Glycation-induced inactivation and loss of antigenicity of catalase and superoxide dismutase

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Oxidative mechanisms are thought to have a major role in several biological phenomena, including cataract formation and diabetic complications. Here we investigate the inactivation of catalase and superoxide dismutase, both powerful antioxidant enzymes, by sugars of different glycating abilities, and the loss of antigenicity that was monitored by the loss of activity after immunoprecipitation with monospecific antibodies. The antigenicity of non-glycated or glycated enzymes separated by affinity chromatography were determined by dot-blotting. Incubation with sugars resulted in a time-dependent inactivation of the enzymes. Ribose and fructose inactivated them more rapidly than glucose and glucose 6-phosphate. Glycation induced losses of antigenicity and inactivation simultaneously. The glycated enzymes had entirely lost their antigenicity compared with non-glycated enzyme. These results further support the idea that inactivation of enzyme and loss of antigenicity are simultaneous. This might occur in the pathogenesis of diabetic complications and aging.

INTRODUCTION

Many reports have pointed out that oxidative damage and disturbance in antioxidant defence systems of the lens might have an important role in the development of cataract [1–3]. H_2O_2 was raised in the aqueous and vitreous humours of human eyes associated with cataract [4,5] and in the aqueous humour of diabetic patients with cataract [6]. H_2O_2 can lead to cortical opacities in the rabbit lens in organ culture [7] and to interference with the function of DNA [8], cytoskeletal proteins and susceptible enzymes such as glyceraldehyde-3-phosphate dehydrogenase [9]. Cation-transport systems in the lens are also sensitive to H_2O_2-induced damage [10]. Subcapsular cataract can be regarded as the result of oxidative damage [11]. In cataractous lenses the enzymic defences against reactive species of oxygen were impaired, as evidenced by the significant decrease in activities of superoxide dismutase (SOD), catalase and glutathione peroxidase [1]. SOD is a dimeric enzyme of molecular mass 32 kDa that destroys the free radical superoxide by converting it to peroxide that can in turn be destroyed by catalase or glutathione peroxidase reactions [12]. It contains copper and zinc atoms at the active site. SOD converts the highly reactive superoxide radical to the less reactive H_2O_2 [13]. Catalase reacts with H_2O_2 to form water and molecular oxygen. Although glutathione peroxidase shares the substrate H_2O_2 with catalase, it alone can react effectively with lipid and other organic hydroperoxides [14]. The glutathione redox cycle is a major source of protection against low levels of oxidant stress, whereas catalase becomes more significant in protecting against severe oxidant stress [15].

The high level of glucose is thought to be the primary cause not only of cataract in diabetes but of all diabetic complications [16]. One major factor in this slow degradative process is thought to be glycation in which sugars, behaving as aldehydes, can react with amino groups on proteins to produce adducts. This is the non-enzymic modification of proteins by sugars by which not only the structural but also the biological properties of protein are altered. This can lead on to a variety of chemical entities and induce structural changes in enzymes starting from conformational alterations, progressing to thiol oxidation, aggregation, formation of disulphide and other covalent cross-links, and inactivation of enzymes [17]. The glycation and inactivation of catalase and SOD by different sugars would therefore greatly weaken the systems for detoxifying superoxide and H_2O_2 and could be one of the mechanisms of cataractogenesis. The human erythrocyte Cu/Zn-SOD undergoes a gradual decrease in activity on incubation with glucose and the proportion of glycated SOD is considerably higher in diabetic patients [18–20]. The inactivation of SOD might be caused by non-enzymic glycosylation (glycation), because a negative correlation has been observed between the activity of SOD and glycated haemoglobin in erythrocytes [21]. Glycation of proteins, a factor possibly contributing to tissue damage in diabetes mellitus, has been extensively modelled by the exposure of proteins to glucose *in vitro*. The activity of SOD was not greatly affected, suggesting that the glycation site was rather far from the active site. However, glycation affected the affinity for heparin, which could decrease the amount of SOD bound to the cell surface, which in turn would increase the susceptibility of cells to superoxide radicals produced in the extracellular space [20].

In further studies it has been shown that the primary glycation sites in extracellular SOD are lysine-211 and lysine-212 in the putative heparin-binding domain at the C-terminal end [22]. Inactivation *in vitro* of purified bovine erythrocyte Cu/Zn-SOD led to a decrease in the enzymic activity, an increase in the carbonyl groups and the modification of one histidine residue [23]. Either SOD lost its enzymic activity but still retained its antigenicity, or it lost both enzymic and antigenic sites [24]. Although SOD activity was decreased in the erythrocytes of diabetic patients, no difference was observed by using an ELISA

Abbreviations used: G6P, glucose 6-phosphate; SOD, superoxide dismutase.

