Inactivation and loss of antigenicity of esterase by sugars and a steroid

Hong Yan 1, John J. Harding *

Nuffield Laboratory of Ophthalmology, University of Oxford, Walton St., Oxford OX2 6AW, UK

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Abstract

Glycation, the non-enzymic reaction of sugars with proteins, has an important role in the complications of diabetes. It has been studied mostly in structural proteins but more recently has been shown to inactivate enzymes. Previous evidence from our laboratory indicated that glycation-induced inactivation and loss of antigenicity of catalase and superoxide dismutase are simultaneous. Esterase, which decreases activity in the lens in senile cataract and diabetes, was measured by a spectrophotometric assay using p-nitrophenyl acetate as the substrate. Here we investigated the inactivation of carboxylesterase (EC 3.1.1.1) by sugars of different glycating power and prednisolone-21-hemisuccinate while simultaneously monitoring the loss of antigenicity. Antigenicity was assessed by immunoprecipitation and by dot-blotting the glycated and non-glycated fractions of enzymes separated by affinity chromatography. Ribose and fructose inactivated more rapidly than glucose and glucose 6-phosphate. The esterase was progressively inactivated by prednisolone-21-hemisuccinate at a lower concentration. Activity and antigenicity were lost simultaneously. The glycated enzyme had entirely lost its antigenicity. These results further support the idea that inactivation of enzyme and loss of antigenicity are simultaneous. ß 1999 Elsevier Science B.V. All rights reserved.

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1. Introduction

Four types of esterase activity occur in mammalian serum: pseudocholinesterase, carboxylesterase, arylesterase and paraoxonase [1]. Esterase activity is abundant in human monocytes, present in T-lymphocytes and equally divided between CD4 and CD8 T-lymphocyte subsets. It is expressed in human liver, colon, stomach, breast and brain tissues [2].

Sequence analysis of human liver carboxylesterase revealed two different iso-types. It inhibited the invasion of Plasmodium falciparum sporozoites into primary human hepatocytes [3].

Carboxylesterase is important in detoxification and extracellular surfactant metabolism [4–8]. The α-naphthyl-acetate-esterase activity of peripheral blood lymphocytes, significantly reduced in diabetic patients as compared to controls, was related to the decreased cell-mediated immunity and the enhanced risk of infections in these patients [9]. The plasma levels of esterase was decreased in diabetic patients [10] and streptozotocin-diabetic rats [11]. Acetylcholinesterase activity of the erythrocyte membrane is significantly reduced in alloxan-induced diabetes in rats [12].
Esterase activity increases with age in some tissues [2]. The carboxylesterase activity in plasma increased from negligible (5 days old) to adult male value (31 days old), and in liver increased markedly during the same period [13]. Two fractions of esterase, with molecular masses approximately 200 kDa (esterase-I) and 30 kDa (esterase-II), were partially purified from the soluble fraction of a normal human lens [14]. The activity of esterase was decreased in the senile cataractous lens compared to a normal lens of a similar age, suggesting that esterase plays a role in cataractogenesis, unless cataract formation has a deleterious effect on the catalytic activity of esterase in the lens [14].

The post-translational modification of structural proteins has been widely studied as a possible mechanism in the long-term complications of diabetes and cataracts. Clearly cataracts qualify as a conformational disease. The conformational change leads to the formation of aggregates which scatter light to produce opacification [15]. Glycation, the reaction of protein with sugars, is an age-dependent process thought to be important in all diabetic complications. The steroid-induced cataract is well known and is positively correlated with the average annual dosage of corticosteroids, although a clear correlation between the duration of treatment or dosage and the incidence of opacity has not emerged [16,17]. The prevailing hypothesis involves the formation of a Schiff base between the steroid C-20 carbonyl group and epsilon-amino groups of crystallin lysine residues. The mechanism of steroid cataract formation holds that glucocorticoids are covalently bound to lens proteins resulting in the destabilisation of the protein structure allowing further modification leading to cataract. Mechanisms other than the covalent binding of steroids to lens proteins may have a role in glucocorticoid-induced cataracts [18]. Sugars and corticosteroids react with lysine residues of protein eliminating its charge [19], and inactivate important metabolic enzymes in the lens.

Glycation-induced inactivation and loss of antigenicity of superoxide dismutase and catalase occur simultaneously [20]. The glycation and inactivation of enzymes could be accompanied by an accumulation of antigenically reactive but catalytically impaired enzyme molecules. Either the enzyme lost its enzymic activity but still retained its antigenicity, or it lost both enzymic and antigenic sites.

