Magnesium-dependent Phosphatase-1 Is a Protein-Fructosamine-6-phosphatase Potentially Involved in Glycation Repair*  

Received for publication, December 12, 2005, and in revised form, April 20, 2006 Published, JBC Papers in Press, May 1, 2006, DOI 10.1074/jbc.MS13208200

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Fructosamine-3-kinase (FN3K) is a recently described protein-repair enzyme responsible for the removal of fructosamines, which are the products of a spontaneous reaction of glucose with amines. We show here that, compared with glucose, glucose 6-phosphate (Glu-6-P) reacted 3–6-fold more rapidly with proteins and 8-fold more rapidly with N-α-t-Boc-lysine, being therefore a more significant intracellular glycating agent than glucose in skeletal muscle and heart. Fructosamine 6-phosphates, which result from the reaction of amines with Glu-6-P, were not substrates for FN3K. However, a phosphatase that dephosphorylates protein-bound fructosamine 6-phosphates was found to be present in rat tissues. This enzyme was purified to near homogeneity from skeletal muscle and was identified as magnesium-dependent phosphatase-1 (MDP-1), an enzyme of the haloacid dehalogenase family with a putative protein-tyrosine phosphatase function. Human recombinant MDP-1 acted on protein-bound fructosamine 6-phosphates with a catalytic efficiency >10-fold higher than those observed with its next best substrates (arabinose 5-phosphate and free fructoselysine 6-phosphate) and >100-fold higher than with protein-phosphotyrosine. It had no detectable activity on fructosamine 3-phosphates. MDP-1 dephosphorylated up to ~75% of the fructosamine 6-phosphates that are present on lysozyme after incubation of this protein with Glu-6-P. Furthermore, lysozyme glycated with Glu-6-P was converted by MDP-1 to a substrate for FN3K. We conclude that MDP-1 may act physiologically in conjunction with FN3K to free proteins from the glycation products derived from Glu-6-P.

Aldoses spontaneously react with amino groups of proteins and of low M, compounds to form Schiff bases, which slowly undergo an Amadori rearrangement and thereby become ketoamines. This non-enzymatic reaction of amines with sugars, known as glycation, takes place with all reducing sugars, and glucose, the most abundant aldose in tissues, is usually considered as the most important glycating agent under physiological conditions. However, glucose, which gives rise to fructosamines, is intrinsically much less reactive than other monosaccharides such as ribose, galactose, and mannose (1) or sugar phosphates such as ribose 5-phosphate (2, 3), and glucose 6-phosphate (Glu-6-P)3 (4–6).

Fructosamines are partially removed from proteins as a consequence of their phosphorylation by fructosamine-3-kinase (FN3K). This enzyme phosphorylates accessible fructosamines on their third carbon, leading to their destabilization and spontaneous shedding from the protein surface (7–9). The physiological occurrence of deglycation has been proven by showing that protein-bound fructosamines are present at higher concentrations in tissues and erythrocytes of FN3K-deficient mice than of control mice.4 FN3K is present in many tissues including, intriguingly, skeletal muscle and heart (10), where the intracellular glucose concentration is more than 10-fold lower than in plasma (11–14). Furthermore, FN3K deficiency is accompanied by an increase in protein-bound fructosamines in skeletal muscle.5 These observations suggest that fructosamines may be formed from another glycating agent than glucose. A logical candidate is Glu-6-P, a ubiquitous intracellular glycolytic intermediate, which is known to be a more powerful glycating agent than glucose (4–6).

In the present work, we have measured the glycation rate of Glu-6-P with two proteins and with N-α-t-Boc-lysine and concluded that, at its prevailing intracellular concentration in skeletal muscle and heart, this aldose phosphate is a more significant glycating agent than glucose. We explored the possibility that a deglycation mechanism exists to remove fructosamine 6-phosphates (FN6P) from protein. As protein-bound FN6P were not substrates for FN3K, we looked for an enzyme that converts them to fructosamines. A phosphatase carrying out this reaction was detected in rat tissues, characterized, and identified. The purpose of the present article is to report these findings.

