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Glycation-induced inactivation of malate dehydrogenase protection by aspirin and a lens molecular chaperone, α-crystallin

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Abstract

Non-enzymic glycosylation (glycation) of structural proteins has been widely studied as a possible mechanism in the long-term complications of diabetes. Here we show that glycation inactivates malate dehydrogenase. Aspirin affords some protection against the glycation, but α-crystallin, a lens protein which appears to act as a molecular chaperone in other systems, is much more effective. For example, 5 mM glucose completely inactivates malate dehydrogenase in four days, and 5 μg α-crystallin/ml provides complete protection against this inactivation. Fructose, a superior glycating agent, inactivates the enzyme in 24 hours but even so the same low concentration of α-crystallin is able to protect 80% of the activity. Other proteins provide no protection at the same concentration. The inactivation of malate dehydrogenase and other enzymes by glycation could play a role in diabetic complications, and molecular chaperones like α-crystallin could serve to protect them.

Keywords: Aspirin; Chaperone; α-Crystallin; Glycation; Malate dehydrogenase

1. Introduction

Diabetes is associated with a number of long-term complications including neuropathy, retinopathy, cataract, nephropathy and angiopathy. For example diabetic patients are four to six times more likely to develop cataract compared with non-diabetics [1].

One suggested mechanism for the formation of cataract and other complications in diabetes is the non-enzymic binding of sugars with proteins; this is a condensation reaction between the carbonyl group of the sugar and free amino groups at the NH_2-terminus or ε-amino groups of lysine residues of the proteins. The product of this reaction is a Schiff base, which is then converted to the more stable Amadori product [2]. Reaction of carbonyl groups on these Amadori products results in the formation of intermolecular cross-links which lead to the formation of high-molecular weight aggregates that scatter light [3,4]. Haemoglobin was the first protein shown to be glycated in vivo; glycation shifts the haemoglobin-oxygen dissociation curve, which could compromise oxygen delivery to the cells [5,6]. The ligand binding properties of albumin are also altered by glycation [7].

The lens is insulin-independent and therefore the intracellular glucose concentration reflects that of the extracellular environment [8]. A possible consequence of this increase in glucose in the lens is greater glycation of the lens proteins. The concentrations of other sugars in the lens are also increased in diabetes [9]. Glucose 6-phosphate (G6P) has been found to cause conformational changes by non-enzymic reactions with α- and γ-crystallins [2,10]. Fructose, galactose and glucosamine, a metabolite of glucose, all cause structural changes of lens proteins through reaction with their amino groups [11–13]. A number of studies investigating glycation of structural proteins have been carried out, but this has not been done to the same degree for glycation of enzymes. Enzymes play a vital role in the body and any change to their activity, structure or affinity for substrate could have profound effects on their efficiency. The enzymes RNase [14], liver glucokinase [15], β-galactosidose, alkaline phosphatase [16], glucose-6-phosphate dehydrogenase [17], and Cu-Zn-superoxide dismutase [18–21] are inactivated by glycation. Glycation also alters the kinetics of Na^+,K^+-ATPase [22]. In this study we examined the effect of glycation on malate

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dehydrogenase, an enzyme with an important role in energy provision.

Proteins in the centre of the lens, unlike other proteins, are not turned over [23]. This could result in the accumulation of modified proteins over a very long period of time, resulting in the loss of structural integrity of the lens; which in turn would affect its ability to focus light on the retina. Transparency of the lens is maintained by the short range order of the lens proteins as has been shown by phase separation experiments [24].

Aspirin, paracetamol and ibuprofen protect against cataract development in diabetic rats [25]; and are associated with a protective effect against cataract in man [26,27]. In vitro aspirin and other analgesics protect against lens opacification [28,29]. Aspirin and ibuprofen also display an in vitro protective effect against the non-enzymic modification of lens proteins [29], and protect against glycation of lens proteins by glucosamine [13], galactose [12,29], glucose and fructose [11]. Apart from the effects on glycation, aspirin has also been shown to decrease the incidence of stroke and myocardial infarction [30].

The major lens protein, α-crystallin, is able to suppress thermally induced aggregation of enzymes, and of β- and γ-crystallins [31,32]. The molecular chaperone GroEL has also shown this ability to suppress thermally induced aggregation of enzymes; the kinetic behaviour of the α-crystallin system being very similar to that of GroEL. Previous studies have shown that the amino-acid sequence of α-crystallin is homologous to the small heat-shock proteins (hsps) [33]. Mouse hsp 25 and α-crystallin share other structural characteristics and can form mixed aggregates providing more evidence that α-crystallin may have functions in the lens additional to that of its refractive function; α-crystallin may function as a molecular chaperone in the lens preventing the aggregation of enzymes and other crystallins. Very recently we showed that α-crystallin could protect glucose-6-phosphate dehydrogenase against glycation-induced inactivation [17].

