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# Human Erythrocyte Bisphosphoglycerate Mutase: Inactivation by Glycation *In Vivo* and *In Vitro*[1]

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**2,3-Bisphosphoglycerate mutase (BPGM) [EC 5.4.2.4] is a multifunctional enzyme that catalyzes both the synthesis and the degradation of 2,3-diphosphoglycerate (2,3-DPG) and contains three types of activities in that it functions as a 2,3-DPG synthetase, a phosphoglycerate mutase and a 2,3-DPG phosphatase. In humans, BPGM occurs only in erythrocytes and plays a pivotal role in the dissociation of oxygen from hemoglobin *via* 2,3-DPG. The present study shows that the specific activity of BPGM in erythrocytes of diabetic patients is decreased, compared to normal controls as judged by 2,3-DPG synthetase activity and immunoreactive contents. To understand the mechanism by which the enzyme is inactivated, the enzyme was purified from pooled erythrocytes from diabetic patients and subjected to a boronate affinity column. The flow through fraction was active while the bound fraction was completely inactive. The bound fraction was reactive to an anti-hexitollysine antibody, indicating that the enzyme had undergone glycation and inactivation. The primary glycated site of the enzyme was found to be Lys158 as judged by amino acid sequencing and the reactivity with an anti-hexitollysine IgG, after reverse-phase HPLC of the lysyl-endopeptidase-digested peptides. Extensive glycation of recombinant BPGM *in vitro* indicated that the glycation sites were Lys2, Lys4, Lys17, Lys42, Lys158, and Lys196. From these results, the loss of enzymatic activity appears to be due to the glycation of Lys158 which may be located in the vicinity of the substrate binding site.**

**Key words: 2,3-bisphosphoglycerate mutase, diabetes, 2,3-DPG, glycation site, phosphoglycerate mutase.**

A number of enzymes in human erythrocytes undergo glycation as a result of contact with high levels of blood glucose. Among these are nucleoside diphosphokinase (*1*), purine nucleoside phosphorylase (*1*), δ-aminolevulinate dehydratase (*2*), carbonic anhydrase (*3*), Cu,Zn-superoxide dismutase (*4*), and $Na^+$,$K^+$ ATPase (*5*). The glycation of these enzymes has a significant effect on their biological functions and physiological properties. Glycation also has been implication in the formation of reactive oxygen species such as superoxide and as a result, this reaction is an important contributor to the pathogenesis of diabetes (*6-13*).

2,3-Bisphosphoglycerate mutase (BPGM) [EC 5.4.2.4] is a multifunctional enzyme which catalyzes both the synthesis of 2,3-diphosphoglycerate (2,3-DPG) *via* its synthetase activity and its degradation *via* its phosphatase activity (*14-17*). In humans, BPGM occurs only in erythrocytes and plays a pivotal role in the dissociation of oxygen from Hb *via* 2,3-DPG (*18*), and is directly associated with the affinity of Hb for oxygen molecules (*19*). It is well known that human Hb A undergoes glycation at higher than normal levels in diabetes and that the primary glycation site is the $NH_2$-terminal valine residue of the β-chain, which is related to 2,3-DPG binding pocket. This may alter the conformation of the protein and decrease its binding affinity to 2,3-DPG, thus reducing the ability of the erythrocyte to release oxygen, resulting in tissue hypoxia. However, the reported levels of 2,3-DPG in diabetes are controversial and serious attempts to precisely determine such levels are lacking.

In earlier studies, we found that the total BPGM activity in erythrocytes of patients with diabetes was decreased but the immunological BPGM contents as judged by enzyme immunoassay were normal, suggesting that the specific

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Abbreviations: BPGM, bisphosphoglycerate mutase; 2,3-DPG, 2,3-diphosphoglycerate; ELISA, enzyme-linked immunosorbent assay; pA(AB)$_4$, an artificially produced immunoglobulin G-binding protein with 4 domains A-B, which are linear polymerized; PTH, phenylthiohydantoin.

activity of BPGM was decreased. The aim of the present study was to investigate the mechanism of inactivation of BPGM and to examine the sites of glycation of BPGM *in vitro* and *in vivo*.

## EXPERIMENTAL PROCEDURES

*Purification of BPGM from Pooled Erythrocytes from Diabetic Patients*—A hemolysate (one liter; assigned percentage Hb A1c, 10.4%; lot no. PB9604) prepared from fresh red blood cells from diabetic patients by Aalto Scientific (CA, USA) was diluted to 4 liters with 5 mM K-$PO_4$ buffer (pH 7.2) containing 5 mM EDTA, 1 mM 2-mercaptoethanol, and 0.02% $NaN_3$. BPGM was purified from the diluted hemolysate, essentially according to the method of Calvin *et al.* (*20*), with the following modifications. The diluted hemolysate was subjected to a DEAE-Sepharose CL-6B (Pharmacia Biotech) column (250×100 mm) equilibrated with 5 mM K-$PO_4$ buffer (pH 7.2) containing 5 mM EDTA, 1 mM 2-mercaptoethanol, and 0.02% $NaN_3$. The column was washed with the same buffer, and then eluted with the same buffer containing 100 mM NaCl. The BPGM fractions were concentrated and then chromatographed on a Blue-Sepharose CL-6B (Pharmacia Biotech) column (250×224 mm), followed by further purification. To the eluent from the Blue-Sepharose column was added 1.5 M ammonium sulfate and the resulting solution was subjected to hydrophobic chromatography on a TSKgel Phenyl-TOYOPEARL 650C column (60×110 mm; Tosoh) equilibrated with 100 mM Na-$PO_4$ buffer (pH 7.0) containing 1.5 M ammonium sulfate, and the adsorbed enzyme was eluted with a linear gradient of the same buffer and 100 mM Na-$PO_4$ buffer (pH 7.0) containing 5% isopropanol.