EXPERIMENTAL PROCEDURES

Materials—Reagents, of analytical grade whenever possible, were from Sigma, Acros, or Merck. DEAE-Sepharose, Q-Sepharose, Blue-Sepharose, Sephacryl S-200, NAP-5, PD-10, [γ-32P]ATP, and [U-13C]glucose were purchased from Amersham Biosciences; Biogel P2 and AG 50W-X4 (100–200 mesh) were from Bio-Rad. Dowex 1-X8-200 was purchased from Acros, and Vivaspin-2 centrifugal concentrators were from Roche Molecular Biochemicals, and v-abl protein-tyrosine kinase was from Calbiochem. Fructoselysine 6-phosphate, fructosevaline 6-phosphate, Escherichia coli fructoselysine-6-kinase (FL6K), and Bacillus subtilis fructosamine-6-kinase were prepared as previously described (15–17).

The abbreviations used are: Glu-6-P, glucose 6-phosphate; BSA, bovine serum albumin; FL6K, fructoselysine-6-kinase; FN3K, fructosamine-3-kinase; FN6P, fructosevaline 6-phosphate; FN6Pase, fructosamine-6-phosphatase; MDP-1, magnesium-dependent phosphatase-1; M6P, 4-morpholineethanesulfonic acid.

1 This work was supported in part by grants from the European Foundation for the Study of Diabetes, the Juvenile Diabetes Foundation, the Interuniversity Attraction Poles Programme-Belgian Science Policy, and the Actions de Recherches Concertées for the Agricultural Development.  
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3 The abbreviations used are: Glu-6-P, glucose 6-phosphate; BSA, bovine serum albumin; FL6K, fructoselysine-6-kinase; FN3K, fructosamine-3-kinase; FN6P, fructosevaline 6-phosphate; FN6Pase, fructosamine-6-phosphatase; MDP-1, magnesium-dependent phosphatase-1; M6P, 4-morpholineethanesulfonic acid.

4 M. Veiga da Cunha and E. Van Schaftingen, manuscript submitted for publication.
Phosphate was purified on AG 50W-X4 (H+) and the sample was diluted 5-fold with water. Fructoselysine 6-phosphate was eluted with 6 ml of 1 M NaCl and desalted on Biogel P2. The other compounds were eluted with 3 ml of 0.1 M NaCl and 6 ml of water. After washing, fructoselysine 6-phosphate was eluted with 6 ml of 1 M NaCl, and desalted on Biogel P2.

To prepare [32P]hexitotamine 6-phosphates, BSA-[32P]FN6P was incubated in 1 ml of a medium containing 50 mM Tris-Cl, pH 8, and 50 mM NaBH4. After 20 h at 4 °C, 80 μl of 1 N HCl was added to destroy the remaining borohydride, and the sample was gel-filtered on Biogel P2.

Low M, 32P-labeled substrates were synthesized with 0.1 mM concentration of the corresponding unphosphorylated compound under the general conditions described above. Fructoselysine was phosphorylated with E. coli FL6K (0.3 units), fructoselysine and fructosegycine were phosphorylated with B. subtilis fructosamine-6-kinate (0.3 units), ribose, and arabinose, respectively, with 5 and 25 milliunits of human recombinant ribokinase, and fructose and glucose were phosphorylated with yeast hexokinase (0.3 units). In all cases, the reaction was stopped by the addition of 100 μl of ice-cold HClO4, washed with 2% (w/v) HClO4, and the proteins were resuspended in 0.5 ml of water and neutralized with KHCO3. After neutralization with KHCO3, the salts were removed by centrifugation, and the sample was diluted 5-fold with water. Fructoselysine 6-phosphate was purified on AG 50W-X4 (H+ form; 1 cm3), equilibrated with water. After washing, fructoselysine 6-phosphate was eluted with 6 ml of 1 mM NaCl and desalted on Biogel P2. The other compounds were purified on Dowex 1-X8 (Cl- form; 1 cm3), equilibrated with water. The column was washed with water (3 ml), and the elution was performed with 2 ml of 0.5 M NaCl.