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method with a monoclonal antibody [21]. It therefore appears that the activity *in vivo* might be more susceptible to glycation than the antigenicity. This possibility is explored in the present paper. However, if the enzyme is more seriously damaged, its antigenicity is also lost.

The glycation and inactivation of the enzyme could be accompanied by an accumulation of antigenically reactive but catalytically impaired enzyme molecules. It has been reported that when active enzyme lost its activity the degradation or modification by denaturation could be so extensive that it completely lost its antigenic determinants and could not be recognized by anti-native enzyme antibody [24,25]. Alternatively the enzyme that had lost its activity on modification of the enzyme molecules could be less severely damaged so that the inactive enzyme only partly lost its antigenic determinants. Antibody would then recognize antigenically reactive material that was catalytically inactive.

The aim of the present study was to determine whether inactivation of catalase and SOD and the loss of antigenicity were simultaneous. For this purpose catalase and SOD were incubated with sugars of different glycating abilities and the extent of the loss of antigenicity of catalase and SOD was monitored by the loss of enzyme activity after immunoprecipitation with monospecific antibody. The antigenicity of non-glycated and glycated enzymes separated by affinity chromatography was determined by dot-blotting.

The results of this study should help our understanding of the role of glycation in the complications of diabetes, increasing the incentive to approach normoglycaemia as closely as possible. It should add to evidence for the role of structural changes in enzyme proteins as revealed by their loss of antigenicity.

MATERIALS AND METHODS

Materials

Catalase (bovine hepatic, thymol-free; EC 1.11.1.6), SOD (bovine erythrocyte; EC 1.15.1.1), fructose, glucose, ribose, glucose 6-phosphate (G6P) and all other chemicals were obtained from Sigma Chemicals (Poole, Dorset, U.K.). Antibodies against catalase and SOD (both rabbit) were obtained from Chemicon International. Affi Gel 601 was from Bio-Rad Laboratories.

Incubation with sugar

Catalase (525 units, 25 μg) was incubated in 50 mM sodium phosphate buffer, pH 7, with and without 5 mM fructose or 10 mM glucose for between 1 and 8 days in a final volume of 1 ml at 37 °C in the first set of experiments. In a second set of experiments 5 mM fructose, 5 mM ribose and 5 mM G6P were used. Stock solutions were made of the enzyme with and without sugar and divided into separate small sterilized glass vials with rubber tops (Whatman) through a sterilized Millipore filter (0.2 μm pore size). The vials were placed in a shaking water bath (110 strokes/min) at 37 °C. Individual vials were removed at zero time and various times thereafter for assay in triplicate. Incubations were performed over 8 days, with measurements taken after 1, 2, 3, 4, 6 and 8 days.

SOD (220 Sigma units, 50 μg) was incubated in a shaking water bath at 37 °C in 0.12 M potassium phosphate buffer (containing 100 μM EDTA), pH 7.8, with or without 50 mM fructose, 50 mM glucose and 50 mM G6P for between 1 and 8 days in a final volume of 1 ml in the first set of experiments.

Stock solutions of SOD were made with or without sugar, then divided into separate vials, sterilized and incubated as above. Individual vials were removed at zero time and various times thereafter for dialysis with three changes of buffer in 3 h at 4 °C.

Catalase activity

The enzymic activity was assayed by monitoring the decrease in absorbance at 240 nm resulting from the decomposition of H_2O_2 for 1 min at 37 °C. The assay mixture consisted of 2.98 ml of H_2O_2 solution from a stock solution of 0.1 ml of 30 % (w/v) H_2O_2 diluted in 50 ml of sodium phosphate buffer, pH 7, and 20 μl of the incubation solution (0.025 mg of enzyme/ml of buffer). All assays were performed in triplicate. Activity is expressed relative to the control activity at each incubation time, which is set at 100 %.

SOD assay

Cu/Zn-SOD was assayed as described by McCord and Fridovich [26]. All assays were performed in triplicate. Activity is expressed relative to the control activity at each incubation time, which is set at 100 %.

Immunoprecipitation

Immunoprecipitation experiments were performed as described by Dovrat and Gershon [24]. The first experiments were to determine the amounts of antibody (anti-catalase and anti-SOD) that were sufficient to precipitate approx. 50 % of the activity of a fixed amount of enzyme during incubation. Various amounts of antibody antiserum (ranging from 5 to 100 μl) were added to preparations of catalase (0.025 mg/ml) and SOD (0.05 mg/ml) in a total volume 0.15 ml. The mixture was adjusted to a constant volume with 10 mM sodium phosphate buffer. After incubation overnight at 4 °C, the mixture was centrifuged at 3000 ***g*** for 30 min and the residual activity in the supernatant was assayed. In the control tubes antiserum was replaced by 10 mM sodium phosphate buffer. In the second set of experiments, antibody (1:100 for catalase and 1:10 for SOD) ranging from 0 to 50 μl was mixed with a fixed amount of enzyme during incubation with 5 mM fructose, 10 mM glucose, 5 mM ribose and 5 mM G6P for catalase and 50 mM fructose and 50 mM G6P for SOD.

