Isolation and some properties of glycated D-glyceraldehyde-3-phosphate dehydrogenase from rabbit muscle

Rong-Qiao HE,*†‡ Ming-Dong YANG,* Xing ZHENG* and Jun-Xian ZHOU*

*National Laboratory of Biomacromolecules, Institute of Biophysics, Academia Sinica, 15 Da Tun Road, Bei-Sha-Tan, Chaoyang District, Beijing 100101, People's Republic of China, and †Department of Biochemistry, University of Cambridge, Tennis Court Road, Cambridge CB2 1QW, U.K.

Glycated D-glyceraldehyde-3-phosphate dehydrogenases (GAPDH) from rabbit muscle and human erythrocytes have been investigated. The specific activity of the non-glycated GAPDH from rabbit muscle is approx. 180 units. (One unit is defined as the specific activity required to convert 1 μ M of substrate/min per mg of enzyme.) The activity of the glycated enzyme, consisting of two sugars per tetramer, is lower than that of the non-glycated GAPDH. Non-enzymic transamination of the N-termini of glycated GAPDH (gGAPDH) indicates that they are not blocked by glycation. The rate of modification of thiols (Cys-149) with 5,5'-dithiobis-(2-nitrobenzoic acid) was

greater for the glycated than the non-glycated enzymes. The rate of modification of amino groups of Lys residues of gGAPDH with o-phthalaldehyde was greater for the non-glycated enzyme. In 0.18 M guanidine-HCl solution, the emission intensity at 410 nm of a fluorescent NAD⁺ derivative introduced into the active site decreased to 80 %, whereas that of gGAPDH decreased to 50 %. This suggests that the glycated sites are near the active site; glycation of the enzyme leads to a change of the micro-environment of Cys-149, alters the conformation of the active site and decreases the activity.

INTRODUCTION

Glycation of proteins is of great importance in many areas of biology and biotechnology. The destruction of lysine and the formation of brown colours upon heating or during long-term storage of proteins in the presence of reducing sugars was recognized as a serious problem by food scientists many years ago [1]. In some cases, glycation may be associated with medical problems. For example, in 1966 Holmquist and Schroeder [2] demonstrated the presence of an N-terminal blocking group involving a Schiff base in haemoglobin A1c and some symptoms of diabetes may correlate with this modification. By the mid-1970s it was recognized that the blocking group involved is glucose, or a glucose-derived product, added non-enzymically as a post-synthetic modification. The Schiff base, which is labile and dissociable, undergoes an Amadori rearrangement to form a more stable keto-amine linkage. Glycation of haemoglobin occurs at various sites in both its α - and β -chains [3]. The amount of this glycated haemoglobin formed *in vivo* is related to the free glucose available in the blood serum. At about the same time it was discovered that levels of other glycated proteins, such as the glycated derivatives from collagen, are elevated in diabetic patients [4]. Other examples of proteins whose glycated derivatives are present in increased amounts in diabetic patients are erythrocyte membrane proteins [5] and lens crystallins [6]. Later, glycated serum proteins [3–5] and glycated plasma and structural proteins were discovered [6–7], and some of them have been used to diagnose and monitor diabetes. Glycated high- and low-density lipoproteins have been investigated in recent years and it has been found that the glycation affects markedly the activity of the proteins.

D-glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (EC 1.2.1.12) catalyses the oxidation and phosphorylation of GAP to 1,3-diphosphoglycerate [8]. It is a key enzyme in the glycolytic conversion of glucose into pyruvic acid, which is an important pathway of carbohydrate metabolism in most organisms. It has been used as a model to study the cooperative binding of NAD⁺ [9]. In our laboratory, Ho and Tsou [10] discovered a fluorescent NAD⁺ derivative that could be used to probe the active site of the enzyme. With this fluorescent probe, we studied the structure–function relationship of this enzyme, particularly, the correlation between conformational changes of the active site disturbed by denaturants and the activity of the molecule [11–16]. It was suggested that the active site was located in a limited region of the molecule, which is more sensitive to the denaturants than the enzyme as a whole, and that a slight perturbation of the spatial geometry of the functional groups responsible for the catalytic mechanism destroys its activity before any gross conformational changes can be detected. Recently, we demonstrated the presence of glycated GAPDH (gGAPDH) in rabbit muscle and human erythrocytes [17,18] and isolated the glycated enzyme. We also demonstrated that glycation of the enzyme may affect its conformation and activity. This paper is concerned with the more detailed characterization of gGAPDH from rabbit muscle and human erythrocytes.

EXPERIMENTAL PROCEDURES

Materials

o-Phthalaldehyde (OPT), triglycine, GAP and 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB) were purchased from Sigma. 3,5-dihydroxytoluene and glyoxylic acid were obtained from Fluka.

Abbreviations used: GAPDH, D-glyceraldehyde-3-phosphate dehydrogenase; gGAPDH, glycated D-glyceraldehyde-3-phosphate dehydrogenase; CM-GAPDH, carboxymethylated GAPDH; GOD-PAP, the method of glucose oxidase coupled with peroxidase; DTNB, 5,5'-dithiobis-(2-nitrobenzoic acid); OPT, o-phthalaldehyde; GuHCl, guanidine-HCl; APBA, m-aminophenylboronic acid.

‡ To whom correspondence should be addressed at the Beijing address.