*Separation of Glycated and Nonglycated BPGM*—The purified BPGM was applied to a TSKgel Boronate-5PW column (7.5×75 mm; Tosoh), which had been equilibrated with 50 mM HEPES buffer (pH 8.5). The nonglycated enzyme was washed through a column with above buffer and the glycated enzyme was eluted with 100 mM Tris-HCl buffer (pH 7.5) containing 200 mM sorbitol. The flow rate was 1.0 ml/min. The eluent was monitored at 280 nm. Each fraction was assayed for BPGM activity. In order to map the lysyl-endopeptidase fragments of the glycated BPGM which bound to the boronate column, the glycated enzyme was collected and reduced by treatment with 100 mM $NaBH_4$, followed by dialysis against pure water and lyophilization.

*Preparation of Expression Plasmid pTRP-BPGM and Purification of BPGM from Transformed Escherichia coli*—The recombinant DNA which encodes for human erythrocyte BPGM was amplified by modified PCR (*21*) with genomic DNA derived from human placental tissues as a template. Four oligonucleotides, 5′-CCGGAATTCATGTCCAAGTACAAACTTAT-3′, 5′-CTTCATCTGAGATACCTTCCAGGTGTTTTAGGAGT-3′, 5′-GTATCTCAGATGAAGACATC-3′, and 5′-CCCGGATCCCTATTTTTTTAGCTTGTTTCA-3′ were used as primers. The amplified DNA was ligated into pTZ19U vectors (Toyobo) for cloning. The cloned DNA coding BPGM was inserted into a pTRP expression vector (*22*), and transformed to *E. coli* JM109. However, the transformant showed a very low level of expression of recombinant BPGM. Thus, the oligonucleotide sequence corresponding to 12 amino acid residues in the $NH_2$-terminal region of BPGM was changed to "ATGTCAAAATACAAACTGATCATGTTAAGACATGGA" by PCR using 5′-CCGGAATTCATGTCAAAATACAAACTGATCATGTTAAGACATGGA-3′ as a 5′-primer. The codon usage in the sequence was exchanged from native codons into "AT rich" codons. The amino acid sequence, however, was not altered. The mutated DNA which encodes for BPGM was cloned and ligated into pTRP, and then was transformed to *E. coli* JM109. The culturing of the transformant was performed as previously described (*22*). BPGM was purified from *E. coli*, essentially according to the method of Calvin *et al.* (*20*). A cell free extract from 500 g (wet weight) of *E. coli* was treated for 30 min at 55°C and then chromatographed on a Blue-Sepharose CL-6B column (250×224 mm). Further purification was performed in the same manner as that for the BPGM from hemolysate. Finally, the BPGM fraction was dialyzed against 50 mM K-$PO_4$ buffer (pH 7.4) containing 150 mM NaCl and 0.02% $NaN_3$.

*Assay of Enzymatic Activity*—Three enzymatic activities of BPGM were spectrophotometrically determined by measuring the rate of NADH or $NAD^+$ formation at 37°C using 1.0 ml of the reaction mixture. Assays of 2,3-DPG synthetase and 2,3-DPG phosphatase activities were carried out using the method of Calvin *et al.* (*20*) with minor modifications, and the assay of phosphoglycerate mutase activity was carried out by a modification of the method of Bergmeyer *et al.* (*23*). The components contained in the reaction mixtures for the activity assays were as follows. For 2,3-DPG synthetase, 50 mM Tris-HCl buffer (pH 8.0), 1 mM $NAD^+$, 7 mM fructose-1,6-diphosphate, 7 mM $KH_2PO_4$, 2 mM 3-phosphoglycerate, 0.2 unit/ml aldolase, 0.8 unit/ml triosephosphate isomerase, and 0.15 unit/ml glyceraldehyde-3-phosphate dehydrogenase. For glycolate-stimulated 2,3-DPG phosphatase, 50 mM triethanolamine-HCl buffer (pH 7.5), 10 mM $MgCl_2$, 3 mM ATP, 0.2 mM NADH, 0.8 mM 2,3-DPG, 3.3 units/ml glyceraldehyde-3-phosphate dehydrogenase, 2.3 units/ml phosphoglycerate kinase, and 1 mM 2-phosphoglycolate. For phosphoglycerate mutase, 100 mM HEPES buffer (pH 7.7), 10 mM $MgSO_4$, 40 mM KCl, 1.4 mM 3-phosphoglycerate, 0.16 mM 2,3-DPG, 0.28 mM NADH, 4 mM ADP, 2.4 units/ml lactate dehydrogenase, 1.6 units/ml pyruvate kinase, and 1.6 units/ml enolase.

