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# Glycation of human erythrocyte glutathione peroxidase: Effect on the physical and kinetic properties

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ABSTRACT

*Background:* Glutathione peroxidase (GPx) is a significant antioxidant enzyme that plays a key role in protecting the body from reactive oxygen species (ROS) and their toxicity. As a biocatalyst, the enzyme has been shown to reduce hydrogen peroxide to water and lipid hydroperoxides to their respective alcohols. The increased levels of ROS in patients with diabetes have been speculated to arise, in part, from alterations in the activity of glutathione antioxidant enzymes, perhaps, by mechanisms such as the glycation of the protein, in vivo.
*Methods:* Under physiological conditions of temperature and pH, we investigated the susceptibility of human glutathione peroxidase to glycation, determined the effects of glycation on the physical and kinetic properties of the enzyme, and identified the protein's vulnerable amino acid sites of glycation.
*Results:* Circular dichroism, UV and mass spectrometry studies revealed that methylglyoxal and DL-glyceraldehyde are potent glycators of glutathione peroxidase; destabilizing its structure, altering its pH activity and stability profiles and increasing its Km value.
*Conclusions:* In comparison to DL-glyceraldehyde, methylglyxol was a more potent glycator of the enzyme and was found to nonenzymatically condense with Arg-177, located near the glutathione binding site of GPx.


## 1. Introduction

The Maillard reaction or glycation is an intricate set of non-enzymatic reactions that occur between amino groups of proteins and the carboxyl group of reducing sugars or their metabolites [1]. In the initial step of glycation, sugars form a Schiff's base typically with the lysyl groups on a protein. This Schiff's base then undergoes an Amadori rearrangement to form a stable ketoamine product that with time generates a heterogeneous group of compounds commonly referred to as 'Advanced Glycation End products' or AGEs [2]. Once formed, AGEs can yield brown or Maillard pigments with characteristic UV or fluorescence signals [3]. The formation of AGEs can also promote the crosslinking of proteins leading to alterations in their structure or function [4–6]. The in vivo accumulation of AGEs has been shown to adversely impact the health and function of many tissues and organs including the heart, kidneys, eyes, and brain [7,8]. AGEs have been also implicated in the process of aging and many of the chronic complications of diabetes [9–11].

Increased levels of reactive oxygen species (ROS) can lead to conditions of oxidative stress that with time can cause tissue damage including alterations to DNA, RNA and proteins [12]. In patients with diabetes, higher than normal levels of ROS have been reported encouraging the speculation that this phenomenon may be due to alterations in the function and activity of anti-oxidant enzymes [13,14].

Glutathione peroxidase (GPx) belongs to a family of enzymes that catalyze the breakdown of lipid hydroperoxides to their respective alcohols and free hydroperoxides to water [15]. In 1957, Mills et al. demonstrated the presence of the enzyme in human erythrocytes and other mammalian tissues [16]. Following their report, numerous studies showed that this enzyme serves as the first line of defense in protecting erythrocytes from oxidative damage [16–21].

The objectives of this study were threefold: 1) to investigate the nonenzymatic modification of human erythrocyte glutathione peroxidase under physiological temperature and pH with D-glucose, D-fructose, D-galactose, DL-glyceraldehyde, glyoxal and methylglyoxal, 2) to determine the effect of glycation on the physical and kinetic characteristics of the enzyme, and 3) to identify the vulnerable amino acid sites of glycation in the protein.

D-glucose, D-fructose, D-galactose, DL-glyceraldehyde, glyoxal and methylglyoxal were chosen as the model glycating agents for two reasons: first, because they are abundant in nature and second, because they have been shown to nonenzymatically condense with proteins in vivo altering their structure and/or function [22–30].

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## 2. Materials and methods

### 2.1. Chemicals and reagents

Glutathione peroxidase from human erythrocytes, glutathione reductase from Baker's yeast, reduced glutathione, β-nicotinamide adenine dinucleotide phosphate (β-NADPH), hydrogen peroxide 30% (w/w), sodium phosphate monobasic, dithiothreitol (DTT), ethylenediaminetetraacetic acid (EDTA), D-glucose, D-fructose, D-galactose, DL-glyceraldehyde, glyoxal 40% (w/w), methylglyoxal 40% (w/w), sinapic acid, α-cyanohydroxycinnamic acid (CHCA), bovine serum albumin (BSA) and analytical grade solvents for HPLC were from Sigma Chemical Company (St. Louis, MO). Quartz cuvettes were obtained from Hellma (Mullheim, Germany). Unless otherwise indicated, all other reagents and supplies were from Fisher Scientific (Waltham, MA).

### 2.2. Protein determination

Protein content was determined by the Bradford method [31] with bovine serum albumin serving as the standard.

### 2.3. Glycation of glutathione peroxidase with sugars and sugar like compounds

A typical incubation mixture contained 10 mmol/l sodium phosphate buffer pH 7.0, 0.03 mg glutathione peroxidase and a set amount of "sugar" [5 and 10 mmol/l]. Unless otherwise indicated, the term "sugar" is hereby used loosely to refer to D-glucose, D-fructose (Fru), D-galactose (Gal), DL-glyceraldehyde (GA) and the sugar behaving like aldehydes, methylglyoxal (MG) and glyoxal (Gly). The reaction mixtures containing the individual sugars and enzyme were then placed in the dark in sealed micro-centrifuge tubes for 5 days at 37 °C, and thereafter periodically removed and frozen until analysis. Prior to their freezing, an aliquot from each sample was assayed for glutathione peroxidase activity.