In the present work we investigated whether the glucose, fructose and glucose 6-phosphate (G6P) used had any adverse affects on the activity of MDH. We then determined whether either aspirin or ibuprofen were able to exert a protective effect against any inhibitory action of the sugars. We then studied the protective properties of α-crystallin in this system and compared it with four protein standards, human albumin, bovine serum albumin, egg albumin and lysozyme, to help in determining whether α-crystallin can be classified as a molecular chaperone.

2. Materials and methods

2.1. Materials

Porcine heart malate dehydrogenase was obtained from Sigma Chemicals (Poole, Dorset, UK). All other chemicals were obtained from either Sigma or BDH (Lutterworth, Leics., UK). Bovine lenses were obtained from the local slaughterhouse.

2.2. Malate dehydrogenase assay

The enzyme activity of porcine heart malate dehydrogenase (MDH) was determined by monitoring the decrease in absorption at 340 nm as oxaloacetate was reduced by NADH, using a Kontron 930 spectrophotometer. The enzyme was prepared in 0.02 ml of 0.05 M potassium phosphate buffer (pH 7.5) incubated in the spectrophotometer at 37°C in a quartz cuvette with 2.85 ml of the above buffer and 0.1 ml of 15 mM oxaloacetate for 1 min, before the addition of 0.03 ml of 12 mM NADH. The mixture was stirred rapidly and the decrease in absorption was monitored over 1.5 min against a water blank.

2.3. Incubation with sugars

To determine whether fructose, glucose and G6P had an affect on MDH activity, the incubation solution above was incubated with these sugars, (which were dissolved in 0.05 M sodium phosphate buffer (pH 7.0) to a final concentration of 5 mM). Stock solutions of the incubation solution with and without the sugars were made, and divided up into small separate sterilized glass vials (3 vials per stock solution) with rubber tops through a sterilized 0.2 μm-pore-size Millipore filter. The vials were placed in a shaking water bath at 37°C. Zero-time readings were taken in triplicate, and samples were taken from the set of three vials per stock experiment and assayed at 2, 4, 6, 8, 24 h, and then every day for 4 days. The graphs are produced from a minimum of three values per point.

2.4. Protective effect of aspirin / ibuprofen against sugar inhibition

Both aspirin and ibuprofen were dissolved in the 0.05 M sodium phosphate buffer (pH 7.0) and incorporated into the incubation solution with or without sugars (5 mM). Aspirin concentrations of 10 and 20 mM and an ibuprofen concentration of 10 mM were used. Incubation and assay of enzyme activity was as described above.

2.5. Isolation and purification of bovine lens α-crystallin

Lenses were removed from fresh bovine eyes and stored at −20°C. Five lenses (approx. 1.95 g per lens) were thawed, decapsulated and homogenized with elution buffer (0.05 M sodium phosphate, 0.2 M KCl, 1 mM EDTA, 1 mM EGTA; pH 6.7). The mixture was then centrifuged at $12\,000 \times g$ for 20 min.

The lens structural proteins, crystallins, were separated by gel chromatography on Sephacryl 300HR by the method of Slingsby and Bateman [34]. The supernatant was loaded

onto a 980 by 75 mm column packed with Sephacryl 300HR eluted at a flow rate of 100 ml/h. The eluent was collected in 10 ml aliquots, fractions were taken and read at 280 nm.

The peaks that came off the column were separately pooled, the first being α-crystallin, and were dialyzed until there was no salt present in the surrounding water. The dialyzed protein was freeze-dried and stored at −20°C. SDS-PAGE was performed on the pooled fraction, showing that α-crystallin is free from contamination by the other crystallins (results not shown).

2.6. Protective effect of α-crystallin against sugar inhibition

The α-crystallin, isolated as described above was dissolved in 0.05 M sodium phosphate buffer (pH 7.0) and added to the enzyme solution to give a final concentration of 0.005 mg/ml and 0.5 mg/ml in the incubation solution, with or without sugar. Incubation and assay of enzymic activities was as described above. To further investigate the protective properties of α-crystallin, several protein standards were also used. Human serum albumin and bovine serum albumin have lysine contents double that of α-crystallin [35–37], whereas those of egg albumin and lysozyme are similar to α-crystallin [38,39]. The concentrations of standards used were 0.005 mg/ml and 0.05 mg/ml.

2.7. Incorporation of radio-labelled fructose into lysozyme, bovine serum albumin, and α-crystallin

Each protein (100 mg) was incubated in 5 mM fructose (containing 10 μCi [^{14}C]fructose) in 100 mM sodium phosphate (pH 7.4) at 37°C for up to 6 h (total volume 10 ml). Aliquots (0.5 ml in quadruplicate) were removed at 1, 3, and 6 h. They were deproteinized using 1 ml 15% trichloroacetic acid. Bovine serum albumin (5 μl of 5 mg/ml solution) was added to aid precipitation which proceeded for 18 h at 4°C. The precipitates were collected on fibre glass filters, dried and radioactivity measured on a scintillation counter [40].