One unit (U) of activity is defined as the amount which catalyzes the formation of 1 $\mu$mol of $NAD^+$ or NADH per min. Protein was determined spectrophotometrically at 280 nm using $A1\%\cdot cm^{-1}=16.0$ for purified BPGM (*17*), or, alternatively, was determined by ELISA analysis of the crude proteins from erythrocytes. The specific activity was expressed as U of 2,3-DPG synthetase activity per mg of BPGM protein.

*In Vitro Glycation of BPGM*—The purified recombinant BPGM was incubated with reducing sugar at a protein concentration of 0.7–1.0 mg/ml in 50 mM K-$PO_4$ buffer (pH 7.4) containing 150 mM NaCl and 0.02% $NaN_3$ at 37°C for the indicated times, after which the incubation mixture was sterilized by ultrafiltration through a Millipore 0.22 mm filter. The reaction was then quenched by freezing the mixture, or aliquots of the incubation mixtures were examined for enzymatic activity. The glycation of BPGM was measured by the thiobarbituric acid reaction as described previously (*13*). In order to map the lysyl-endopep-

tidase fragments of the glycated BPGM, after a 1-day and a 7-day glycation with 1 M glucose at 37°C, the glycated BPGM was reduced with 100 mM $NaBH_4$, followed by dialysis and lyophilization.

*Preparation of Polyclonal Anti-Human Erythrocyte BPGM Antibody*—An anti-human erythrocyte BPGM antiserum was obtained from several rabbits which had been immunized with highly purified BPGM according to Harlow and Lane (*24*). The IgG fraction was prepared by affinity chromatography on a $pA(AB)_4$ (*25*) column, which is an artificial IgG-binding protein coupled to activated CH-Sepharose 4B (Pharmacia Biotech). The resulting IgG fraction was passed through a column of human phosphoglycerate mutase [EC 5.4.2.1] (B type isozyme) coupled to Formyl-Cellulofine (Tosoh), in order to remove the antibody populations which are reactive to phosphoglycerate mutase whose amino acid sequence was 53.8% homologous to that of BPGM (*26*).

*SDS-PAGE and Immunoblot Analysis*—SDS-PAGE was performed by the method of Laemmli using a 10-20% (w/v) gradient acrylamide gel (*27*). BPGM was previously reduced with 100 mM $NaBH_4$. After electrophoresis, one gel was stained with Coomassie Brilliant Blue R-250, and the other gel was electrophoretically transblotted to a polyvinylidene difluoride membrane. The glycated BPGM was immunostained using a rabbit anti-hexitollysine IgG which recognizes proteins which are glycated by glucose, but not fructose, galactose, or ribose (*28*) and a horse radish peroxidase conjugated goat anti-rabbit IgG antibody.

*Enzyme Immunoassay*—ELISA analysis was performed as previously described (*29*). In brief, for assay of immunoreactive contents of BPGM protein, each well of a microtiter plate was incubated with 50 $\mu$l of an anti-BPGM IgG (10 $\mu$g/ml). After washing, the wells were incubated with 50 $\mu$l of the BPGM preparations or the standard protein (purified BPGM). The washed wells were then reacted with 50 $\mu$l of a biotinylated anti-BPGM IgG (20 $\mu$g/ml). The washed wells were reacted with 50 $\mu$l of a streptoavidin-alkaline phosphatase conjugated (0.5 unit/ml), and were washed, followed by reaction with 100 $\mu$l of a mixture of 10 mM *p*-nitrophenyl phosphate and 2.5 mM $MgCl_2$ in 1 M diethanolamine-HCl buffer (pH 9.8) for 10-20 min. The reaction was terminated by adding 25 $\mu$l of 2 N NaOH to each well and the absorbance at 415 nm against that at 630 nm was measured with a microplate reader. The glycated peptides obtained from reverse-phase HPLC were detected with a microtiter plate using an anti-hexitollysine IgG as a primary antibody and a horseradish peroxidase conjugated goat anti-rabbit IgG antibody as a secondary antibody.

*Proteolytic Cleavage of BPGM with Lysyl-Endopeptidase and Purification of Peptide Fragments*—*S*-Pyridylethylation of BPGM was performed at 25°C by the method of Cavins and Friedman (*30*). The pyridylethylated BPGM (200 $\mu$g) was digested in 200 $\mu$l of 50 mM ammonium hydrogen carbonate buffer (pH 8.0) containing 4 M urea and lysyl-endopeptidase (10 $\mu$g) for 15 h. The fragments were separated by reverse-phase HPLC on a Wakosil-II 5C18 HG column (4×250 mm; Wako Chemical), and eluted with a linear gradient of 0-60% solution of acetonitrile containing 0.1% trifluoroacetic acid. The flow rate was 0.7 ml/min. The eluent was monitored at 220 nm. In re-chromatography on the same column, the peptides were eluted with a linear gradient of a 0-12.5% solution of acetonitrile containing 0.1% trifluoroacetic acid.