Affinity chromatography of glycated enzymes

Glycated and non-glycated catalase formed during incubation with ribose and fructose at 2 days, and with G6P at 5 days, were separated on a pre-equilibrated Affi Gel 601 column. A column was packed with aminoethyl succinyl aminophenylboronate covalently attached to the matrix. The gel has an affinity for sugar groups and should pull out fructose-labelled proteins from the pool of unlabelled proteins [27]. A buffer containing 0.25 M ammonium acetate, pH 8.5, was used for column equilibration and for eluting non-glycated proteins at a rate of 18 ml/h. The bound proteins were eluted first with 0.1 M acetic acid and then with 0.3 M acetic acid (30 ml of each); 1.5 ml fractions were collected, 20 μl of each was taken for measurement of activity and the absorbance of each was read at 240 nm as this wavelength gave the lowest background absorbance from the buffer. Fractions contributing to activity peaks were freeze-dried. After most bound proteins had been eluted, 0.5 M acetic acid was used to regenerate the column.

Glycated and non-glycated SOD were separated in a similar way, on the same column.

Determination of protein concentration

The protein concentration was determined by a bicinchoninic acid protein assay, by Sigma procedure no. TPRO-562.

Dot-blotting

Glycated and non-glycated protein solutions were applied as a dot to a sheet of nitrocellulose. Dot-blotting was performed as described previously [28].

Analysis of the data

All measurements were made in triplicate. Parameters were expressed as means ± S.E.M. and Student's *t* test was used.

RESULTS

Inactivation and loss of antigenicity of catalase

The extent of the loss of antigenicity was monitored by the immunoprecipitation of catalase with monospecific anti-catalase in which the concentration (1:100) of antibody was sufficient to precipitate catalase activity. There was 100.4 ± 3.8 %, 93.1 ± 4.8 %, 75.6 ± 9.7 %, 18.7 ± 8.9 % and 5.5 ± 1.7 % of the activity remaining after immunoprecipitation with 10, 20, 30, 40 and 50 μl of antibody respectively. Catalase activity was decreased by incubation with fructose, glucose, ribose and G6P, after which the antigenicity of catalase was demonstrated by a decrease in activity after incubation with antibody, which is reactive with the major antigenic determinant of catalase (Figures 1 and 2).

The marked inactivation of catalase was observed during incubation with 5 mM fructose. Fructose had significantly lowered enzyme activity after 1 day (Figure 2A; $P < 0.01$). The inactivation of catalase was greater after 2 days ($P < 0.001$), 3 days and 4 days (Figure 2A). At zero time, immunoprecipitation of enzyme activity was observed equally with or without fructose and glucose (Figure 1A). Catalase was inactivated by incubation with fructose; after immunoprecipitation with increasing amounts of antibody increasing significant losses of catalase activity were seen (Figure 1B; note the error bars). Glucose was less effective. Up to 2–3 days the same amount of antibody precipitated an equal amount of activity, indicating that antibody was precipitating only active enzyme (Figure 2A). The remaining activity after incubating with 30 μl of antibody after 1, 2 and 3 days indicated that 30 μl of antibody precipitated approx. 18.4 %, 15.5 % and 7.6 % of active enzyme at 1, 2 and 3 days. At first the catalase lost activity and antigenicity simultaneously but after 3 days some inactivated enzyme had retained its antigenicity. However, the activities of control samples after immunoprecipitation were approx. 54.9 ± 9.4 %, 55.2 ± 7.3 % and 47.1 ± 6.8 % at the same times, indicating that after 3 days the inactive enzyme has a minor fraction that was recognized by antibody.

Glucose displayed a very much slower inhibitory effect (Figure 2B), which was not significant until 3 days ($P < 0.05$) and was more striking at 6 days ($P < 0.01$). After incubation with 30 μl of antibody, the remaining activity decreased (Figure 2B); the difference between the curves shows that antibody precipitated approx. 54.2 %, 57.1 % and 64.1 % of activity after 1, 3 and 6 days respectively. This constant precipitation indicates that inactive catalase had lost its antigenicity. The inactivation of enzyme and the loss of antigenicity in the enzyme incubated with glucose was less and slower than that with fructose.