NAD⁺ was purchased from Boehringer Mannheim and from Merck. Guanidine-HCl (GuHCl) was obtained from a local source: the A_{250} of a 6 M solution was less than 0.05 after purification [19]. The *m*-aminophenylboronic acid (APBA)-Sephacrose CL 6B mini-column used for affinity chromatography of glycated protein was purchased from Seikagaku Corporation (Japan). The other reagents were of analytical grade and were used without further purification.

Purification and assay of the enzyme

Preparation of GAPDH from rabbit muscle, recrystallized six times, was as described before [20] and the specific activity of the enzyme was approximately 110 units. (One unit is defined as the specific activity required to convert 1 μ M of substrate/min per mg of enzyme.) Absorption coefficients at 280 nm of 149 and 121 $\text{mM} \cdot \text{cm}^{-1}$ were used for concentration determinations of the holo- and apo-enzymes respectively. Both the holo- and the apo-enzyme showed a single band in PAGE and in SDS/PAGE. Measurement of activity was carried out using a mixture of 0.1 M NaHCO₃, 0.02 M NaCl, 0.001 M NAD⁺ and 0.001 M GAP, pH 8.5, at 25 °C. The removal of tightly bound NAD⁺ at the active site was essentially as described before [10,20]. GAPDH prepared by this method contains glycated and non-glycated enzyme.

Preparation of the fluorescent NAD⁺-enzyme adduct

A photochemically induced reaction between NAD⁺ and the carboxymethylated enzyme is known to produce a fluorescent adduct. Carboxymethylation of SH groups at the active site and the photochemical linkage of NAD⁺ at the same site were essentially as described for the enzymes from muscle [10,20] and from *Bacillus stearothermophilus* [21]. The holo-enzyme (0.2 mM) was treated with 6 mM iodoacetate for 10 min at 0 °C, which resulted in an inactivation of approximately 98%. The solution was then passed through a Sephadex G-50 column to remove the excess iodo-acetate. Modification with DTNB showed that only the thiol at the active site of each subunit had been modified. The carboxymethylated enzyme (30 μ M) dissolved in 0.1 M phosphate buffer (pH 7.0) containing 0.6 mM NAD⁺ was placed in a 3 ml cuvette situated about 8 cm in front of a 150 W xenon lamp and irradiated for 20 min. The solution was occasionally stirred and cooled with an electric fan during the irradiation.

PAGE

Enzymes were dissolved in 0.01 M phosphate buffer containing 1% SDS and 0.1% 2-mercaptoethanol, before being boiled for 2–5 min. A 10 μ g portion of both the glycated and non-glycated enzyme was used for electrophoresis with 5% polyacrylamide gel containing 0.1% SDS and 0.1 M phosphate buffer at pH 8.3. After electrophoresis, the gels were dyed using a 0.05% aqueous solution of Coomassie Brilliant Blue R250 [22]. The gels were destained in dilute acetic acid.

Ion-exchange chromatography of GAPDH

The glycated and non-glycated forms of GAPDH can be separated by ion-exchange chromatography. Rabbit muscle GAPDH (100 μ g), dissolved in 100 μ l of 0.02 M Tris-HCl buffer at pH 8.5, containing 0.001 M EDTA, was passed through a column of Mono Q 5/SHR for anionic exchange and the protein fractions were monitored by their A_{280} using an FPLC system (Pharmacia). The mobile-phase buffer included 0.15 M NaCl. gGAPDH, for use as a control, was prepared *in vitro* according to the following method. GAPDH, purified according to Ho and

Tsou [20], was incubated at room temperature for 4 days with 1.0 M glucose in a solution of 0.1 M NaHCO₃ at pH 8.5, containing 0.02 M NaCl and 0.001 M EDTA, followed by anion-exchange chromatography.

Affinity chromatography of the enzyme

All the affinity separations reported in this paper were carried out using CL 6B mini-columns from Seikagaku. These columns consist of an agarose matrix coupled to APBA, as first described by Millia [23]. The pH was adjusted to 9.2 using a buffer comprising 0.02 M asparagine/0.05 M MgCl₂/0.001 M Na₂EDTA/0.02% NaN₃ (pH 9.2). The enzyme (50 μ l; 0.8 mg) was added to the column, followed by addition of 0.1 ml of mobile buffer (0.01 M asparagine/0.1 M MgCl₂/0.001 M Na₂EDTA/0.02% NaN₃, pH 9.2). The column was allowed to stand at room temperature for 10 min and the mobile buffer (2.9 ml) was used to elute the non-glycated enzyme. Then, 2 ml of the elution buffer (0.01 M Tris-HCl/0.2 M sorbitol/0.01 M asparagine/0.001 M EDTA/0.02% NaN₃, pH 9.2) was used to displace gGAPDH. The affinity gel was regenerated with 0.1 M HCl and stored in a solution of 0.001 M HCl for repeat procedures. For separation of the gGAPDH of human erythrocytes, 1% Triton X-100 was used to lyse the cells. The enzyme in the extract was separated by affinity chromatography and the two major fractions were used for activity measurements.