*Amino Acid Sequencing*—Automated sequence analysis of the derived peptides was performed using an Applied Biosystems model 477A protein sequencer equipped with a model 120A PTH derivative analyzer.

## RESULTS

*Specific Activity of BPGM from Diabetic Patients*—Hemolysates of three females and six males patients with non-acidotic and non-ketoacidotic diabetes aged 61±10 years (mean±SD) were subjected to assay for 2,3-DPG synthetase activity of BPGM and to ELISA analysis for measurement of BPGM protein. Their blood glucose levels were 178±51 mg/100 ml and the percentage Hb A1c ranged from 8.1 to 13.1%, with a mean value of 10.2%. The control group was comprised of five normal, healthy volunteers (two females and three males). The specific activity of BPGM from diabetic patients was 13.8±0.4 U/mg and the normal healthy controls gave 20.2±4.8 U/mg. This indicates that the 2,3-DPG synthetase activity of BPGM in patients with diabetes is decreased and approximately 70% that of normal subjects. No difference was found in blood Hb concentrations for normal subjects (140±18 mg/ml) *vis-a-vis* diabetic patients (138±14 mg/ml).

*Purification of BPGM from Human Erythrocytes of Diabetes and Affinity Chromatography on Boronate Column*—In order to better understand the mechanism by which 2,3-DPG synthetase activity of BPGM is decreased in diabetic patients, the enzyme was purified from pooled erythrocytes from diabetic patients and subjected to a boronate affinity column which preferentially binds glycat-

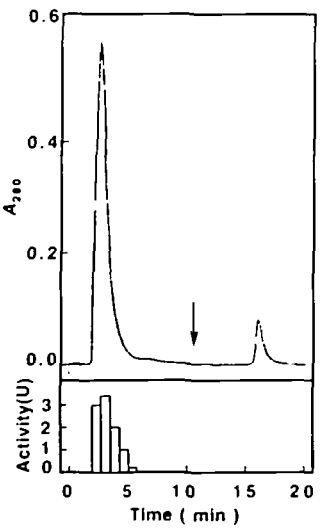


Fig. 1. **Boronate affinity chromatography of the purified BPGM from pooled erythrocytes of diabetic patients, *i.e.*, *in vivo* glycation.** The purified BPGM (500 $\mu$g) from pooled erythrocytes of diabetic patients was applied to a TSKgel Boronate column (7.5×75 mm) that was equilibrated with 50 mM HEPES buffer (pH 8.5). After washing the column with the same buffer, the bound enzyme was eluted with 100 mM Tris-HCl buffer (pH 7.5) containing 200 mM sorbitol (arrow). The flow rate was 1.0 ml/min. The eluent was monitored at 280 nm (upper panel). Each fraction was assayed for BPGM activity. The lower panel indicates 2,3-DPG synthetase activity of BPGM.

ed proteins. The BPGM purified from diabetic patients had a specific activity of 15 U/mg which was lower than that of normal subjects. As shown in Fig. 1, BPGM underwent glycation *in vivo*, as judged by boronate affinity chromatography as well as by reactivities to thiobarbituric acid and an anti-hexitollysine antibody with the bound protein to the column. The bound enzyme had no BPGM activity (Fig. 1, lower panel), while the flow through fractions contained active in BPGM.

*Inactivation of BPGM during Incubation with Reducing Sugar*—In order to determine the mechanism by which BPGM activity is decreased in diabetic patients, the effect of glycation on enzymatic activity of the purified recombinant BPGM relative to its 2,3-DPG synthetase, phosphoglycerate mutase, and 2,3-DPG phosphatase activities were studied *in vitro*. The above three enzymatic activities of BPGM decreased in a time-dependent manner on incubation with reducing sugar, whereas incubation with a non-reducing sugar, sorbitol resulted in no decrease in activity (Table I and Fig. 2A). The inactivation of BPGM by fructose was more rapid than that by glucose. BPGM which had been incubated with a sugar was subjected to SDS-PAGE and immunoblot analysis. The dye-stained band of each protein on SDS-PAGE gel showed no change in mobility (Fig. 2B). BPGM which had been incubated only with glucose reacted with an anti-hexitollysine antibody (Fig. 2C). The reactivity of the thiobarbituric acid reaction which provides a measure of the formation of ketoamine adducts in the glycation reaction with glucose also paralleled with the loss of enzymatic activity (Fig. 2A).