A rapid loss of enzymic activity occurred after incubation of catalase with 5 mM ribose, and was statistically significant even after 1 day ($P < 0.001$), and up to 3 days (Figure 2C). The rate

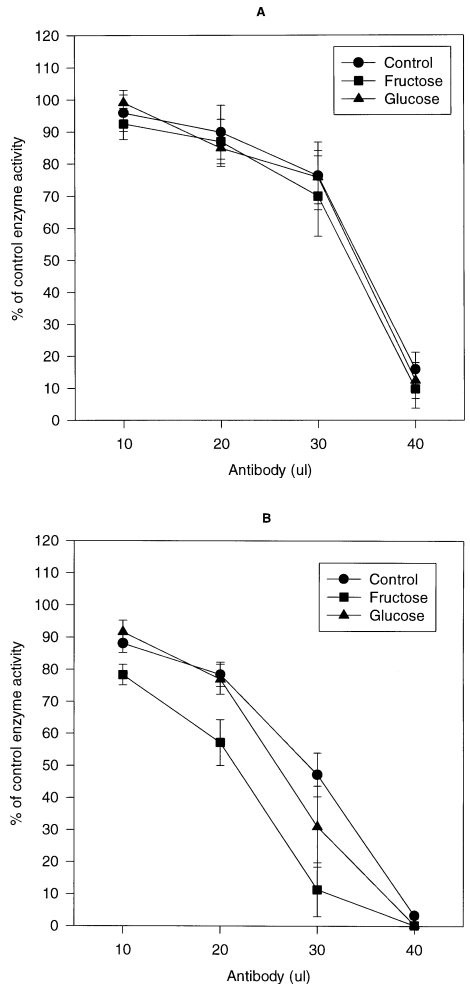

Figure 1 Immunoprecipitation of catalase incubated with 5 mM fructose or 10 mM glucose

(**A**) Incubation day zero; (**B**) incubation day 3. Abbreviation: ul, μl.

of inactivation by fructose seemed slightly faster than by ribose but different preparations of catalase had been used. For this experiment, G6P displayed a slower inhibitory effect than ribose and fructose [significant after 2 days ($P < 0.05$); more so after 3 days ($P < 0.001$), 4 and 6 days] (Figure 2D). The catalase incubated with ribose was examined by immunoprecipitation. A larger amount of antibody (30 or 40 μl) precipitated so much activity at the beginning that it was bound to decrease with time as the total activity falls. The results with 20 μl of antibody are shown (Figures 2C and 2D). Here the amount of activity precipitated by 20 μl stayed constant (approx. 25–30 %) from day 0 to day 3 (ribose) and to day 5 (G6P). So the antibody was precipitating only the active enzyme, indicating that catalase lost activity and antigenicity simultaneously.

Inactivation and loss of antigenicity of SOD

The inactivation of SOD was also investigated by incubating the enzyme with different sugars. There was no immediate inhibition of enzyme activity at zero time but it was slowly inactivated by fructose, glucose and by G6P. This clearly indicated that the inactivation was due to slow post-translational modification of SOD by the sugars. They displayed a time-dependent inhibition