Determination of glycation sugar

Two methods were used for the determination of the glycation sugar: (1) phenol sulphuric acid reagent [24,25] and (2) 3,5-dihydroxytoluene sulphuric acid reagent [17,18,26,27]. For method (1), 1.0 ml of the enzyme (final concentrations: apo-enzyme, 0.018 mM; holo-enzyme, 0.03 mM) was mixed first with 1.0 ml of 5% v/v phenol (in water), then with 5.0 ml of conc. sulphuric acid ($d = 1.84$). The solutions were allowed to stand at room temperature for 20 min. The A_{480} was measured after the solutions had cooled to 25 °C. For method (2), 0.1% (w/v) 3,5-dihydroxytoluene in water (1.0 ml) was mixed with 1.0 ml of GAPDH (2.0 mg), followed by the addition of 5 ml of conc. sulphuric acid ($d = 1.84$) at room temperature and the solutions were allowed to stand for 20 min before measurement of the A_{480} . It has been demonstrated that proteins do not interfere with the results of the methods [26,27]. In order to estimate the amount of glucose (if any) present free in solution, an assay system based on glucose oxidase coupled with peroxidase (GOD-PAP) was used. The GOD-PAP kit was purchased from Zhongsheng High-Tech Bioengineering Company (Beijing, China).

Modification of the enzyme with OPT

Before modification of the enzyme, carboxymethylated GAPDH (CM-GAPDH) should be prepared (as described above) to protect the thiol of Cys-149 from reaction with OPT. The enzyme (4×10^{-6} M CM-GAPDH or CM-gGAPDH, dissolved in 0.1 M NaHCO₃/0.02 M NaCl/0.001 M EDTA, pH 8.2) was incubated with 2-mercaptoethanol (50-fold excess) at room temperature for about 30 min, after which a 50-fold excess of OPT was added to the cuvette and the progress of the modification reaction was monitored by measuring the fluorescence at 450 nm. The sample temperature was held at 15 °C and the reaction proceeded to completion in about 15 min.

Modification of the enzyme with DTNB

DTNB (0.010 M; 10 μ l) was added to a cuvette containing GAPDH or gGAPDH (1.0–2.0 mg/ml dissolved in 0.1 M

NaHCO₃/0.02 M NaCl/0.001 M EDTA, pH 8.2, at 1 °C) and the kinetics of modification were followed by measuring the A_{412} .

Transamination of N-terminal amino groups of the enzyme

GAPDH and gGAPDH (40 mg) were dissolved in water (2.0 ml) before mixing with 2 ml of nickel sulphate–glyoxylate transaminating solution [28] at room temperature for 30 min. In order to remove the residual sodium glyoxalate, the samples were then dialysed against 50 times the sample volume of doubly distilled water at 4 °C; this procedure was repeated five times. After drying at –70 °C under vacuum, the enzymes were dissolved in 1 M NaOH at room temperature. Fluorescence at 405 nm of the two enzymes in NaOH solution was measured by excitation at 320 nm. The fluorescence intensity is enhanced under basic conditions.

Denaturation of the enzyme with GuHCl

The enzymes (final concentration 5.0×10^{-7} M for GAPDH and 2.0×10^{-7} M for gGAPDH) were dissolved in 0.1 M phosphate buffer, containing 0.001 M EDTA at pH 7.0 and GuHCl at the desired concentrations. The solutions were allowed to stand at room temperature, usually around 20 °C, for 24 h, when the reaction reached completion, as indicated by activity loss or fluorescence changes. The enzymes were completely stable in the absence of denaturant under similar conditions. Aliquots were then taken for activity and fluorescence measurements. The enzyme from rabbit muscle was found to be poorly soluble in guanidine solutions of concentrations of around 1 μ M and this precluded measurement of conformational changes by other methods, such as CD or UV-difference spectroscopy. At a concentration of 0.2 μ M, the enzyme was soluble throughout the concentration range of GuHCl solutions employed.

Scatchard plot

In order to measure the quenching of the intrinsic fluorescence of the enzyme by NAD⁺, a solution of NAD⁺ was added stepwise, by means of a burette, to the enzyme solution; after each addition the solution was mixed using a small plastic rod. The fluorescence intensity was measured for a set of NAD⁺ concentrations and a Scatchard plot was drawn as described previously [29].

Absorbance measurements were carried out with either a Shimadzu UV-250 or UV-3000 spectrophotometer. Fluorescence measurements were made using a Hitachi MPF-4 and a Hitachi-850 fluorophotometer. In all cases the samples were thermostated at a specified temperature.

RESULTS

Determination of the glycyating sugar

Although we protected the enzyme from contamination by any reducing sugar, it was nevertheless necessary to investigate whether there existed a trace of glucose in the solutions in contact with the enzyme during purification [20]. However, we did not detect any trace of glucose by the GOD-PAP method. Before determination of the glycyating sugar with phenol–sulphuric acid [method (1) [24,27]], it was necessary to remove NAD⁺ from holo-GAPDH by incubating the enzyme with activated charcoal in 0.1 M phosphate buffer, pH 7.0, at 35 °C for 10 min. The absorbance, A_{280}/A_{260} , was then found to be in the range 2.00–2.10. This showed that the enzyme contained little NAD⁺. We found that one mole of apo-GAPDH contained

Table 1 Some properties of GAPDH and gGAPDH after fractionation by affinity chromatography

* The first fraction was eluted by 2.9 ml of the mobile buffer (0.01 M asparagine/0.1 M MgCl₂/0.001 M Na₂EDTA/0.02% NaN₃, pH 9.2) so that it consisted of the non-glycated GAPDH. Then 2.0 ml of the elution buffer (0.01 M Tris-Cl/0.2 M sorbitol/0.01 M asparagine/0.001 M EDTA/0.02% NaN₃, pH 9.2) were added to elute gGAPDH (the second fraction) at room temperature [23]. ** GAPDH and gGAPDH were passed through a sieve-chromatography column of Sephadex G50 before absorbance was measured. *** GAPDH and gGAPDH isolated by affinity chromatography were dialysed against doubly distilled water (1:50 v/v five times at 4 °C). Then the glycyating sugar was determined.