*Mapping of Lysyl-Endopeptidase Fragments from Non-glycated and In Vitro Glycated BPGMs*—Human erythrocyte BPGM is comprised of 258 amino acids, contains 16 lysine residues, and no intramolecular disulfide bridges among cysteine residues. When the non-glycated recombinant BPGM was digested with lysyl-endopeptidase, reverse-phase HPLC yielded 12 major peaks corresponding to its amino acid sequence (Fig. 3A). Peak I, which eluted from a reverse-phase column at 11 min, was pooled and subjected to re-chromatography with a linear gradient of 0–12.5% solution of acetonitrile, since it contained three or more fragments, judging from amino acid sequencing. A partial elution profile of peak I obtained by re-chromatography, which is shown in the inset in Fig. 3A, revealed the presence of 4 peaks. Each collected peak was subjected to amino acid sequencing, and the results are shown in Table II. The sequence analyses data, confirmed that fragments from 1 to 12 all contained fragments derived from BPGM. In the case of BPGM, the second lysine residues from both $NH_2$- and COOH-terminals, which are in a Ser1-Lys2 sequence and in a Lys257-Lys258 sequence, respectively, and Lys254 were likely not to be cleaved with lysyl-endopeptidase. In addition, the $NH_2$-terminal sequences of fragments 11 and 12 were identical, for the first 12 residues. This suggests that lysyl-endopeptidase is capable of cleaving Arg226, in an Arg-Ala sequence (*31*), releasing a fragment containing 30 amino acid residues from His197 to Arg226, thus accounting for peak 11. Peaks from 1 to 12 were not found to be reactive with an anti-hexitollysine antibody.

The glycated recombinant BPGM *in vitro*, which had been incubated for 7 days with 1 M glucose was subjected to amino acid analysis and as a result, 4 mol of lysine residues were found to be glycated per mol of enzyme. No arginine modification (*32*) was detected. BPGM glycated with 1 M glucose for 1 day or 7 days at 37°C was digested with lysyl-endopeptidase, and the digests were subjected to reverse-

TABLE I. **Changes in activities of 2,3-DPG synthetase, phosphoglycerate mutase, and 2,3-DPG phosphatase of BPGM during incubation with glucose.** The purified recombinant BPGM (1 mg/ml) was incubated with or without (control) 0.1 M glucose at 37°C. The reaction was quenched by freezing the mixture, and aliquots of the incubation mixtures were then subjected to assay of BPGM activity. The values in parentheses indicate each relative activity (%).

| | Activity (U/ml) | | |
|---|---|---|---|
| | Incubation time (day) | | |
| | 0 | 3 | 7 |
| 2,3-DPG synthetase | | | |
| Control | 21.1 (100) | 19.0 (90.0) | 18.2 (86.3) |
| + Glucose | 21.2 (100) | 16.3 (76.9) | 10.7 (50.5) |
| 2,3-DPG phosphatase | | | |
| Control | 5.1 (100) | 4.4 (86.3) | 4.1 (80.4) |
| + Glucose | 5.1 (100) | 3.7 (72.5) | 2.1 (41.2) |
| Phosphoglycerate mutase | | | |
| Control | 2.8 (100) | 2.5 (89.3) | 2.4 (85.7) |
| + Glucose | 2.8 (100) | 2.1 (75.0) | 1.3 (46.4) |

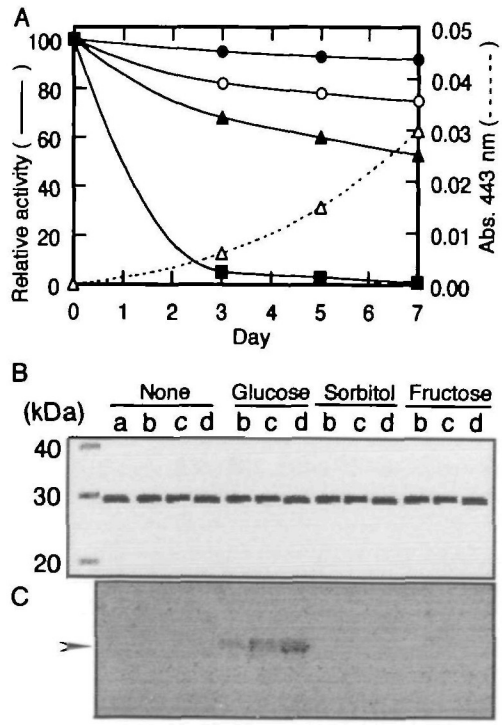


Fig. 2. **Inactivation of BPGM during incubation with various carbohydrates at 37°C.** (A) After incubation of 0.7 mg of the purified recombinant BPGM with buffer (○), 100 mM sorbitol (●), 100 mM glucose (▲), or 100 mM fructose (■) at 37°C, each remaining 2,3-DPG synthetase activity of BPGM was assayed. The reactivity of thiobarbituric acid with BPGM incubated with glucose is given on the right (△ in A). After incubation at conditions of (A), the reaction mixtures were loaded directly on SDS-PAGE. Each lane contained 2 μg of BPGM. Lanes a, b, c, and d indicate incubation times of 0, 3, 5, and 7 days, respectively. Proteins were stained with Coomassie Brilliant Blue R-250 (B) or indirect immunostaining using an anti-hexitollysine IgG as a primary antibody (C). The molecular masses in kDa are given on the left in (B) and arrowhead indicates the material which reacted with an anti-hexitollysine IgG in (C).