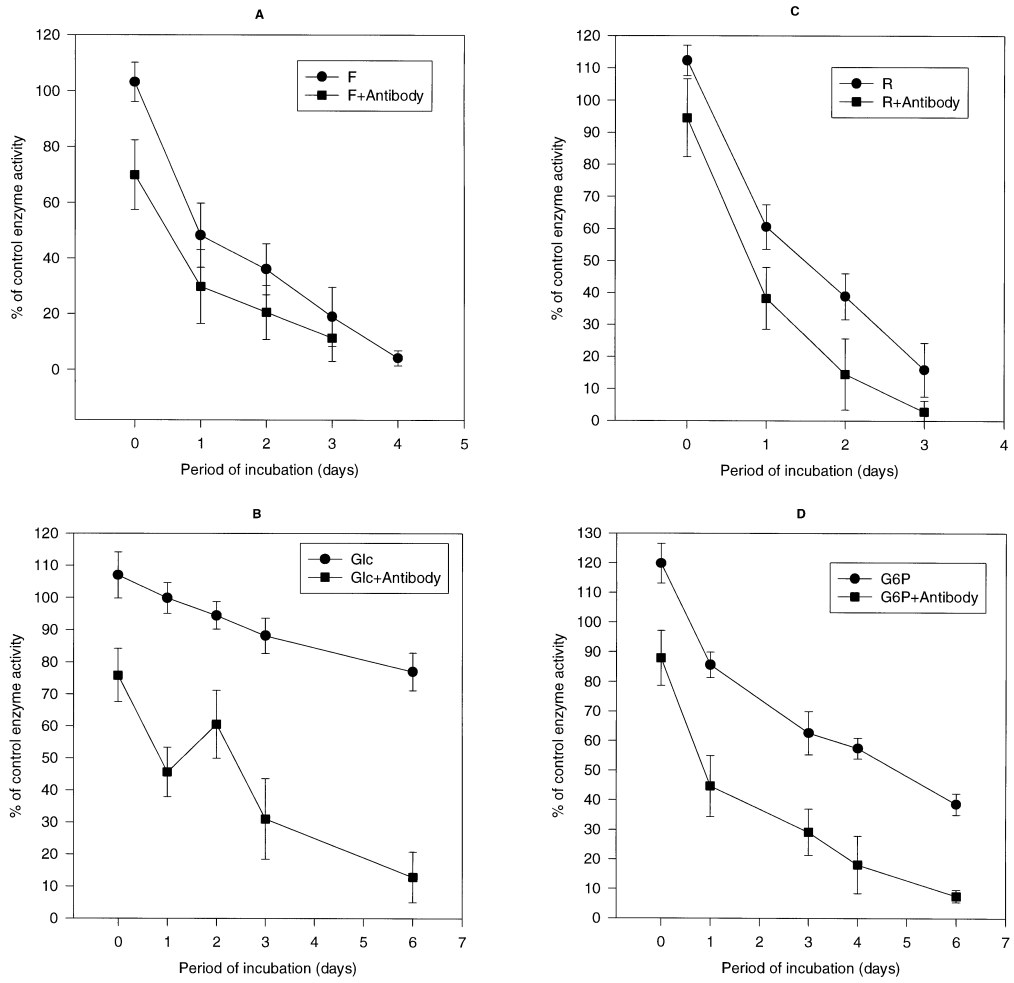

Figure 2 Immunoprecipitation of catalase incubated with (A) 5 mM fructose (F) or (B) 10 mM glucose (Glc), both by 30 μl of antibody; or (C) 5 mM ribose (R) or (D) 5 mM G6P, both by 20 μl of antibody

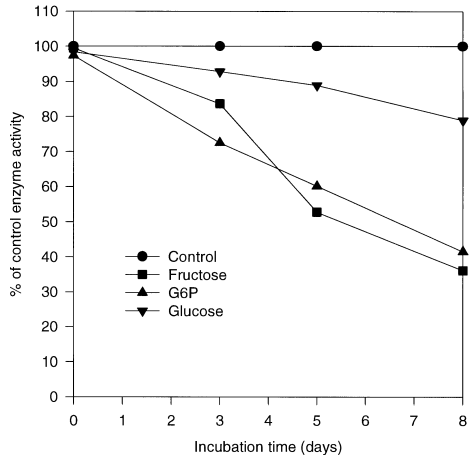

Figure 3 Inactivation of SOD incubated with 50 mM sugars

of enzyme activity (Figure 3). Fructose and G6P were more effective than glucose. Both fructose and G6P had not significantly lowered the enzyme activity after 3 days ($83.4 \pm 13.4\%$ of control activity, $P = 0.121$; $72.4 \pm 19.3\%$, $P = 0.06$ respectively). After 8 days the inactivation of SOD was more clear ($37.3 \pm 11.6\%$ of control activity remaining, $P = 0.0003$; $41.5 \pm 14.4\%$, $P = 0.0021$ respectively). Glucose displayed a slower inhibitory effect such that the remaining activity of SOD was $79.6 \pm 9.5\%$ ($P = 0.07$) after 8 days, not significantly different from the control. The more reactive a sugar was in glycation the more rapidly it caused inactivation, suggesting non-enzymic binding to the protein molecule.

The activity of a fixed amount of SOD was measured before and after immunoprecipitation with monospecific anti-SOD antibody, of which 40 μl was sufficient to precipitate nearly 50% (49.2%) activity of native SOD (results not shown). The antigenicity of SOD was demonstrated by a decrease in SOD activity in both the absence and the presence of fructose or G6P after the incubation of SOD products with commercial antibody (Figures 4A and 4B). At zero time the immunoprecipitation was not affected by the presence of sugar (Figure 4A). There was more inactivation of SOD incubated with fructose or with G6P than the control, especially after 7 days of incubation (Figure 4B). For at least 3 days the antibody precipitated approx. 50% of the original activity, that is the same as for the control. Therefore for 3 days there was no loss of antigenicity, because antibody was precipitating only active enzyme.