	First fraction	Second fraction
Protein content (mg)*	0.32	0.28
Relative protein content (%)*	65	35
Specific activity (μ M NADH $\cdot$ min ⁻¹ $\cdot$ mg of enzyme)*	180	40
A_{280}/A_{260} **	1.20	1.16
Glycyating sugar***	—	2

Figure 1 Comparison of subunit masses of the gGAPDH with the non-glycated GAPDH

The glycated and non-glycated enzymes (5 μ g of each) from rabbit muscle isolated by affinity chromatography were used for 0.1% SDS/PAGE after the enzymes had been boiled with 1% SDS at 100 °C for 2–5 min. Bands A and B represent the non-glycated enzyme and the glycated enzyme respectively.

approximately 0.65 moles of glucose. Determination of the glycyating sugar by using the 3,5-dihydroxytoluene reagent [method (2)] showed that gGAPDH isolated by affinity chromatography consisted of 1.89–2.10 moles of glucose per mole of the glycated tetramer (see Table 1). It was also found that holo-GAPDH does not contain any sugar, as determined by measurement using method (2). Care was taken to make the sugar determinations on holo- and apo-enzymes prepared from the same sample.

Both GAPDH and gGAPDH, isolated by affinity chromatography, ran as a single band in PAGE. Moreover, 0.1% SDS/PAGE gave similar subunit masses for the two tetramers (Figure 1).

Ion-exchange chromatography of GAPDH

Anion-exchange chromatography of GAPDH gave two elution peaks absorbing at 280 nm when eluted by a linear NaCl gradient

Figure 2 Ion-exchange chromatography of GAPDH

Enzyme (100 μ g) dissolved in 100 ml of 0.02 M Tris-Cl buffer, pH 8.5, containing 0.001 M EDTA was passed through a column of Mono Q 5/5 HR for anionic exchange with an FPLC system (Pharmacia). The mobile phase was the same buffer except that it contained 0.15 M NaCl. Rabbit muscle GAPDH was incubated with 1 M glucose in 0.1 M NaHCO_3 , pH 8.5, containing 0.02 M NaCl and 0.001 M EDTA, at room temperature for 4 days. Curves represent the A_{280} of the gGAPDH *in vitro* (A), that of GAPDH (B) purified by the method of Ho and Tsou [10,20] and (C) using a linear NaCl gradient. Peaks 1, 2 and 3 represent respectively GAPDH, gGAPDH *in vivo* and gGAPDH *in vitro*.

(0–0.15 M NaCl in 0.02 M Tris-HCl buffer, pH 8.5) through an FPLC column of Mono Q 5/5 HR at 25 °C (see Figure 2, curve B). The two fractions (peaks 1 and 2 respectively) showed GAPDH activity. The specific activity of the enzyme in the second fraction was as low as approx. 20% of that in the first fraction. Peak 3 (Figure 2, curve A), whose position corresponded to fraction 2, showed a low activity of GAPDH, while peak 1 disappeared after the enzyme had been pre-incubated at room temperature for 4 days in a solution of 1.0 M glucose dissolved in 0.1 M NaHCO_3 containing 0.02 M NaCl and 0.001 M EDTA, pH 8.5. Simultaneously, the intensity of the intrinsic fluorescence at 335 nm decreased after the incubation. Thus, glycation of the enzyme *in vitro* appears to cause decreased activity and to alter the molecular conformation.

Affinity chromatography on APBA–Sephacrose-CL 6B

APBA bound to agarose gel combines specifically with mono-saccharides in proteins [23]. We know that reducing mono-saccharides react spontaneously with the amino groups of a protein. This affinity method makes it possible to separate apo-GAPDH samples into two parts, the glycosylated and non-glycosylated enzyme. The activity of gGAPDH isolated by affinity separation was found to be as low as 25–35% of that of the non-glycosylated enzyme. For holo-GAPDH, similar results were obtained, thus excluding the possibility that the ligand of APBA could combine with the ribose in NAD^+ -bound to the enzyme. Pace and Pace [30] reported APBA as a ligand that could combine with RNA but, in the present instance, the NAD^+ may be bound to the enzyme in such a way as to make the ribose molecules inaccessible to the APBA. The APBA ligand also combines weakly with amino groups, so asparagine was dissolved in the mobile solution to prevent the APBA from combining with amino groups in the protein. This resolution of GAPDH into glycosylated and non-

Figure 3 Scatchard plot showing changes in intrinsic fluorescence of GAPDH and gGAPDH during binding of NAD^+

apo-GAPDH (final concentration, 5.0×10^{-7} M) and apo-gGAPDH (2.0×10^{-7} M) were dissolved in 0.1 M phosphate buffer, pH 7.0, containing 0.001 M EDTA to which NAD^+ was added to reach the desired concentrations, at 25 °C, followed by measurement of the intrinsic fluorescence at 335 nm, with excitation at 292 nm [29]. Curves 1 and 2 are Scatchard plots representing respectively, changes in emission intensities (ΔF) with the concentrations of free NAD^+ ($[\text{NAD} +]_f$) for the glycosylated and non-glycosylated forms. ΔF is the emission intensity of the apo-enzyme.

glycosylated fractions suggests that GAPDH purified instead by recrystallization [10,20] probably contained gGAPDH.