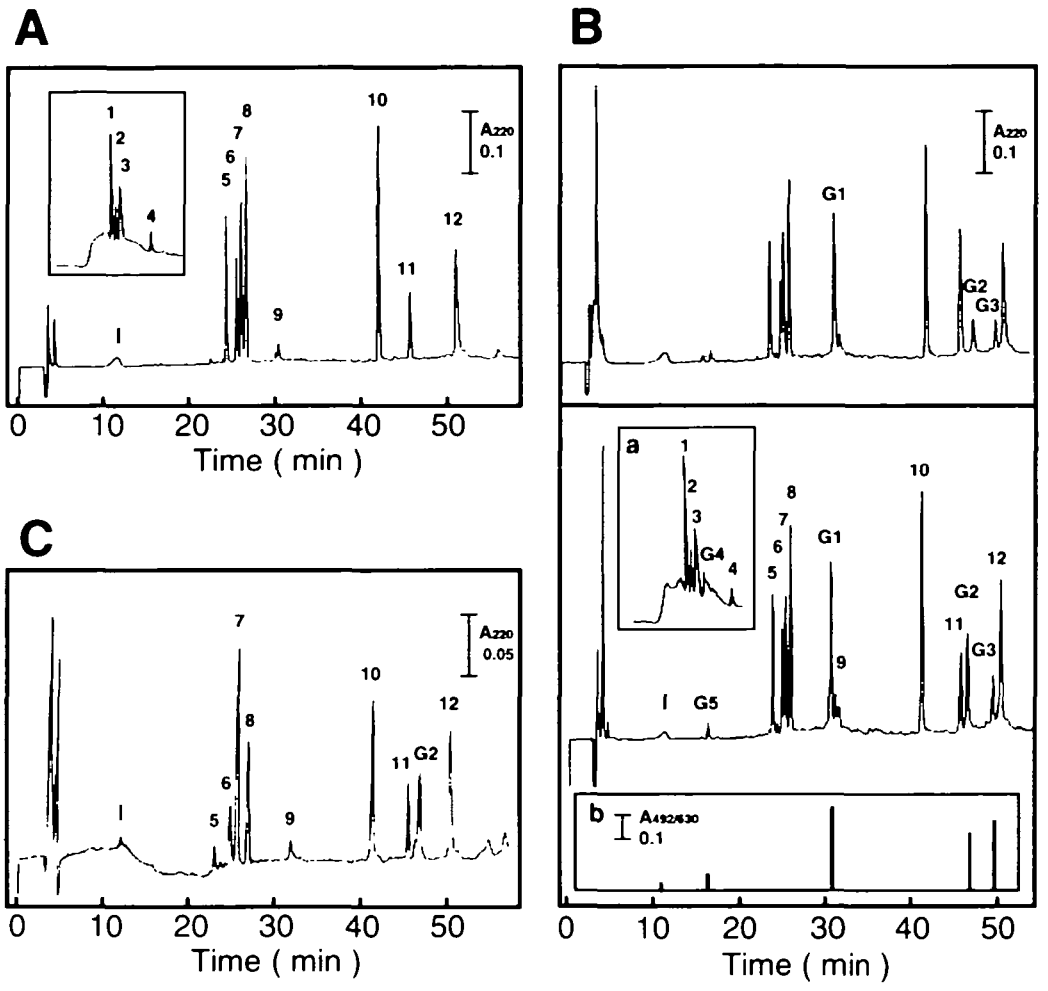


Fig. 3. **Peptide mapping of the lysyl-endopeptidase fragments from the non-glycated, *in vitro* glycated, and *in vivo* glycated BPGMs.** Elution profiles of the lysyl-endopeptidase-digested peptides derived from the purified recombinant BPGM (A), *in vitro* glycated BPGM (B), and *in vivo* glycated BPGM (C) on reverse-phase HPLC with a Wakosil-II 5C18 HG column (4×250 mm) are shown. The fragments were eluted with a linear gradient of a 0-60% aqueous solution of acetonitrile containing 0.1% trifluoroacetic acid. The upper and lower panels in (B) show the elution profiles for a 1-day and a 7-day incubation of the purified recombinant BPGM with 1 M glucose for at 37°C, respectively. The insets in (A) and (a) in (B) show the re-chromatography, on the same column indicated above, of peak I which eluted at 11 min from reverse-phase HPLC, respectively. A linear gradient of acetonitrile 0-12.5% was used. The flow rate was 0.7 ml/min. The eluent was monitored at 220 nm. All the main peaks are numbered. The inset (b) in (B) shows the reactivity of each peak to an anti-hexitollysine IgG. Peaks from G1 to G5 indicate new peaks containing the glycated fragments.