Proteins glycated with [32P]Glu-6-P were prepared by incubating, for 1 day at 50 °C, hen egg lysozyme or BSA (60 mg/ml) in 1 ml of a solution containing 0.5 mM Glu-6-P and 15.106 cpm [32P]Glu-6-P. 32P-Labeled proteins were purified by biogel filtration on Dowex 1-X8 (see above). The reaction was incubated with 2% (w/v) HClO4, washed with 2% (w/v) HClO4, and the proteins were resuspended in 0.5 ml of water and neutralized with KHCO3.

The preparation of [U-14C]Glucose and Preparation of [U-14C]Glucose—[U-14C]Glucose was phosphorylated at the 1-C position with Mg-ATP. For the preparation of protein-[14C]FN6P, 15 μg of glycated BSA was incubated with 7 units of E. coli FL6K. To prepare protein-bound [32P]fructosamine-3-phosphates, glycated albumin was incubated with 0.1 milliunits of FN3K. Protein-[32P]phosphotrosine was prepared by phosphorylation of 1 mg of myelin basic protein with 100 units of mouse v-abl tyrosine kinase according to the manufacturer’s instructions. The samples were then diluted with water (to 0.5 ml), applied on Dowex 1-X8 columns (Cl− form; 1 cm3) equilibrated with water. The columns were washed with water (1 ml) to elute the [32P]-labeled proteins, free from unreacted ATP, which was retained on the anion-exchanger. To prepare [32P]hexitotamine 6-phosphates, BSA-[32P]FN6P was incubated in 1 ml of a medium containing 50 mM Tris-Cl, pH 8, and 50 mM NaBH4. After 20 h at 4 °C, 80 μl of 1 N HCl was added to destroy the remaining borohydride, and the sample was gel-filtered on Biogel P2.

Low M, 32P-labeled substrates were synthesized with 0.1 mM concentration of the corresponding unphosphorylated compound under the general conditions described above. Fructoselysine was phosphorylated with E. coli FL6K (0.3 units), fructoselysine and fructosegycine were phosphorylated with B. subtilis fructosamine-6-kinate (0.3 units), ribose, and arabinose, respectively, with 5 and 25 milliunits of human recombinant ribokinase, and fructose and glucose were phosphorylated with yeast hexokinase (0.3 units). In all cases, the reaction was stopped by the addition of 100 μl of ice-cold HClO4, washed with 2% (w/v) HClO4, and the proteins were resuspended in 0.5 ml of water and neutralized with KHCO3.

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Repurification of [U-14C]Glucose and Preparation of [U-14C]Glucose—[U-14C]Glucose was phosphorylated as described previously (17), with some modifications. Briefly, 0.1 mCi (0.3 μmol) of [U-14C]glucose was incubated at 37 °C in 1.5 ml of a solution containing 25 mM Hepes, pH 7.1, and 60 mg/ml BSA. After 4 h, the mixture was gel-filtered on a PD-10 column. The resulting [U-14C]glucose (3.2 ml) was phosphorylated (for 20 min with 0.3 units/ml of yeast hexokinase and 80 μM Mg-ATP) and [U-14C]Glu-6-P was purified on Dowex 1-X8 (see above). A portion (0.5 ml) was incubated for 1 h with 10 μg of alkaline phosphatase. The reaction was stopped by adding HClO4, the sample was centrifuged, and the supernatant was neutralized. It was verified that no [U-14C]Glu-6-P remained in the preparation.