The objective of the current study was to determine if inactivation of esterase by sugars (ribose, fructose, glucose 6-phosphate and glucose) and prednisolone-21-hemisuccinate, was simultaneous with the loss of antigenicity.

2. Materials and methods

2.1. Materials

Esterase (porcine liver, carboxylic ester hydrolase from porcine liver, EC 3.1.1.1), ribose, fructose, glucose 6-phosphate (G6P), glucose, prednisolone-21-hemisuccinate (P-21-H), p-nitrophenyl acetate (PNPA) and acetonitrile were obtained from Sigma (Poole, Dorset, UK). Sephacryl S-300HR was purchased from Pharmacia (Milton Keynes, UK). Rabbit anti-esterase (porcine liver) polyclonal antibody (catalog number AB1217, lot number 16395023) and Promega kit were obtained from Chemicon International. Affi-Gel 601 (boronate-derivatised polyacrylamide gel) was from Bio-Rad Laboratories.

2.2. Incubation with sugars and P-21-H

Esterase (5 U/30.3 μg) was incubated in a shaking water bath at 37°C in 50 mM sodium phosphate buffer, pH 6.79, with or without 20 mM ribose, fructose, G6P, and 1 mM P-21-H for 0–13 days in a final volume of 10 ml. Stock solutions were divided into separate small sterilised glass vials with rubber tops (Whatman) through a sterilised Millipore filter (0.2 μm pore size). Individual vials were removed at zero time and various times thereafter for assay in triplicate.

2.3. Esterase assay

The enzyme activity was assayed by monitoring the rate of PNPA hydrolysis catalysed by porcine liver esterase using a Hitachi U-2001 spectrophotometer [21]. The PNPA was dissolved in acetonitrile to make a stock solution (60 mM) to prevent slow hydrolysis in water and to achieve higher concentrations of PNPA in the reaction solution. A 1 ml ali-
The quotient of the PNPA solution was diluted with 19 ml sodium phosphate buffer (50 mM, pH 6.79, ionic strength as 0.5 with NaCl) just before the assay. Then 1 ml of this diluted PNPA solution and 0.2 ml of enzyme solution were mixed in a 1.4 ml cuvette for reaction compared against a phosphate buffer blank reference to subtract non-enzymatic background hydrolysis at 28°C. The rate of the enzymatic hydrolysis was followed at 400 nm for a standard period of 100 s with a lag of 30 s at the beginning of the reaction. Rates were calculated from the $p$-nitrophenol absorbance data beginning 30 s after the reaction had started, with measurements at 5 s intervals for 70 s. Activity is expressed relative to the control activity at each respective incubation time, set at 100%.

### 2.4. Immunoprecipitation

Immunoprecipitation experiments were used as described [20]. Firstly the amounts of antibody that were sufficient to precipitate approximately 50% of the activity of a fixed amount of enzyme during incubation were determined. Various amounts of antibody (ranging from 0 to 50 µl) were added to preparations of esterase (3.03 µg/ml) in a total volume 0.6 ml. The mixtures were adjusted to a constant volume with 50 mM sodium phosphate buffer, pH 6.79. After incubation overnight at 4°C, the mixtures were centrifuged at $3000 \times g$ for 30 min and the residual activity in the supernatant was assayed. The 50 mM sodium phosphate buffers replaced antiserum as the control. Then, antibody solution (1:4) ranging from 15 to 30 µl was mixed with preparations of esterase (3.03 µg/ml) for a total volume of 0.6 ml. The mixtures were adjusted to a constant volume with 50 mM sodium phosphate buffer, pH 6.79. After incubation overnight at 4°C, the mixtures were centrifuged at $3000 \times g$ for 30 min and the residual activity in the supernatant was assayed. The 50 mM sodium phosphate buffers replaced antiserum as the control. Then, antibody solution (1:4) ranging from 15 to 30 µl was mixed with preparations of esterase (3.03 µg/ml) during incubation with 20 mM ribose, fructose, G6P, glucose and 1 mM P-21-H.

### 2.5. Affinity chromatography of glycated enzymes

Glycated and non-glycated esterase taken from the incubation medium after the remaining activity was approximately 50% compared with the control, were separated on a pre-equilibrated Afi-Gel 601 column. Afi-Gel 601 boronate-derivatised polyacrylamide gel has an affinity for coplanar adjacent cis-hydroxyl groups (cis-diols), which provides highly efficient separation of low molecular weight compounds such as nucleotides, nucleosides, catecholamines and sugars.