phase HPLC (Fig. 3B). These experiments were based on the assumption that, if the lysine residues are specifically glycated, these modified residues are not digested with lysyl-endopeptidase, and, hence, the digests would give new peaks in an elution profile by reverse-phase HPLC. In a profile of digests obtained from a 1-day glycation, three major peaks (G1, G2, and G3) (Fig. 3B, upper panel), and in a profile of digests from a 7-day glycation, an additional two peaks (G4 and G5) [Fig. 3B, lower panel and inset (a)] were observed, as compared with that of the nonglycated sample, which is shown in Fig. 3A. The peaks comprising G1 to G5, which were eluted from the column, were collected and their reactivities to an anti-hexitollysine IgG was examined by ELISA analysis. The peaks were found to be reactive [Fig. 3B, inset (b) in lower panel]. These results suggest that peaks G1-G5 contain glycated fragments. Glycation of peaks G1, G2, and G3 increased only after 24 h's incubations. This suggests that the fragments in peaks G1 to G3 contain the primary glycation sites which are related to the enzymatic activity of BPGM.

*Sequence Analysis of Lysyl-Endopeptidase Fragments of In Vitro Glycated BPGM*—Peaks from G1 through G5 were subjected to amino acid sequencing. Results of sequence analyses are summarized in Table III. The peaks from 1 to 12, derived from a 7-day glycation of BPGM, showed the same amino acid sequences compared with those of the nonglycated sample shown in Table II. The first peak shown in Fig. 3B, G1, which eluted from a reverse-phase HPLC column at 31 min, was identified as two glycated fragments containing amino acid residues from Ser1 to Lys17 and from Leu5 to Lys28, except that signal corresponding to PTH-lysines at Lys2, Lys4, and Lys17 was absent. The second peak, G2, which eluted at 47 min, was also a glycated fragment which contained a modified Lys158. The third peak, G3, which eluted at 49 min, was also a glycated fragment and contained modified Lys196. Peak G5, which

TABLE II. **Amino acid sequences of the lysyl-endopeptidase fragments of BPGM.** The *S*-pyridylethylated recombinant BPGM was digested with lysyl-endopeptidase, and the resulting digested peptides subjected to reverse-phase HPLC. The elution pattern is shown in Fig. 3A. Sequence analysis of each peak was performed.

| Peaks[a] | Amino acid sequence | Peptide assignment[b] |
|---|---|---|
| 1 | VKQAKK | 253-258 |
| 2 | SKYK | 1-4 |
| 3 | QLK | 43-45 |
| 4 | KVEDQG | 246-252 |
| 5 | ENRFC[c]SWVDQK | 18-28 |
| 6 | TILISAHGNSSRALLK | 181-196 |
| 7 | VC[c]DVPLDQLPRSESLK | 143-158 |
| 8 | LNSEGMEEARNC[c]GK | 29-42 |
| | LIMLRHGEGAWNK | 5-17 |
| 9 | ALNFEFDLVFTSVLNRSIHTAWLIL--- | 46- |
| 10 | DVLERLLPYWNERIAPEVLRGK | 159-180 |
| 11 | HLEGISDEDIINI--- | 197- |
| 12 | HLEGISDEDIINITLPTGVPILLEL--- | 197- |

[a]Numbers of the each peptide are those of the corresponding fractions on reverse-phase HPLC in Fig. 3A. [b]Number of the amino acid residue. [c]Determined as *S*-pyridylethylcysteine.

TABLE III. ***In vitro* glycated BPGM and amino acid sequences.** The *in vitro* glycated recombinant BPGM was *S*-pyridylethylated, digested with lysyl-endopeptidase, and the resulting digested peptides were subjected to reverse-phase HPLC. The elution pattern is shown in Fig. 3B. Sequence analyses of peaks, G1-G5 were performed.

| Peaks[a] | Amino acid sequence | Peptide assignment[b] |
|---|---|---|
| G1 | S( )[c]Y( )[c]LIMLRHGEGAWNK | 1-17 |
| | LIMLRHGEGAWN( )[c]ENRFC[c]SWVDQK | 5-28 |
| G2 | VC[d]DVPLDQLPRSESL( )[c]DVLERLLPY--- | 143- |
| G3 | TILISAHGNSSRALL( )[c]HLEGISDED--- | 181- |
| G4 | S( )[c]YK | 1-4 |
| G5 | LNSEGMEEARNC[d]G( )[c]QLK | 29-45 |

[a]Numbers of the each peptide are those of the corresponding fractions on reverse-phase HPLC in Fig. 3B. [b]Number of the amino acid residue. [c]A "blank" result shown in parentheses indicates that no phenylthiohydantoin derivative was detected. [d]Determined as *S*-pyridylethylcysteine.

eluted at 16 min, also a glycated fragment, contained modified Lys42. Finally, peak G4, which contained a glycated fragment containing modified Lys2 was found. The lysine residues at positions of 2, 4, 17, 42, 158, and 196 were therefore identified as the *in vitro* glycated sites.