3. Results

3.1. Incubations with 5 mM sugar solutions

None of the sugars inhibited malate dehydrogenase at zero time but fructose, G6P and glucose all inactivated the enzyme with time. The degree of inactivation and the time taken for inactivation varied between the sugars. Fructose was the most effective taking only 2 h to inactivate MDH by 38.5% ± 8.3% ($p < 0.01$, Student t-test), and 4 h to inactivate by 68.5% ± 7.9% ($p < 0.001$) (Fig. 1). G6P reduced the activity of MDH by 41% ± 7.4% in 6 h ($p < 0.01$), whereas glucose took much longer; the enzyme activity being reduced by 53% ± 9.7% after 2 days ($p < 0.01$) (Fig. 2).

3.2. Incubations with aspirin and ibuprofen

The incorporation of 10 mM aspirin into the incubation assay did not provide any protection against the inactivation of MDH by glycation by fructose, both incubations with and without aspirin inactivated MDH to the same extent (98 ± 0.23% inactivation after 24 h) (Fig. 3). Incubations with 10 mM aspirin and G6P indicated that the aspirin was having a slight protective affect against glycation by G6P; after 24 h there was 85% ± 9.4% ($p < 0.001$,

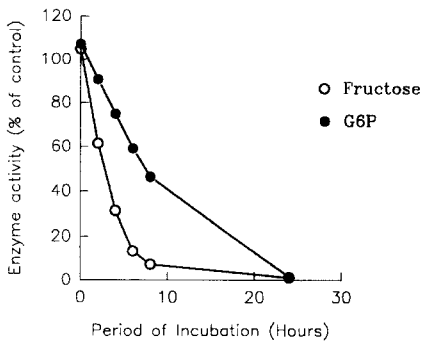

Fig. 1. Inhibition of MDH by 5 mM fructose and 5 mM glucose 6-phosphate (G6P).

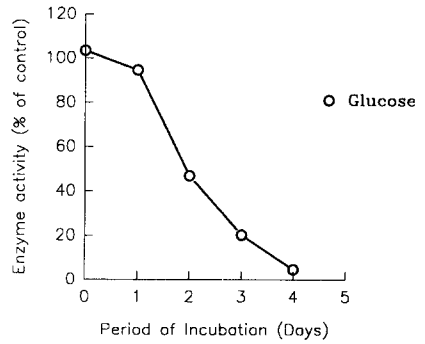

Fig. 2. Inhibition of MDH by 5 mM glucose.

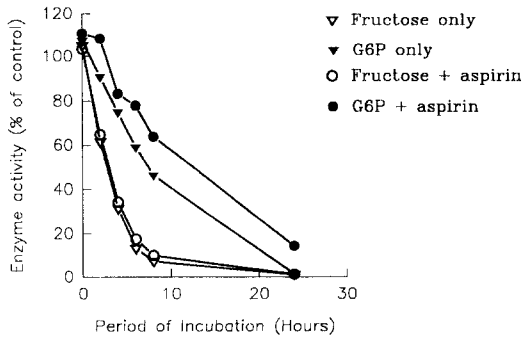

Fig. 3. Protective effect of 10 mM aspirin against inactivation of MDH by G6P and fructose.

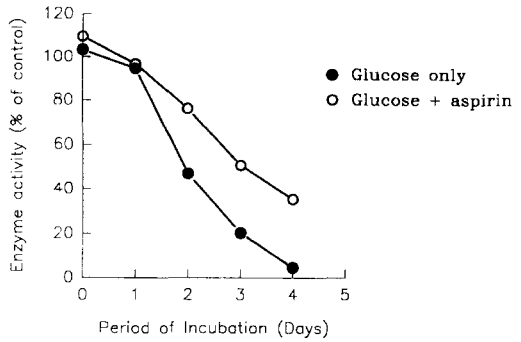

Fig. 4. Protective effect of 10 mM aspirin against inactivation of MDH by 5 mM glucose.

comparing inactivation without aspirin) inactivation of MDH, with aspirin present, as opposed to $98 \pm 0.8\%$ inactivation in incubations with G6P without aspirin (Fig. 3).

The effect of 10 mM aspirin on the glycation of MDH by glucose was more pronounced; after 4 days MDH had been inactivated by $64.5\% \pm 0.5\%$ ($p = 0.002$) (Fig. 4), in comparison to the inactivation of $95.3\% \pm 4\%$ seen in incubations without aspirin.