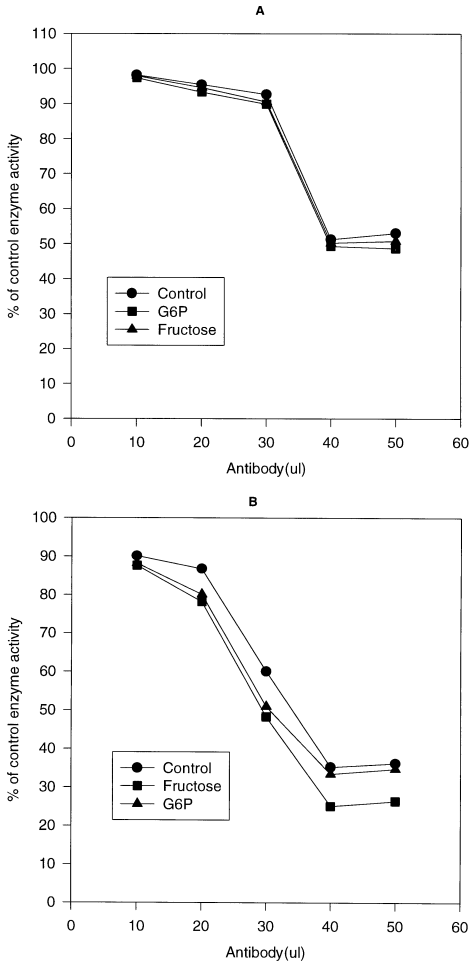

Figure 4 Immunoprecipitation of SOD incubated with 50 mM fructose or 50 mM G6P

(A) incubation day zero; (B) incubation day 7. Abbreviation: ul, μl.

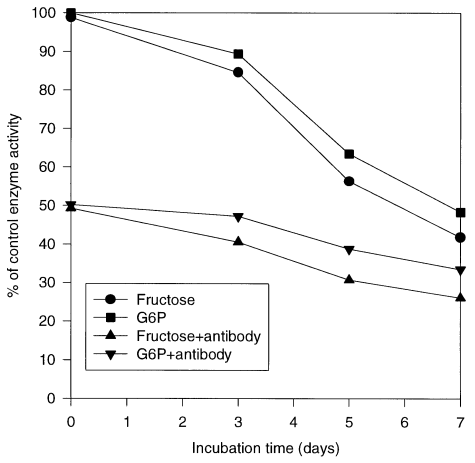

Figure 5 Inactivation of SOD incubated with 50 mM fructose or 50 mM G6P, and immunoprecipitation of SOD by 40 μl of antibody

As shown in Figure 5, a rapid loss of enzymic activity occurred after 3 days of incubation with 50 mM fructose, leading to a decrease to 84.5%, 56.2%, 41.2% of control enzyme activity after 3, 5 and 7 days respectively. The remaining activities after the immunoprecipitation reaction were approx. 49.3%, 40.5%, 30.7% and 25.6% after 0, 3, 5 and 7 days respectively in the same samples. The decreases in activity after imunoprecipitation were 44.0%, 25.5% and 15.6% after 3, 5 and 7 days respectively (Figure 5). The activity of SOD incubated with 50 mM G6P fell