A column (1×13 cm$^2$) was packed with aminoethyl succinyl aminophenylboronate covalently attached to the matrix. The sample was loaded into 0.25 M ammonium acetate buffer, pH 8.5, which was used for column equilibration and for eluting non-glycated proteins at a rate of approximately 25 ml/h. After this the column was eluted sequentially with 0.1 M and 0.3 M acetic acid (30 ml of each). Fractions of 2.2 ml were collected, 20 µl of each was taken for measurement of esterase activity. Fractions contributing to activity peaks were concentrated by rotary evaporation and diluted by PBS, then concentrated again. After most bound proteins had been eluted, 0.5 M acetic acid was used to regenerate the column.

### 2.6. Determination of protein concentration

Protein concentrations were determined by Bradford protein assay with Coomassie blue using bovine plasma albumin as a standard [22]. To 50 µl of standards was added 2.5 ml Coomassie blue protein reagent and mixture, the absorbance at 595 nm was measured after 10 min. A standard protein curve was constructed by absorbance at 595 nm. Then samples were prepared by dissolving in 1 M NaOH and treated as above.

### 2.7. Dot-blotting

Dot-blotting was performed as described [20]. The sample for blotting was applied as a dot to a sheet of nitrocellulose which was treated first with 10% ethanol, then it was blocked by incubation for 1 h at 37°C in PBS/milk. Following the blocking step the nitrocellulose was washed at least three times in a fresh solution of PBS/Tween (0.2% Tween 80 in PBS). The nitrocellulose was then incubated in PBS/milk containing antiserum to esterase (1:1000) for 1 h at 37°C. After careful washing, anti-rabbit conjugate was bound by incubating the nitrocellulose for 1 h at 37°C in a 1/7500 dilution of Promega anti-rabbit serum in PBS/milk. Following incubation the nitrocellulose was washed 3–6 times in PBS/Tween, and incubated at room temperature in the dark in colour development reagent solution (26.4 µl of NBT and 13.2 µl of BCIP were added to 4 ml of reaction buffer (100 mM Tris-HCl, pH 9.5, 100 mM NaCl, 5 mM MgCl$_2$) as described in the Promega kit) for
20 min. When colour development was complete the nitrocellulose was rinsed in PBS and dried on filter paper. Photographs were taken later.

2.8. Analysis

Statistical analysis was performed using Student’s paired t-test.

3. Results

3.1. Inactivation and loss of antigenicity of esterase

The extent of the loss of antigenicity was determined by the immunoprecipitation of esterase with polyclonal antibody to esterase in which the concentration (1:4) of antibody was sufficient to precipitate esterase activity. There was 82.7 ± 8.9%, 75.3 ± 6.8%, 65.3 ± 9.5%, 28.0 ± 5.0% and 21.2 ± 4.2% of activity remaining after immunoprecipitation with 20, 25, 27, 30, 35 μl of antibody respectively (Fig. 1). About 27 μl of antibody was required to precipitate 60% of the activity.

The esterase was inactivated over 4 days incubation with ribose (Fig. 2). The antibody precipitated approximately a further 37.5%, 32.5%, 17.9% and 9.9% of activity after 1, 2, 3 and 4 days respectively.

The esterase was inactivated over 4 days incubation with fructose (Fig. 3). The antibody precipitated approximately a further 37.5%, 32.5%, 17.9% and 9.9% of activity after 1, 2, 3 and 4 days respectively.
Up to day 2 the same amount of antibody precipitated an equal amount of activity, suggesting that the antibody was precipitating only an active enzyme. The course of inactivation was similar with fructose. 27 µl of antibody precipitated approximately 47.8%, 46.0% 29.4% and 30.6% after 1, 2, 3 and 4 days incubation with fructose (Fig. 3). This unchanging precipitation showed that the antibody precipitated only an active enzyme, indicating that activity and antigenicity were lost simultaneously. If the inactivated enzyme had retained its antigenicity, the antibody would have precipitated an inactive as well as an active enzyme and the two lines would not have stayed parallel, but would have converged continuously.