Increasing the concentration of aspirin to 20 mM failed to provide any protection against inactivation of MDH by fructose; there was $99 \pm 0.2\%$ inactivation after 24 h. No additional protection was provided by 20 mM aspirin against inactivation of MDH by G6P; the level of inactivation remaining at around $85 \pm 0.2\%$ ($p < 0.001$). Increasing the concentration of aspirin to 20 mM in incubations with glucose and MDH increased its protective effect; there was $51.5 \pm 0.5\%$ ($p < 0.01$) inactivation of the MDH by glucose after 4 days (results not shown), in comparison to the inactivation of 64.5 + 0.5% with 10 mM aspirin.

No protection against glycation by fructose, G6P or glucose was seen by 10 mM ibuprofen (data not shown).

3.3. Incubation with α-crystallin

α-Crystallin was isolated by gel chromatography (Fig. 5) and was introduced into the incubation vials at two concentrations; 0.005 mg/ml (ratio of MDH: α-crystallin, 1:40) and 0.5 mg/ml (MDH:α, 1:4000). Four protein standards at 0.005 and 0.05 mg/ml concentration were also run in order to investigate whether the protective role of this lens protein was unique.

No protection was provided by the albumins at 0.005 mg/ml and by lysozyme at either 0.005 or 0.5 mg/ml (Fig. 6). Protection against inactivation of MDH by 5 mM fructose was afforded by all three albumins at 0.5 mg/ml, over 8 h incubation (Fig. 6b). There was $72.6 \pm 2.9\%$ inactivation of MDH in the presence of human albumin (HA), $p = 0.005$; $64.7 \pm 3.4\%$, inactivation in the presence of bovine serum albumin (BSA), $p < 0.001$ and $84 \pm 0.5\%$, inactivation in the presence of egg albumin

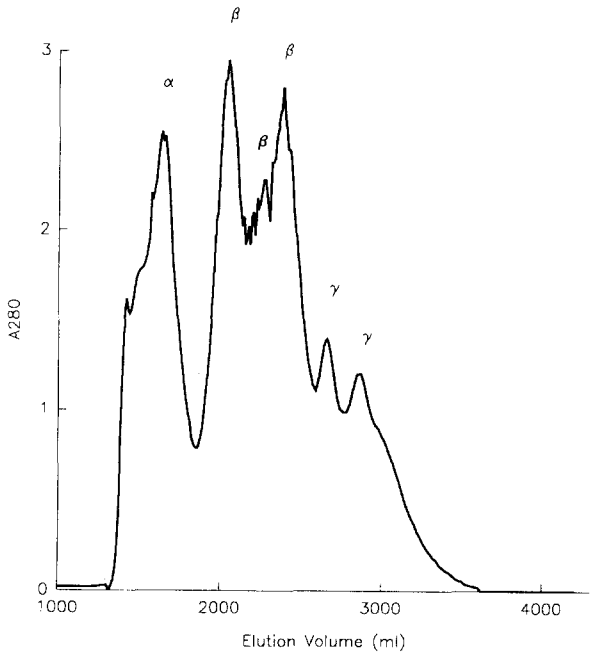

Fig. 5. Elution profile of bovine lens crystallins on Sephacryl 300 HR (980×75 mm) eluted at 100 ml/h. The first peak was pooled as α-crystallin.

(EA), $p < 0.001$ (all t-test values compared to inactivation by fructose only). This result could be explained by the fact that albumins contain a relatively large amount of lysine residues on their surface which are available for

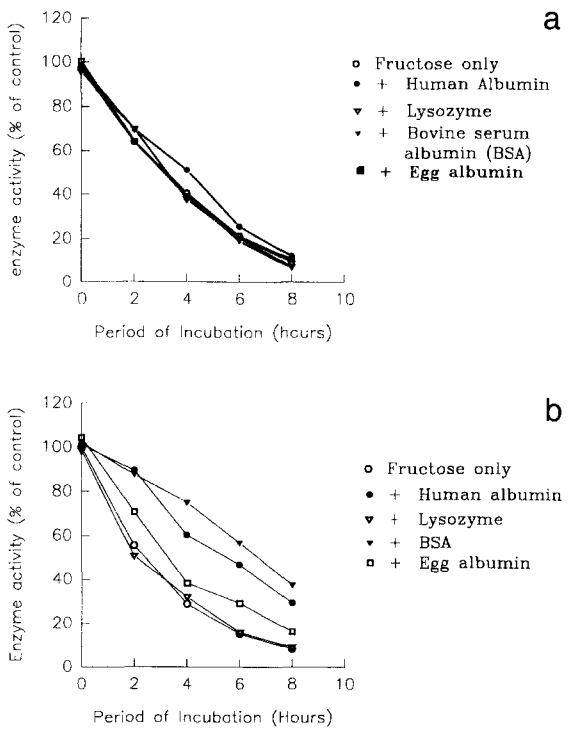

Fig. 6. Protection against inactivation of MDH by 5 mM fructose by (a) 0.005 mg/ml protein standards and (b) 0.5 mg/ml protein.