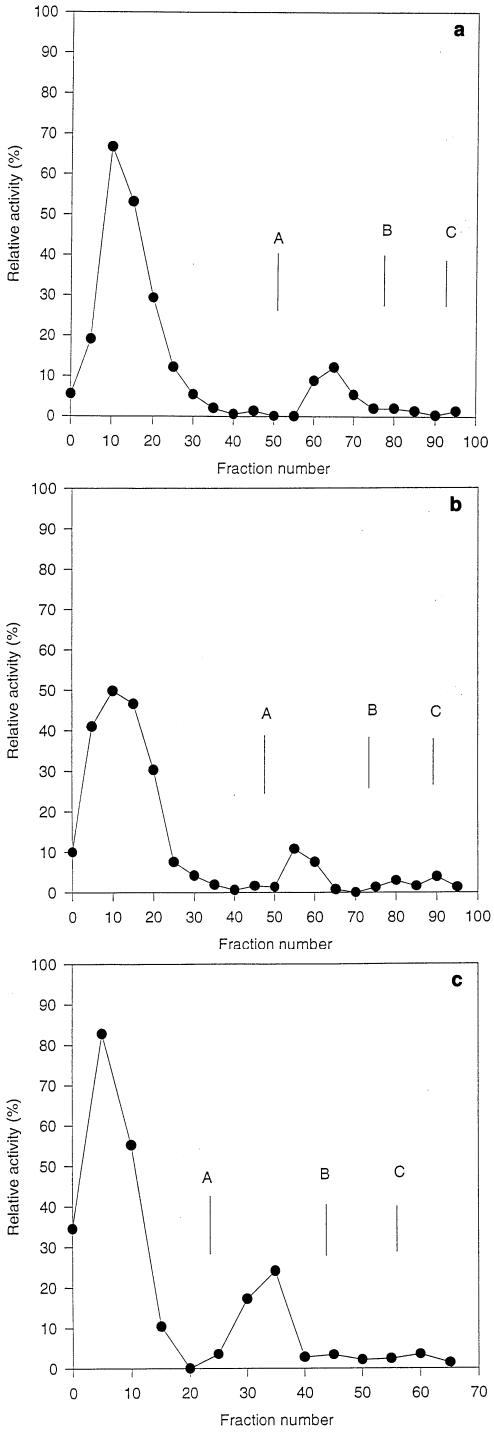

Figure 6 Isolation of glycated and non-glycated catalase incubated with 5 mM fructose (a), 5 mM ribose (b) or 5 mM G6P (c), by affinity chromatography on Affi-gel 601

At the indicated points 0.1 M (A), 0.3 M (B) and 0.5 M (C) glacial acetic acid were used.

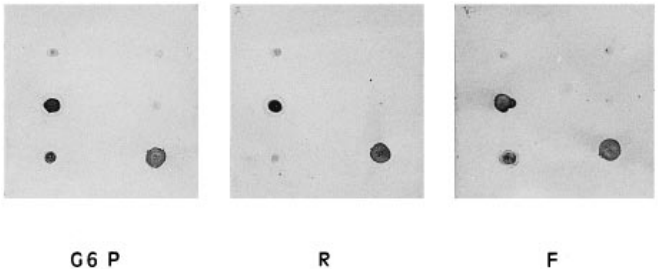

Figure 7 Dot-blotting of glycated and non-glycated catalase incubated with 5 mM G6P, 5 mM fructose (F) or 5 mM ribose (R)

Middle and bottom left dots, non-glycated catalase, main peak and tail; top and middle right dots, glycated catalase; bottom right dot, standard catalase; top left dot, BSA. The weak marks for glycated fractions and BSA were caused by pipette marks in the nitrocellulose layer.

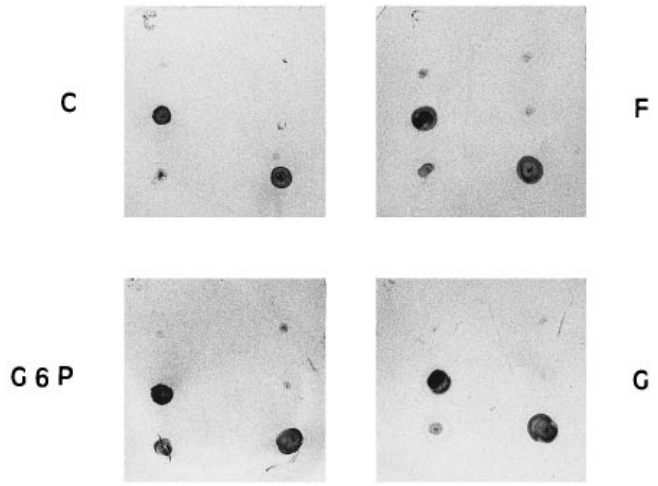

Figure 8 Dot-blotting of glycated and non-glycated SOD incubated with 50 mM fructose (F), 50 mM G6P or 50 mM glucose (G); and the control incubation (C)

All dot-blots were incubated with anti-SOD. On each blot BSA was run as a negative control at top left; the other two dots on the left were two fractions of the unbound protein, main peak and tail; the right-hand side had the bound fractions eluted with 0.1 and 0.3 M acetic acid (top and middle respectively). The bottom right dot was SOD itself. Weak marks for bound fractions and BSA were caused by pipette marks in the nitrocellulose layer.

to 89.3 %, 63.3 % and 48.2 % of control enzyme activity after 3, 5 and 7 days respectively. After incubation with 40 μl of antibody, the remaining activities were approx. 47.2 %, 38.8 % and 33.8 % after 3, 5 and 7 days respectively, so that antibody precipitated approx. 42.1 %, 24.5 % and 14.4 % of activity. After 5 days the same amount of antibody precipitated less activity, indicating that some enzyme protein was present that had lost activity but retained its antigenicity (Figure 5). This is consistent with the finding of cross-reacting SOD in erythrocytes from diabetics.