The specific activity of the non-glycosylated GAPDH, which was eluted by the mobile buffer (cf. Table 1), was approximately 180 units. It was important to investigate the effect of MgCl_2 on the activity of non-glycosylated GAPDH to ascertain whether the increased activity, relative to the activity of the mixture of glycosylated and non-glycosylated enzymes, was attributable to the effects of the MgCl_2 present in the mobile buffer. In fact, the results showed that MgCl_2 (> 0.01 M) decreased the activity of GAPDH. The effect of MgCl_2 on the activity of GAPDH may be due to the effect of salt, as NaCl (> 0.1 M) also decreased the activity. The possibility that MgCl_2 might slightly increase the activity of the enzyme can therefore be excluded.

Glycolytic conversion of glucose into pyruvic acid is the only means of energy generation in human erythrocytes. Isolation of gGAPDH of erythrocytes with affinity chromatography and measurement of the activity of the enzyme, showed that 40–50% of the total activity of the cell is provided by gGAPDH. The GAPDH of erythrocytes is exposed to glucose contained in the blood and thus has an opportunity to be glycosylated, as with the formation of glycosylated haemoglobin.

Binding of NAD^+ to rabbit muscle GAPDH is found to occur with negative co-operativity, i.e. the binding of each molecule of NAD^+ decreases the affinity of the vacant sites of the neighbouring subunits [9]. Moreover, the binding of NAD^+ to GAPDH quenches the intrinsic fluorescence at 335 nm. Changes in the intensity of the intrinsic fluorescence in NAD^+ solutions of different concentrations demonstrated that the binding of NAD^+ occurred with negative co-operativity for both GAPDH and gGAPDH (Figure 3).

Glycosylated sites of the enzyme

Rabbit muscle GAPDH consists of four subunits. The N-terminal residue is valine and its 2-amino group can be specifically removed by non-enzymic transamination; at the same time an oxoacyl group is introduced at the N-terminus [28]). More

Figure 4 Kinetic procedure for modification of the enzymes with DTNB

The enzymes (final concentration, 2 mg/ml) were dissolved in 0.1 M NaHCO_3 /0.02 M NaCl /0.001 M EDTA , pH 8.2, before $10 \mu\text{l}$ of 0.010 M DTNB was added to the solution, followed by measurement of A_{412} at 1°C . The inset is the same data plotted in semilogarithmic form and curves 1–3 represent, respectively, the progress curves for the fast, intermediate and slow phases.

Table 2 Kinetics of modification of Cys-149 with DTNB

Rates (in units of ms) were calculated from data obtained at pH 8.2 and 1°C .

	Rate (ms)		
	Fast phase	Slow phase	Very slow phase
GAPDH	9.8	—	1.23
Modified thiols	2	—	4
gGAPDH	96.80	11.2	1.28
Modified thiols	2	4	10

recently we [14] reported that this 2-oxoacyl group fluoresces at around 400 nm (excitation at 320 nm). The emission intensities of the 2-oxoacyl groups of both the glycosylated and non-glycosylated enzymes were approximately similar. The presence of oxoacyl groups can be detected using 2,4-dinitrophenylhydrazine [31]; this method showed that the N-termini of gGAPDH were converted into 2-oxoacyl groups by transamination. This suggests that the N-termini of gGAPDH are not blocked by glycation.

Each subunit of rabbit GAPDH consists of 12 Cys residues [8]. In the natural state, four of them (Cys-149) can be modified by DTNB at room temperature. Two of the four react with DTNB fast and the other two react slowly [20]. Figure 4 shows changes in the A_{412} during modification of the thiol (Cys-149) with DTNB. We find that the modification of the non-glycosylated enzyme is biphasic, similar to the results reported previously [20]. On the other hand, the modification of gGAPDH is found to be a triphasic process, consisting of fast, slow and very slow phases (see Table 2). This seems to indicate that glycation of the enzyme leads to some conformational changes resulting in the exposure of Cys residues at the surface of the molecule. The biphasic kinetics of modification reported in the work of Harris and Water [8] presumably arises because the non-glycosylated enzyme is the dominant component in the mixed preparations. It is possible that glycation leads to a change in the conformation at the active site, where Cys-149 is situated, as well as a change in the conformation of the molecule as a whole.

Table 3 Emission (em.) and excitation (ex.) of the OPT-modified derivatives

Measurements of fluorescence with slit widths: emission, 5 nm and excitation, 5 nm; at pH 8.5 and 25°C .

Sample	$\lambda_{\text{em.}}/\text{nm}$	$\lambda_{\text{ex.}}/\text{nm}$	
GAPDH	442	284	346
gGAPDH	435	284	346
Triglycine	452	278	345

Figure 5 Kinetic procedure of modification of CM-gGAPDH with OPT

The enzymes (final concentration, 4×10^{-6} M) were dissolved in the same buffer as used for DTNB modification, except that it contained thio-ethanol 50 times in excess of the enzyme, and incubated for 15 min at 25°C . An OPT concentration 100 times in excess of the enzyme was added to the solution, followed by the measurement of the fluorescence at 442 nm with excitation at 346 nm. The inset is the same data plotted in semilogarithmic form.

Since the N-termini of both GAPDH and gGAPDH are not blocked by glycation, OPT can react with the N-terminal, 2-amino groups. OPT can also react with the ϵ -amino groups of residues elsewhere in the enzyme. The residues that have been glycosylated should not become modified by OPT. Table 3 shows the wavelengths of emission and excitation of the OPT-treated derivatives of GAPDH, gGAPDH and triglycine. The emission maxima of these compounds can be seen to be appreciably different; this might be because of different micro-environments in the molecules. The intensity of the intrinsic fluorescence at 335 nm decreased markedly in the OPT-treated derivatives of GAPDH and gGAPDH when they were excited at 292 nm. This suggests that there exists a radiationless energy transfer between Trp residues and OPT-modified residues when emission spectra, with excitation at 292 nm, are measured.