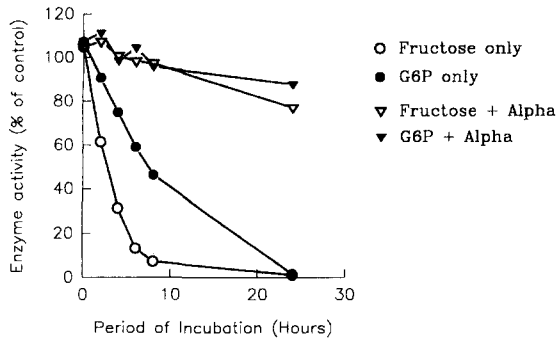

Fig. 7. Protection provided by 0.005 mg α-crystallin per ml against inactivation of MDH by 5 mM fructose and G6P.

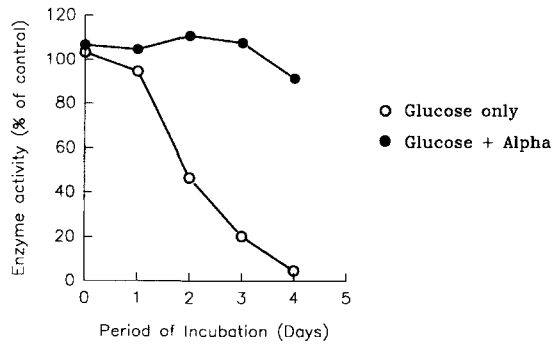

Fig. 8. Protective effect of 0.005 mg α-crystallin per ml against inactivation of MDH by 5 mM glucose.

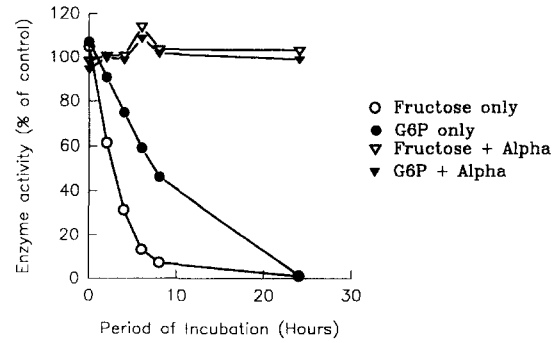

Fig. 9. Protective effect of 0.5 mg α-crystallin per ml against inactivation of MDH induced by 5 mM fructose or G6P.

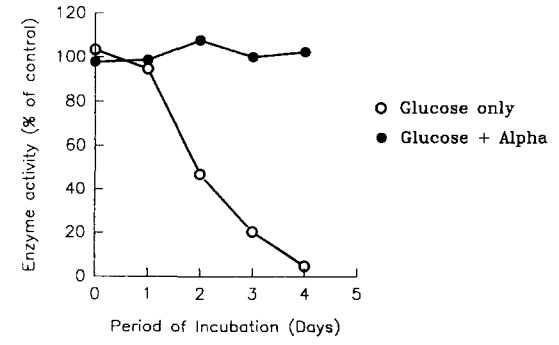

Fig. 10. Protective effect of 0.5 mg α-crystallin per ml against glucose-induced inactivation of MDH.

glycation by fructose; lysozyme, in comparison contains fewer lysines and therefore glycation by fructose is less likely to occur. The glycation of these proteins could protect MDH from complete inactivation by glycation. However, in the incubation with 0.005 mg/ml protein standards, the inactivation of MDH increases and the enzyme is inactivated to the same extent as the incubations with fructose only (Fig. 6a). The proteins at this concentration may no longer be able to protect against the inactivation of MDH. At the higher concentration of protein standards protection is afforded by the albumins throughout the 8 h incubation, this is not seen with lysozyme (Fig. 6b).

The lens protein α-crystallin, at a concentration of 0.005 mg/ml, seemed to protect against glycation of MDH by all three sugars (Figs. 7 and 8). The greatest protection was afforded against inactivation by glucose where MDH had 91.5% ± 3.8% ($p < 0.001$) of the activity of the control after 4 days (Fig. 8); protection against G6P and fructose was very similar to that of glucose, MDH having 87.7% ± 10.6% ($p < 0.001$) and 77% ± 15.8% ($p < 0.001$) of the activity of the control respectively after 24 h (Fig. 7). Increasing the concentration of α-crystallin to 0.5 mg/ml resulted in complete protection against glycation by all three sugars, ($p < 0.001$) ± 0.5% (Figs. 9 and 10). The results with α-crystallin, which has a comparatively low lysine content compared to the albumins and the lack of a free N-terminal α-amino group (unlike in the protein standards), could indicate another mechanism for protection against glycation other than its own glycation ahead of that of MDH.