The antigenicity of glycated and non-glycated enzymes

The catalase samples incubated with fructose (52.4 % activity remaining) and ribose (46.9 %) after 2 days, and with G6P (54.6 %) after 5 days, were separated by affinity gel chromatography into bound and unbound fractions. The amounts of non-glycated fractions were nearly equal to those of glycated fractions except G6P, for which the amount of non-glycated fraction was slightly more. Affinity chromatography of the enzyme gave a single main peak, containing most of the non-glycated enzyme, that was eluted by 0.25 M ammonium acetate. Another, smaller, single peak containing glycated catalase (bound fraction), which had little activity, was eluted by 0.1 M acetic acid (Figure 6).

The dot-blotting shows clearly that glycated enzyme entirely lost its antigenicity compared with non-glycated enzyme and standard protein (catalase). The total activity of the enzyme decreased by approx. 50 % of control activity; most of this active enzyme was in the non-glycated fractions (Figure 7). The immunoprecipitation results show that catalase lost activity and antigenicity simultaneously (Figures 1 and 2). The bound (glycated) protein still had some catalase activity although antigenicity was lost. The active site of catalase might be partly the same as the antigenic site.

Glycated SOD was separated from non-glycated SOD by affinity chromatography as for catalase, with similar results (results not shown). Dot-blotting showed that, whereas the non-glycated fractions gave a strong reaction with the antibody, the glycated fractions did not (Figure 8). With all three sugars the delayed material corresponded to the negative control.

DISCUSSION

The results from the present work show that fructose, ribose, G6P and glucose each inactivate catalase in a time-dependent manner, although the extent of inactivation is different. Ribose and fructose inactivate the enzyme much more quickly than G6P and glucose. Fructose and G6P also inactivate SOD but this enzyme seems more resistant to glycation because it was necessary to use a higher concentration of sugar to achieve inactivation. The relative abilities of the sugars to inactivate catalase and SOD parallel the abilities of these sugars to inactivate glutathione reductase [29], G6P dehydrogenase [30,31], malate dehydrogenase [32] and Ca^{2+}-ATPase [33]. Incubation of myofibrillar proteins with ribose results in the loss of ATPase activity as sugar becomes attached to proteins [34]. The phosphate group of G6P increases the level of disruption of the enzyme, probably through an increase in surface negative charge [35], but possibly by facilitating the Amadori rearrangement [33]. These results suggest strongly that the inactivation of catalase and SOD is a consequence of glycation, where sugar combining with catalase and SOD could lead to their inactivation and thus to accumulating peroxide and superoxide, which might contribute to the overall complications of diabetes and aging.

Oxidative stress has been suggested as an important causative factor in many pathological processes. Oxidative modification of protein might be involved in aging and in the pathogenesis of senile cataract [36]. Catalase is responsible for removing intracellular H_2O_2, which is an inhibitor of SOD activity. This inhibition is potentiated by the catalase inhibitor 3-aminotriazole, which has no direct effect on lens SOD [4]. Inactivation of catalase would lead to oxidative damage not only directly through H_2O_2 and its derivative, but also indirectly through the inhibition of SOD and thus an increased level of superoxide radicals. In contrast, the glycation of Cu/Zn-SOD led to inactivation and fragmentation of the enzyme [37]. The capacity of glycated SOD to enhance damage to DNA that was mediated by thiol-metal-catalysed oxidation was inhibited by catalase [38]. The peroxidative damage to the lens cell membranes has been proposed as a triggering mechanism of cataractogenesis [3], although other mechanisms are undoubtedly involved [17]. The increased sugar levels associated with diabetes could lead to the inhibition of

SOD and catalase; thus the enzymic defence against the toxicity of the excited oxygen species could also be impaired. This mechanism might be partly involved in the pathogenesis of cataract and diabetic complications.

During the early stages of fructation of SOD, when there was relatively little inactivation, the antibody precipitated the same amount of activity as in the control; however, after 5 days it precipitated less, indicating that some inactivated SOD retained its antigenticity. The active site of SOD might be partly similar to the antigenic sites. The antigenicity might be lost through glycation of the antigenic site itself or by glycation elsewhere leading to a conformational change. In the present study the loss of antigenicity of catalase is possibly explained by partial overlap between the active site of catalase and the antibody site. Glycation induced losses of antigenicity and activity as the sugar became attached to the amino groups and disrupted the structure within the enzyme molecule. The disruption of enzyme results in a partial unfolding of its structure, which in turn leads to the progressive losses of its catalytic activity and antigenicity. The catalase is more seriously damaged: its antigenicity is also lost.

The dot-blotting experiments demonstrated clearly that the glycated enzymes had entirely lost their antigenicity. These results further support the idea that inactivation of enzyme and loss of antigenicity are simultaneous. Whether or not these changes are significant in other enzymes and the importance of such changes in the pathogenesis of aging or complications in diabetes are interesting fields for future study.

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