During the OPT modification of CM-gGAPDH and CM-GAPDH, we observed that the formation of the fluorescent derivative was a biphasic process (see Figure 5). The fast phase for CM-gGAPDH was approx. 5 times faster than that for CM-GAPDH. The kinetics of modification of triglycine with OPT differed markedly from that for both CM-GAPDH and CM-gGAPDH; a 30 s time-lag was observed for the reaction of triglycine with OPT, while the enzymes reacted rapidly with OPT with no time-lag (Table 4). The difference between the rates of modification of CM-GAPDH and CM-gGAPDH could well be due to the presence of modified Lys residues. Moreover,

Table 4 The first order rates of the OPT-modified molecules

Rates (in units of 10^{-2} s^{-1}) were obtained from changes in A_{412} while the enzyme was reacting with OPT at pH 8.2 and 15 °C.

Sample	$10^{-2} \times \text{Rate (s}^{-1}\text{)}$	
	Fast phase	Slow phase
GAPDH	2.58	0.95
gGAPDH	12.84	1.23
Triglycine	Relaxation	0.50

fluorescence quenching of the OPT derivatives of both CM-GAPDH and CM-gGAPDH by potassium iodide (0.005–0.1 M) was markedly different, as seen in the Stern Volmer plot [17–18]. This plot showed that fluorescence of the OPT-treated derivative of CM-gGAPDH is much more easily quenched than that of CM-GAPDH, which suggests that OPT modification of the Lys residues in the CM-GAPDH and CM-gGAPDH may have taken place at different sites. In other words, some Lys residues are glycosylated in gGAPDH and are therefore not available to react with OPT. Thus the modification appears to occur at different sites for CM-gGAPDH and CM-GAPDH. The glycosylated sites may be near the active site of the enzyme, because (1) glycosylation *in vitro* decreases the activity markedly, (2) the active site conformation is changed and (3) glycosylation leads to a change in the spatial position of the active thiols.

Conformational changes of the active site

In our laboratory, conformational and activity changes of GAPDH from rabbit, lobster and baker's yeast during thermal- and GuHCl-denaturation have been studied [11–16,32,33]. Inactivation of these enzymes usually occurs before significant conformational changes can be detected by fluorescence, CD, absorbance and exposure of SH groups. It has been suggested that the active sites of these enzymes are more flexible than the molecules as a whole. A slight perturbation of the correct spatial geometry of the functional groups responsible for the catalytic mechanism by heat or denaturants destroys their activity before any gross conformational changes can be detected [34–35]. With a fluorescent probe introduced at the active site of muscle GAPDH [36], it has been shown that low concentrations of GuHCl, leading to complete inactivation, also affect markedly the 410 nm fluorophore emission, indicating conformational changes at the active site. Conformational and activity changes of gGAPDH were different from those of GAPDH in GuHCl solutions of different concentrations [16]. In particular, the conformational changes of the active site of the glycosylated enzyme were more subject to disturbance by the effect of the denaturant than were those of the non-glycosylated enzyme (see Figure 6). The emission intensities for gGAPDH decreased to 50% of the control value in 0.18 M GuHCl solution and the emission maximum shifted to 428 nm, however for GAPDH the decrease was only about 80% and the emission maximum shifted to 412 nm under similar conditions. This suggests that glycosylation causes instability of the active site.

DISCUSSION

Although glycosylated derivatives from haemoglobin, albumin, serum, plasma, collagen, erythrocyte membrane proteins and lens crystallins have been discovered and studied [2–6,37], there

Figure 6 Changes of emission intensity at 410 nm of the fluorescent NAD^+ derivatives at the active site of GAPDH during GuHCl denaturation

Carboxymethylation of SH groups at the active site as well as preparation of the enzyme with the covalently linked fluorescent NAD^+ derivative were as described previously for the muscle enzyme [20]. The fluorescent NAD^+ derivatives from the non-glycosylated GAPDH (final concentration, $5.0 \times 10^{-7} \text{ M}$) and from gGAPDH ($2.0 \times 10^{-7} \text{ M}$) were dissolved in 0.1 M phosphate buffer, pH 7.0, containing 0.001 M EDTA, to which GuHCl was added at the desired concentrations. The curves represent: (1) emission intensities at 410 nm; (2) emission maxima of the non-glycosylated GAPDH; (3) emission intensities at the same wavelength; and (4) emission maxima of gGAPDH.

have been few attempts to study glycosylated enzymes. In our laboratories, GAPDH, which is an important enzyme in the conversion of glucose into pyruvic acid, has been investigated in glycosylated form. Reports which have appeared in the literature include the observations that: glycosylation of low-density lipoprotein decreased its ability to bind to its receptor [38], glycosylated erythrocyte spectrin failed to stiffen the cell membrane [39] and glycosylation of high-density lipoprotein accelerated its catabolism in guinea pigs [40]. Jedziniak [41] observed that the stability of GAPDH from human lenses decreased during cataract formation. The isoelectric range of the enzyme broadened (suggesting increased heterogeneity of the enzyme), there was an increased acidic charge and a decreased stability was observed [41] with increasing age. By analogy, and also based on our experimental results, we suggest that glycosylation of GAPDH decreases its activity and that this may influence the glycolytic conversion of glucose into pyruvic acid *in vivo*.