At the lower concentration it is unlikely that α-crystallin is simply competing for the fructose because it lacks the α-amino group which is usually more reactive with sugars and it has fewer lysine residues than the control proteins. Nevertheless we checked on the reactivity of these proteins with radiolabelled fructose (Fig. 11). Bovine serum albumin, lysozyme and α-crystallin all bind fructose

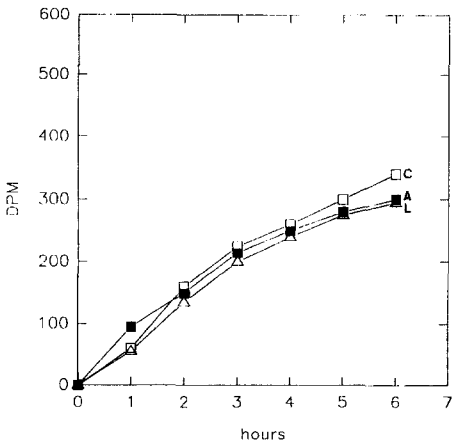

Fig. 11. Glycation of BSA, lysozyme and α-crystallin by radiolabelled fructose.

at a similar rate. α-Crystallin was not significantly more reactive although it was by far the most effective in protecting MDH (Figs. 6–8). In fact at the end of the six hour incubation no more than 0.03% of the fructose was bound to any of the proteins so they cannot be lowering the effective concentration of fructose available for glycation.

The protective effect of α-crystallin in this system appears to be specific when compared to the other proteins supporting the view that it has chaperone-like properties.

4. Discussion

Glycation affects many proteins and is of most interest in diabetes, where excess glycation of many proteins including haemoglobin, albumin, collagen and lens crystallins has been found [41].

The time-dependent inactivation of MDH by fructose, G6P and glucose suggests that these sugars bind non-enzymically to the enzyme; an explanation for the implication of these glycating agents in the formation of cataracts could come from the fact that some of them are metabolic intermediates, and are present in the lens in significant concentrations that increase in diabetes [23]. We used a concentration of sugar (5 mM) that lay within the physiological range of sugar levels in the diabetic lens; the concentration of fructose within the diabetic lens is between 1.2 and 12 mM and that of glucose is between 3 and 4.5 mM [42]; the concentration of G6P has also been shown to increase in the diabetic lens [9]. The rate of glycation is dependent on the percentage of sugar in the open chain form; the carbonyl group is then available for forming a Schiff-base. Fructose, which has a greater reactivity with haemoglobin, has a higher percentage in the open chain form than glucose, 0.7% for fructose and 0.002% for glucose [43]. This could explain the more rapid inhibition of MDH activity by fructose, as the first stage in the Maillard reaction is a nucleophilic attack by the amino group of the protein on the carbonyl group of the sugar; this reaction can only take place when the sugar is in the open chain form. G6P also inhibited MDH activity more rapidly than glucose. This could be due to the phosphate group increasing the level of disruption of the enzyme through an increase in surface negative charge; this idea has been suggested for the unfolding of γ-crystallin by G6P [10]. Near ultra-violet circular dichroism spectra performed in the same paper, indicate that G6P binding to lens proteins resulted in an alteration in the isoelectric point and tertiary/quarternary structure of the protein. The reactivity of the sugar is not solely dependent on the carbonyl content; the position of any phosphate groups present also seems to be an important factor in the reactivity of the sugar [44].

Glycation occurs by reaction of sugars with free amino groups of lysine residues. Malate dehydrogenase has 87 lysine residues/1000 residues [45] as potential glycation sites. Over half of the lysine residues are found within α-helices, and some are involved in hydrogen bonding and the structural stability of the catalytic domain of this globular dimer; 8 of the lysines are in β-sheet formation, a number of which are part of the NAD binding domain, which takes up the first 153 residues. Work carried out on haemoglobin has suggested that it is the higher order structure (tertiary/quarternary) of the protein that determines the Amadori rearrangement activity of the glycation sites, rather than the amino-acid sequence around the non-enzymic glycation site [46]. In vivo glycation of free amino groups of glutathione reductase indicates that there is a preference for lysyl-lysine sequences as opposed to single lysine residues [47]. The concentration of reduced glutathione decreases with cataract [23]; this reducing agent being used to diminish the opacification of a protein solution treated with hexose [2]. The decrease in GSH correlates with the increased opacification in diabetic rats [48]. Glutathione reductase maintains the level of GSH in the cell, it has a lysyl-lysine pair in its catalytic centre. Glycation of this pair could contribute to a decrease in enzyme activity, seen in human cataracts, and hence a lowering of the level of GSH in early diabetic cataract [48]. Porcine cytoplasmic malate dehydrogenase has a lysyl-lysine present at the surface of the protein at positions 120 and 121 [45], and is in an optimum position for being attacked by sugar; this could lead to disruption of the dimer through the prevention of NAD binding and hence a reduction in enzymic activity. The binding of G6P could additionally exert electrostatic repulsion which could disrupt hydrogen bonding and the integrity of the dimer.