*Identification of Glycated Site of BPGM Purified from Patients with Diabetes*—The *in vivo* glycated BPGM purified from human diabetic patients were also subjected to reverse HPLC after lysyl-endopeptidase digestions as shown in Fig. 3C. Peak I, which eluted from a reverse-phase column at 11 min, was not subjected to re-chromatography, since it did not contain the glycated fragments, judging from the reactivity to an anti-hexitollysine IgG. Only peak G2 was observed as a new peak in an elution profile compared with that of the nonglycated sample shown in Fig. 3A. This peak was found to be reactive with an anti-hexitollysine IgG. Peak G2 was subjected to amino acid sequencing. The results of sequence analysis are V143CDVPLDQ-LPRSESL( )DVLE---, and show an amino acid sequence which is identical for the nonglycated and *in vitro* glycated samples. Peak G2, which eluted at 47 min, was the glycated fragment which contained glycated Lys158. These data indicate that the loss of enzymatic activity of *in vivo* glycated BPGM is due to the glycation of Lys158.

## DISCUSSION

Glycation is a common post-translational *in vivo* modification of proteins, and is likely to cause an alteration of, not only physicochemical properties of proteins, but also their functional characteristics (*7, 9, 13, 33-35*). It is also possible that BPGM, as well as several other previously reported erythrocyte enzymes (*1-5*) undergoes functional and conformational changes on exposure to high concentrations of glucose.

The present study shows that human erythrocyte BPGM undergoes glycation and inactivation in diabetic patients and that *in vitro* glycation of the enzyme also leads to a gradual inactivation of the enzyme. The glycation of BPGM was confirmed on the basis of: (i) reactivities to an anti-hexitollysine IgG, (ii) affinity to a boronate column, (iii) reactivities in the thiobarbituric acid reaction, (iv) resistance to digestion by lysyl-endopeptidase, and (v) failure to detect PTH-derivatives at the expected position.

The sites of *in vivo* and *in vitro* glycation were identified by amino acid sequencing of fragments, which were generated by the digestion of the pyridylethylated BPGM, after separation by reverse-phase HPLC. It is generally thought that the site specificity of glycation is dependent on both the equilibrium Schiff base concentration and the rate of Amadori rearrangement at each site. Histidine imidazole groups are effective acid-base catalysts in a neutral solution, and accelerate the glycation of amino groups of nearby lysine residues. Therefore, the interatomic distances between the histidine residue and a nearby lysine in the primary or the three-dimensional structure of protein are important in glycation reactions (*36, 37*).

The *in vivo* glycated site was found to be Lys158, and other sites of *in vitro* glycation of BPGM were Lys2, Lys4, Lys17, Lys42, Lys158, and Lys196. Considering the positions of the sites of *in vivo* and *in vitro* glycation of BPGM, it is interesting that two sites are located in the primary sequence of acidic amino acids: Lys17 in a Lys-Glu sequence and Lys158 in a Lys-Asp sequence. The other three sites are located in a sequence of basic amino acids: Lys2 and Lys4 in a Lys-Tyr-Lys sequence, and Lys196 in a Lys-His sequence. From the computer image (Brookhaven National Laboratory, Protein Data Bank, January 1997, release #79, volumes 970130.d4) of the X-ray crystallographic model of the yeast phosphoglycerate mutase [EC 5.4.2.1] (*38, 39*), which contains an amino acid sequence and functional properties which are highly similar to those of human erythrocyte BPGM (*40*), the glycated Lys4, Lys17, Lys42, Lys158, and Lys196 in BPGM are likely to be located on the surface of the BPGM molecule. These sites appear to be easily attacked by glucose, and are near His197, His10, His10, His197, and His197, respectively.

It is conceivable that erythrocyte BPGM in diabetic patients is glycated, but possibly not as extensively as was observed for the intensive 7-day *in vitro* incubation with very high levels of glucose (1 M), which results in extensive glycation at all potential sites. No enzymatic activity was detected for the *in vivo* glycated form. Therefore, this indicates that the loss of enzymatic activity by glycation of

BPGM both *in vitro* and *in vivo* is due to glycation of the lysine residue at position 158. Lys158, which is the glycated site, must be located in an important catalytic region, and the glycation of this residue must affect enzymatic activity by influencing substrate binding or the conformation of the enzyme. From the structure of yeast phosphoglycerate mutase, it is likely that Lys158 is located in the vicinity of positively charged residues, arginine residues at positions 89, 115, and 192, the entrance of the active site which may serve as a binding site for substrates (*40*).

In conclusion, the present findings provide evidence that BPGM activity in erythrocytes of diabetic patients decreases as the result of glycation and that the loss of activity by glycation of BPGM both *in vitro* and *in vivo* is due to glycation of the lysine residue at position 158. Three enzymatic activities of BPGM play an important role in the regulation of 2,3-DPG and in this study all three activities were decreased as a result of glycation. Therefore glycation of BPGM is likely to be one of the determining factors for the controversial fluctuation of the levels of 2,3-DPG in diabetic conditions. A complete three-dimensional structure of BPGM will be useful in the explanation of the mechanisms and extent of inactivation of the three activities of BPGM by glycation.

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