### 2.4. Determination of glutathione peroxidase activity

Glutathione peroxidase activity was assayed with modifications of the procedure of Wendel [32]. In brief, to 1 mg of β-NADPH, 9.2 ml of 50 mmol/l sodium phosphate containing 0.04 mmol/l EDTA and 1 mmol/l sodium azide pH 7.0, 100 μl of glutathione reductase enzyme solution (100 units/ml) and 50 μl of 200 mmol/l glutathione reduced solution were added, mixed by inversion and pH was adjusted to 7.0 with 1 mol/l HCl or 1 mol/l NaOH (if necessary). Of this mixture, 3.0 ml was taken into a quartz cuvette and to it 50 μl of glutathione peroxidase (0.075 units) was added. Blank incubation mixtures included 1) all the aforementioned constituents of the assay except for hydrogen peroxide, or 2) all the constituents of the assay with glutathione peroxidase excluded. Once prepared, the mixtures were placed in Spectramax M2 Multi-mode spectrophotometer (Molecular Devices, Sunnyvale, CA) equipped with temperature controlled cuvette holders set at 25 °C. After the absorbance stabilized, 50 μl of 0.042% $H_2O_2$ was added to the mixture and the disappearance of β-NADPH was monitored at 340 nm. One unit of glutathione peroxidase activity is defined as the amount of enzyme catalyzing the oxidation of 1 μmol of reduced glutathione/min in the presence of saturating levels of $H_2O_2$ at pH 7.0 and 25 °C. Unless otherwise indicated, all reactions were run in triplicate.

### 2.5. Determination of Km and Vmax

Apparent Michaelis constants and maximal velocities were determined by the Eisenthal et al. direct linear plot method [33] using reduced glutathione as substrate. Aliquots of enzyme (0.075 units) were incubated in substrate of varying concentrations (0.1–10 mmol/l) and assayed as described previously. Blank incubation mixtures included: 1) all the aforementioned constituents of the assay except for hydrogen peroxide, or 2) all the constituents of the assay with glutathione peroxidase excluded. Control tubes included all the components of the assay in the presence or absence of the glycating agent (20 mmol/l).

### 2.6. Monitoring glycation of glutathione peroxidase by UV spectroscopy

The acquisition of UV spectra was performed on Spectramax M2 Multi-mode micro plate reader (Molecular Devices) spectrophotometer equipped with SoftMax Pro software. Every reaction mixture was run in triplicate using Corning 96-well plates (Corning, NY) at 320 nm.

### 2.7. Determination of pH activity profiles of glycated and non-glycated glutathione peroxidase

Aliquots (50 μl) containing 3 U of the glycated enzymes and the non glycated enzyme were each brought up to a final volume of 100 μl with a citrate/phosphate/carbonate buffer containing 50 mmol/l of each salt, with pH's varying from pH 6.0 to 11.0 and assayed for activity as previously described. Reaction mixtures were prepared in triplicate, and all values were compared relative to the highest reading set at 100%. Each glycated enzyme preparation used in this experiment was glycated with 20 mmol/l sugar for 5 days at 37 °C in dark.

### 2.8. Determining the effect of ph and temperature on the stability of glycated and non-glycated glutathione peroxidase

#### 2.8.1. Preparation of glycated and non glycated enzyme

Glycated enzyme (3 U/ml) was prepared by incubating the protein with 20 mmol/l methylglyoxal or DL-glyceraldehyde at 37 °C for 5 days. Unless otherwise indicated, all incubations were performed in 10 mmol/l phosphate buffer, pH 7.0. The resulting samples were then dialyzed overnight at 4 °C with 2 changes of 1 l of distilled water to remove the excess sugars. The non glycated glutathione peroxidase samples (3 U/ml each) were processed similar to the glycated enzyme preparations, but were not reacted with methylglyoxal or glyceraldehyde.

The control enzyme preparations included samples of glutathione peroxidase (3 U/ml each) dispensed in the presence and absence of the sugars not incubated at 37 °C. Other than this difference, the processing and dialysis of control samples were similar to that of the glycated and non glycated enzyme preparations (hereinafter also referred to as the 'native' enzyme).

#### 2.8.2. Generating pH stability profiles

pH stability studies were performed on dialyzed enzyme preparations that had the same protein content as follows: an aliquot from each dialysate (50 μl) was first separately pre-incubated at 37 °C for 30 min in 5 mmol/l citrate/phosphate/carbonate buffer (250 μl) of varying pH ranging from pH 6.0 to pH 9.0 at 0.5 pH intervals. Samples were then adjusted to neutral pH and assayed for their activity as previously described. Every reaction was conducted in triplicate and all values were compared relative to the sample with the highest activity set at 100%.

#### 2.8.3. Generating heat stability profiles

Heat stability studies were performed on glycated and non glycated enzyme samples prepared in the 10 mM phosphate buffer, pH 7.0 dialyzed as described previously. All samples were adjusted for their protein content with BSA to the same value prior to heat exposure. Glycated and non glycated glutathione peroxidase preparations (1.5 U/ml) were pre-incubated at 20, 25, 37, 45 and 50 °C for 30 min and assayed for their activity as specified earlier. Each assay was