These glycation studies indicate that sugars bind to enzymes and other proteins in tissues exposed to sugars leading to unfolding inactivation and aggregation. Most of the work on glycation has been done on structural proteins, however, alterations in enzyme activity as a result of glycation have been shown. Incubations of three enzymes; β-galactosidase, alkaline phosphatase and G6P dehydrogenase with glucose over a period of 6 days demonstrated an increase in K_m for all three enzymes after glucosylation and competitive inhibition of β-galactosidase was induced by glucosylation [16]. Glycation studies in the past have focused on the glucosylation of lens proteins as a cause of diabetic cataracts. The discovery that fructose and G6P are more reactive than glucose regarding interaction with lens proteins and enzymes, implies that they may play an important role in the development of diabetic cataracts particularly as fructose is present at a higher concentration than glucose in diabetic human lenses [42]. Fructose is often recommended as a substitute for sucrose for diabetics, and many diabetic foods contain fructose. In the light of the results obtained for the fructosylation of lens proteins and enzymes, diabetic foods containing fructose could be having a detrimental effect on the patient.

If glycation of enzymes and proteins is important in the

aetiology of cataract and other tissue damage, measures that prevent the reaction may prevent the tissue damage. Aspirin has a protective effect against glycosylation by galactose [12] and glucosamine [13]. The mechanism of protection is believed to be acetylation, as work on the prevention of non-enzymic carbamylation of proteins by aspirin indicated that acetylation was important in the protective effect [28]. Work on protection against glycosylation by glucosamine [13] provides further evidence that it is acetylation that protects the proteins; protection against glucosamine was afforded by aspirin (acetysalicyclic acid) but not by salicyclic acid, which is structurally identical except for the absence of the acetyl group. The acetylation by aspirin of amino groups of a variety of proteins has been seen, including haemoglobin, albumin and immunoglobulins [41].

Incubation of aspirin with the sugars and MDH resulted in aspirin displaying protection against glycation by glucose and to a lesser degree against glycation by G6P. Aspirin, at concentrations of 10 and 20 mM was unable to prevent glycation of MDH by fructose. Fructose inactivates MDH much more quickly than glucose; fructose reducing the enzyme's activity to 65.5% of the control in 2 hours, glucose taking 2 days to reduce the activity to 47%. Aspirin's inability to protect against glycation by fructose could be a result of the fast rate of reaction between fructose and MDH; in the experiments with glucose aspirin has more time to react with MDH as glucose is slower at glycating the enzyme.

Acetylation may not be the only mechanism for protection against glycation of proteins. The analgesic ibuprofen, unlike aspirin, does not have an acetyl group. Ibuprofen, however, has been shown to protect against cataract in epidemiological studies [27] and in the diabetic rat [25], where it decreased the amount of glycation. It protected against glycation of bovine lens proteins by glucosamine [13] and fructose [11]. Ibuprofen (10 mM) did not afford any protection of MDH against glycation by any of the three sugars used. Binding studies were performed between lens proteins and ibuprofen, to examine the mechanism of the protective action against cataract but only weak binding could be demonstrated [49]. There must be another mechanism whereby ibuprofen protects against cataract. This weak binding cannot account for the therapeutic effect of the drug; as in order to protect the proteins, the drug must be able to stay on the protein molecule tightly enough to compete with any cataractogenic agents for binding sites. It is possible that cataract may be prevented not through ibuprofen itself, but through a metabolite of ibuprofen which might have an additional hydrogen-bonding species; this metabolite could be produced elsewhere in the body and then travel to the lens. Thus the two anti-inflammatory drugs have rather limited benefits especially when it is recalled that the concentrations used were greatly in excess of levels achieved in tissues during treatment.

α-Crystallin is affected by non-enzymic chemical modification by sugars and other substances, e.g. cyanate; but there is now growing evidence that α-crystallin itself can provide protection against modification of proteins. The work performed so far has examined the protective effect α-crystallin has on heat-induced aggregation of its fellow lens crystallins and various enzymes [31,32]. Horwitz found no effect on enzyme inactivation. The in vitro work performed here, investigated the possibility that α-crystallin could prevent the reduction in activity of MDH caused by glycation — a more gradual process than thermal denaturation. A similar protection has been found for glucose-6-phosphase dehydrogenase [17].

