377
- 33 Cleland, W. W., Frey, P. A. and Gerlt, J. A. (1998) The low barrier hydrogen bond in enzymatic catalysis. *J. Biol. Chem.* **273**, 25529–25532
- 34 van den Ijssel, P. R., Overkamp, P., Knauf, U., Gaestel, M. and de Jong, W. W. (1994) α A-crystallin confers cellular thermoresistance. *FEBS Lett.* **355**, 54–56
- 35 Plater, M. L., Goode, D. and Crabbe, M. J. (1996) Effects of site-directed mutations on the chaperone-like activity of α B-crystallin. *J. Biol. Chem.* **271**, 28558–28566
- 36 Klemenz, R., Frohli, E., Steiger, R. H., Schafer, R. and Aoyama, A. (1991) α B-crystallin is a small heat shock protein. *Proc. Natl. Acad. Sci. U.S.A.* **88**, 3652–3656

Received 14 June 2002/20 August 2002; accepted 30 August 2002

Published as BJ Immediate Publication 30 August 2002, DOI 10.1042/BJ20020924