Glycosylation can occur at the 2-amino group on the N-terminus of a protein and the ϵ -amino group of lysine residues. Although the rate of reaction of reducing sugar with 2-amino groups on N-terminal residues is faster than with ϵ -amino groups [28], 2-amino groups of gGAPDH were not found to be blocked by glycosylation. One subunit of rabbit muscle GAPDH contains 22 lysine residues. Lys-183 is acetylated irreversibly when apoenzyme is allowed to react with either *p*-nitrophenylacetate or acetyl phosphate at alkaline pH [8]. N-Acetylation occurs not by direct reaction with Lys-183 but as the result of an S to N migration of acetyl groups from Cys-149. This implies that Lys-183 is close to Cys-149 in the quaternary structure of the apoenzyme, an observation that was confirmed by the use of cross-linking reagents [42]. Acetylation of Lys-183 was found to inhibit NAD^+ binding as well as dehydrogenase activity. It has been suggested that Lys-183 is itself involved in coenzyme binding, but is not conserved in the primary structure for the other species [8].

Reaction of the rabbit muscle holo-enzyme with pyridoxal phosphate, which results in total inactivation, is specific for Lys-191 and Lys-212. As glycation decreases the activity of the GAPDH, affects the conformation of the active site and changes the spatial position of Cys-149, the glycated sites might be near the active site. But, so far we do not know whether glycation involves the non-enzymic reaction of an amino group with a reducing sugar or is a post-translational enzymic modification of the protein.

He [17,18] reported that changes in the intrinsic fluorescence of the non-glycated GAPDH during GuHCl denaturation were different from those observed for the glycated enzyme. Trp-193 is essential for the activity of this dehydrogenase from yeast [43]. It is also known from X-ray crystallographic studies that Trp-193 of the lobster enzyme is situated not very far from the active site [44]. However, this Trp residue is not conserved in the sequence of the residues of GAPDH from thermophilic bacillus [45]. On the other hand, quantitative studies on the energy transfer between Trp and fluorescent NAD⁺ derivatives have shown that, for both the muscle [20] and the bacillus enzyme [33] at least one of the Trp residues is situated fairly close to the nicotinamide. The results obtained for both lobster [33] and rabbit muscle [32] enzymes show concomitant exposure of the Trp residue near the active site and inactivation of the enzyme with increasing GuHCl concentrations. This suggests that a Trp residue plays a role in the catalytic mechanism of this enzyme. Giovanni and Edi [46] reported that the region situated near Trp-310, which is conserved in the amino acid residue sequence, correlated with the co-operativity of the enzyme for binding NAD⁺. Kinetic studies of the course of inactivation and unfolding of the rabbit muscle enzyme produced results somewhat different from those obtained previously for the lobster and rabbit muscle enzyme which were not purified by affinity chromatography [33,34]. Even at low GuHCl concentrations, the inactivation reaction seems to be as fast as the unfolding process, as indicated by the exposure of the Trp residue at or near the active site, which was shown by changes in the intrinsic fluorescence of the molecule. We have observed radiationless energy transfer from Trp residues to the OPT-modified derivatives. For lobster GAPDH, positioned around Trp-83 there are amino groups of Lys-2, 85, 114 and the N-terminus. Lys-183 and 191 are near Trp-193. So, some Lys residues near Trp residues were not glycated. We were only able to demonstrate that Lys-306 is unglycated, as this is the only residue within 15 Å (1.5 nm) of Trp-310. We cannot exclude the possibility that other Lys residues are glycated.

Glycation leads not only to a change in the conformation of the active site, but also to a change in the conformation of the molecule as a whole. Differences in the far-UV CD spectra (220–250 nm) between GAPDH and gGAPDH are not observed, but some differences between the two enzymes were detected in the near-UV region. It appears that the glycation of the enzyme affects the tertiary structure of the molecule more markedly than the secondary structure. We may conclude that glycation affects the function of the protein by disturbing the native spatial structure of the active site and of the molecule as a whole. Whether the glycation of GAPDH *in vivo* involves reaction with ADP-ribose [47–49], needs to be investigated further.

We gratefully acknowledge the director of the Institute of Biophysics, Academia Sinica, Beijing, China, for providing financial support for this work. We thank Dr. W. G. Griffin for his help in writing this manuscript and Professor Chen-Lu Tsou for his advice about this work.