We found that at low concentrations (0.005 mg/ml) α-crystallin seemed to provide protection against inactivation of MDH by all three sugars, the greatest protection being afforded against inactivation by glucose. When the concentration of α-crystallin was increased to 0.5 mg/ml, the lens protein provided complete protection against the inactivation of MDH. The possibility that this protection could be solely due to the sugars glycating α-crystallin rather than MDH can probably be discounted from the results of the experiments with the protein standards and the fact that MDH has nearly twice as many lysine residues per 1000 residues as α-crystallin [50,51] and so do some of the control proteins.

The results of the glycation experiments with the protein standards was that even the lysine-rich plasma albumin was unable to protect MDH from glycation, despite having over twice the number of lysine residues when compared to α-crystallin. This therefore indicates that there is a specific protective role for α-crystallin and its role may be in the form of a molecular chaperone. The effect of α-crystallin is all the more striking when the lower concentration used (0.005 mg/ml) is compared with the concentration in vivo, which can be 160 mg/ml in bovine lens and only slightly less in human lens.

One of the functions of the proteins synthesized by cells in response to heat shock, is to minimize the denaturing effects of heat shock upon the native structure of the proteins [52]. It appears that these heat-shock proteins (hsps) preferentially bind to partially denatured forms of polypeptides, this protects them from further denaturation and aggregation and may enable a more rapid renaturation of the denatured protein. The changes resulting from heat-induced denaturation could also occur as a result of other chemical changes, e.g., osmolarity changes and post-translational modification (e.g., glycation); these processes have been implicated in complications of diabetes including cataractogenesis. There is no turnover of proteins in the centre of the lens, making them very susceptible to any modifications known to occur in vivo [23]. It is therefore possible that the lens contains proteins with a similar role to the hsps.

There are sequence similarities between hsp 27 in *Drosophila* and mammalian α-A and α-B crystallins [33].

Anfinsen's basic idea that all the information needed for protein folding is held in the amino-acid sequence still holds true in principle. In vitro, some isolated proteins can be denatured and refolded, usually very slowly, in the absence of other macromolecular cellular components; in vivo, however, the folding and assembly of polypeptides requires other proteins known as molecular chaperones [53]. Their function is thought to be to temporarily stabilize unfolded or partially folded structures and to keep them in a form that will enable them to subsequently fold and assemble. The main role of molecular chaperones appears to be to prevent the incorrect intermolecular association of unfolded polypeptide chains, which would result in their aggregation. Two distinct mechanisms of chaperone action have evolved; the first is to prevent aggregation by the shielding of hydrophobic surfaces, this is carried out by the Hsp 70 family of chaperones; Dna K, Dna J and GrpE. The second mechanism involves the removal of a complete but as yet unfolded protein from the cellular environment to prevent aggregation, and simultaneously allow folding to the native state to proceed; this is performed by the Hsp 60 family of chaperones, GroEL and GroES. These two mechanisms can act together in a sequential pathway of folding [53].

Work carried out on the possible chaperone properties of α-crystallin have shown that it is able to protect against thermal aggregation of enzymes, e.g., the rate and amount of aggregation of yeast α-glucosidase was reduced on titration with bovine α-crystallin [32]. α-Crystallin also had a similar effect on enolase, glutathione-*S*-transferase, alcohol dehydrogenase, carbonic anhydrase, lactate dehydrogenase, aldolase and phosphoglucose isomerase. α-Crystallin also suppressed the aggregation of β_H-crystallin and of bovine γ-crystallin [32]. The addition of an equimolar concentration of α-crystallin to a denatured sample of γ_S-crystallin resulted in 95% of the original sample being recovered; α-crystallin therefore aiding the refolding of the denatured γ_S.

In the central region of the mammalian lens (the lens nucleus) there is no detectable protein synthesis and yet there are measurable activities of a variety of enzymes [23]. It is often pointed out that the enzyme activity here is less than in the outer part of the lens (the cortex) but it was difficult to explain how there could be any activity in cells that had not synthesized protein for decades. The demonstration that α-crystallin can act as a molecular chaperone provides an explanation for this enigma. Thirty or 40% of lens protein is α-crystallin. Lens enzymes are more heavily chaperoned than those in any other tissue.

α-Crystallin is a very good example of gene sharing, where a protein has two functions in a certain tissue. α-Crystallin is expressed at high levels in the lens where it contributes to the refractive properties and serves as a molecular chaperone, protecting enzymes and other proteins in the lens against aggregation that will lead to light scattering and loss of function. The fact that α-crystallin appears to protect lens proteins from denaturation and light scattering, leads to the proposal that the age-related deterioration of α-crystallin may have a major role in the onset of cataract. Any post-translational modifications that occur with age — e.g., glycation — may interfere with the chaperone function of α-crystallin and lead to its aggregation and aggregation of other crystallins.

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