Glucose 6-phosphate and glucose inactivated esterase more slowly than ribose and fructose. The amounts of activity precipitated by 27 µl were 37.2%, 46.9%, 48.9%, 45.3% and 33.5% after 1, 4, 7, 9 and 13 days incubation with G6P respectively (Fig. 4). It stayed constant at 35-45% up to 9 days. With glucose the precipitated activities were 41.7%, 44.0%, 48.1% and 47.3% after 4, 7, 9 and 13 days incubation respectively, indicating that esterase lost activity and antigenicity simultaneously (Fig. 5).

3.2. The antigenicity of glycated and non-glycated enzymes

The esterase preparations incubated with ribose (58.4 ± 7.3% activity remaining), fructose (49.0 ±
3.5%), G6P (67.6 ± 3.3%) and glucose (51.2 ± 1.1%) were separated by affinity chromatography into bound and unbound fractions. The amounts of non-glycated fractions were over twice those of glycated fractions by protein assays. Affinity chromatography of the non-incubated enzyme gave a single main peak, containing the non-glycated enzyme (Fig. 6). After elution by 0.1 M and 0.3 M acetic acid, there were no further marked peaks of activity from the incubated samples (Fig. 7; results shown for the ribose incubation, incubations with the other sugars gave very similar results).

Samples of the glycated and non-glycated fractions were dot-blotted. All the unbound fractions (non-glycated) gave a positive reaction with the anti-esterase, but the bound fractions (glycated) were negative (Fig. 8). 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 (non-glycated) after esterase was incubated with 20 mM ribose (R), 20 mM fructose (F), 20 mM G6P and glucose (Glc) respectively. All the unbound fractions gave clear positive dots. The right-hand side had the bound fractions eluted with 0.1 and 0.3 M acetic acid (top and middle respectively). All of them were negative; weak marks for bound fractions were caused by pipette marks in the nitrocellulose layer. The bottom right dot was esterase itself which gave a strong spot compared to BSA and unbound proteins. It appears that glycated esterase lost its antigenicity. The dot-blotting shows clearly that the glycated enzyme entirely lost its antigenicity compared with non-glycated enzyme (Fig. 8).

4. Discussion

Esterase has been considered to be linked with diabetes and ageing because of its decreased activity
in diabetic patients, experimental rats and the senile cataractous lens [1,9–12,14]. Glycation-induced inactivation of enzymes, which involves the reaction of a reducible sugar with free N-terminal α-amino groups or ε-amino groups of lysine residues, is an important factor in diabetic complications and cataracts. In the results presented we show that carboxylesterase activity was remarkably decreased by sugars of different glycat ing abilities. Inactivation was more rapid in the presence of ribose and fructose than G6P and glucose which is consistent with previous studies of other enzymes such as glutathione reductase, catalase, superoxide dismutase, glucose 6-phosphate dehydrogenase and malate dehydrogenase [23].

During the inactivation of esterase by sugars, the antibody precipitated similar amounts of activity as the control, indicating that the inactivation of the enzyme was paralleled by the loss of antigenicity. The dot-blotting results demonstrated clearly that the glycated esterase had entirely lost antigenicity, again suggesting that inactivation of the enzyme and its loss of antigenicity are simultaneous. Inactivation of the enzyme could be by reaction of a molecule with its catalytic centre, or indirectly following reaction elsewhere on the protein surface. The active sites of enzymes often contain their most reactive residues. The disruption of the surface charge network of enzymes by glycation may result in conformational changes which in turn lead to the progressive loss of their catalytic activity and antigenicity. The antigenicity might be lost through glycation of the antigenic site itself or by glycation elsewhere leading to a conformational change. The active site of esterase might be partly similar to the antigenic sites. However, enzyme activity may be compromised before gross conformational changes of proteins take place although inactivation can also be induced by modification away from the active site.

Corticosteroids, often taken for prolonged periods of time, are a risk factor for cataracts and systemic disease. Enzyme levels fall during the onset of steroid-induced cataracts [15,24]. P-21-H inactivated catalase and glyceraldehyde 3-phosphate dehydrogenase in vitro [25,26]. In the case of esterase, P-21-H led to significant loss of enzyme activity during 9 days incubation. The activities precipitated by the antibody are similar over 9 days except at time zero, indicating that inactivation of esterase and loss of antigenicity are simultaneous.

A significant association between inactivation and loss of antigenicity of enzyme was identified. These data, when considered with the previous evidence of catalase and superoxide dismutase for inactivation and loss antigenicity [20], indicate that glycation- or steroid-induced inactivation of enzymes may contribute to the immunological difficulties of patients with diabetes and age-related diseases.

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References