REFERENCES

- Patton, A. R. and Hill, H. G. (1948) *Science*, **107**, 68–69
- Holmquist, W. R. and Schroeder, W. A. (1966) *Biochemistry*, **5**, 2489–2503
- Shapiro, R., McManus, M. J., Zalut, C. and Bunn, H. F. (1980) *J. Biol. Chem.* **255**, 3120–3127
- Robins, S. P. and Bailey, A. J. (1972) *Biochem. Biophys. Res. Commun.* **48**, 76–84
- Bailey, A. J., Robins, S. P. and Tanner, M. J. A. (1976) *Biochim. Biophys. Acta*, **434**, 51–57
- Stevens, V. S., Rouzer, C. A., Monnier, V. M. and Cerami, A. (1978) *Proc. Natl. Acad. Sci. U.S.A.* **75**, 2918–22
- Kennedy, L., Mehl, I. D., Ider, E., Varghese, M. and Merimee, I. J. (1982) *Diabetes*, **31**, 52–56
- Harris, J. I. and Water, M. (1976) *The Enzymes* **3**, 1–49
- Conway, A. and Koshland, D. E., Jr. (1968) *Biochemistry*, **7**, 4011–4022
- Ho, Y. S. and Tsou, C. L. (1979) *Nature (London)* **277**, 245–246
- He, R. Q. and Tsou, C. L. (1991) *Prog. Nat. Sci., (Engl. Ed.)* **1**, 315–322
- He, R. Q. and Tsou, C. L. (1991) *Acta Biophys. Sin.* **7**, 126–131
- He, R. Q. and Tsou, C. L. (1991) *Acta Biophys. Sin.* **7**, 25–31
- He, R. Q. and Tsou, C. L. (1992) *Biochem. J.* **287**, 1001–1005
- He, R. Q., Zhao, K. Y., Yan, Z. Z. and Li, M. (1993) *Biochem. Biophys. Acta* **1163**, 315–320
- He, R. Q. (1993) *Acta Biochim. Biophys. Sin.* **25**, 348–353
- He, R. Q. (1994) *Acta Biophys. Sin.* **10**, 361–366
- He, R. Q. (1994) *Acta Biophys. Sin.* **10**, 519–524
- Nozaki, Y., (1992) *Methods Enzymol.* **7**, 43–50
- Ho, Y. S. and Tsou, C. L. (1979) *Sci. Sin.* **22**, 478–489
- Ho, Y. S., Liang, S. J. and Tsou, C. L. (1988) *Biochim. Biophys. Acta* **613**, 249–255
- Weber, K. and Osborn, M. (1975) *The Proteins*, (Heurath, J., Hill, R. L. and Boeder, C.-L., eds.), vol. 1, 3rd edn., pp. 180–206, Academic Press, New York
- Millia, A. K. (1981) *Anal. Lett.* **14**, 649–661
- Chen, G. S., Li, L. G., Zhang, Q. X. and Fan, H. Y. (1983) in *Methods in Microbiological Experiments: Determination of Sugar with Sulfuric acid* (Wang, H. J., ed), 1st edn., pp. 198–200, Academia Press Sinica, Beijing
- He, R. Q. (1986) *J. Luzhou Med. Coll.* **9**, 160–164
- Francois, C. (1962) *Biochem. J.* **83**, 335–341
- Marshall, R. D. (1972) *Glycoproteins* (A. Golfschalk, ed.), pp. 283–287, Elsevier, Amsterdam
- Dixon, H. B. F. (1972) *Biochem. J.* **129**, 203–208
- Zhou, J. M. and Tsou, C. L. (1985) *Acta Biochim. Biophys. Sin.* **17**, 463–468
- Pace, B. and Pace, N. R. (1980) *Anal. Biochem.* **107**, 1621–1634
- Fields, R. and Dixon, H. B. F. (1971) *Biochem. J.* **121**, 587–589
- Ju, M. and Tsou, C. L. (1988) *Adv. Membr. Biochem. Bioenerg.* (Kim, C. H., Tedeschi, H., Diwan, J. and Salerno, J. C., eds.), pp. 507–515, Plenum Publishing Co., New York
- Xie, G. F. and Tsou, C. L. (1987) *Biochim. Biophys. Acta* **911**, 19–24
- Liang, S. J., Lin, Y. Z., Wu, P. Q., Zhou, J. M. and Tsou, C. L. (1990) *Biochim. Biophys. Acta* **1038**, 240–247
- Lin, Y. Z., Liang, S. J., Wu, P. Q., Zhou, J. M. and Tsou, C. L. (1990) *Biochim. Biophys. Acta* **1038**, 425–429
- Tsou, C. L., Xu, G. Q., Zhou, J. M. and Zhao, K. Y. (1986) *Biochem. Soc. Trans.* **11**, 425–429
- Bunn, H. F., Gabbay K. H. and Gallop P. M. (1978) *Science* **200**, 21–27
- Kraemer, F. B., Chen, Y. D. I., Chueng, R. M. C. and Reaven, G. M. (1982) *Diabetologia* **23**, 28–33
- McMillan, D. E. and Brooks, S. M. (1982) *Diabetes*, **31**, Suppl. 3, 64–69
- Witztum, J. L., Fisher, M., Pietro, T., Steinbrecher, U. P. and Elam, R. L. (1982) *Diabetes* **31**, 1029–1032
- Jedziniak, J. A. (1986) *Curr. Eye. Res.* **5**, 119–126
- Shaltiel, S. and Tauber-Finkelstein, M. (1971) *Biochem. Biophys. Res. Commun.* **44**, 484–490
- Heilmann, H. D. and Pfeleiderer, G. (1987) *Biochim. Biophys. Acta* **384**, 331–341
- Moras, D., Olsen, K. W., Sabesen, M. N., Buehner, M., Ford, G. C. and Rossmann, M. G. (1975) *J. Biol. Chem.* **250**, 9137–9162
- Hocking, J. D. and Harris, J. I. (1980) *Eur. J. Biochem.* **108**, 567–579
- Giovanni, B. S. and Edi, G. (1989) *Biochemistry* **28**, 160–166
- Daniel, C.-L., David, E. M., Elaine, L. J. and Myron, K. J. (1993) *Biochemistry* **32**, 1528–1534
- Lee, J. M. and Joel, M. (1993) *Proc. Natl. Acad. Sci. U.S.A.* **90**, 6238–6241
- Lee, J. M. and Joel, M. (1993) *J. Biol. Chem.* **268**, 17878–17882