5 E. Van Schaftingen, unpublished results.

This purification of [U-14C]glucose, involving its enzymatic phosphorylation and the isolation of Glu-6-P, was found necessary to eliminate completely a radiochemical impurity of unknown structure that reacts more rapidly than glucose and causes non-linear kinetics of incorporation of radioactivity into proteins, as previously reported by others (17, 18).

Incubation of Proteins with Labeled Glucose or Glucose 6-Phosphate—Solutions containing 60 mg/ml of protein (hen egg lysozyme or BSA), 50 mM Hepes, pH 7.1, 1 mM EGTA, 0.5 mM AlF3 (to inhibit phosphatases that otherwise slowly hydrolyze Glu-6-P), 100 units/ml penicillin, 100 μg/ml streptomycin, 106 cpm (repurified) [U-14C]glucose or [U-14C]Glu-6-P, and 10 or 20 mM unlabeled compound were filtered on a 0.22-μM membrane and incubated at 37 °C. At the indicated times, aliquots were taken and spotted on 3MM papers, which were immersed in ice-cold 2% (w/v) trichloroacetic acid. The papers were then washed three times in diluted trichloroacetic acid and counted for radioactivity. The proportion of radioactivity present on the paper, corresponding to the glucose or Glu-6-P incorporated into protein, was used to calculate the glycation level.

Incubation of N-α-t-Boc-lysine with Glucose or Glucose 6-Phosphate—Solutions containing 0.2 M N-α-t-Boc-lysine, 50 mM Hepes, pH 7.1, 100 mM NaCl, 1 mM EGTA, and different concentrations of glucose or Glu-6-P were filter-sterilized and incubated at 37 °C for 2 days. The t-Boc group was removed by overnight incubation in 1 N HCl at room temperature, and the samples were neutralized with NaOH. To separate fructosamine 6-phosphate from unreacted Glu-6-P, samples (70 μl) were diluted 15-fold with 10 mM MES, pH 6, and loaded onto Dowex 1-X8 columns (Cl− form, 1 cm3) equilibrated with the same buffer, which were washed with 1.5 ml of MES buffer. Fructoselysine and fructoselysine 6-phosphate were assayed as described (15).

Preparation of Tissue Extracts and Purification of Fructosamine-6-phosphatase—Rat tissue homogenates and erythrocyte lysates were prepared as described previously (10). For the purification of F6Pase, rat skeletal muscle (130 g) was homogenized in an Ultra Turrax with 260 ml of buffer B (25 mM Hepes, pH 7.1, 1 mM dithiothreitol, 2 μg/ml leupeptin, and 2 μg/ml antipain), and the homogenate was centrifuged for 15 min at 11,000 × g. The supernatant (250 ml) was diluted 2-fold with buffer B and applied to a DEAE-Sepharose column (200 cm3) equilibrated with the same buffer. The column was washed with 400 ml of buffer B and developed with a 0.2–0.5 M NaCl gradient in 100 ml of buffer B. The most active fractions were pooled (32 ml), brought to 200 ml with buffer C (20 mM Tris-Cl, pH 8, 1 mM dithiothreitol, 2 μg/ml leupeptin, and 2 μg/ml antipain), and applied to a Q-Sepharose column (12 cm3) equilibrated with buffer C containing 10 mM NaCl. The column was washed with 25 ml of equilibration buffer and developed with a 0–100 mM NaCl gradient in 250 ml of buffer B. The most active fractions were pooled (12 ml), and a portion (3 ml) was kept for the kinetic studies and desalted on NAP-5 columns. The remaining 9 ml were concentrated to 0.5 ml (with Vivaspin-2) and applied to a Sephacryl S-200 column (70 cm3) equilibrated with buffer B containing 100 mM NaCl. Fractions of 1 ml were collected. All purification steps were performed at 4 °C, and the preparation was stored at −70 °C between steps. Protein was assayed as described (19) using bovine γ-globulin as a standard.

The band co-eluting with the F6Pase activity in the last purification step was cut out of a 12% (w/v) polyacrylamide–SDS gel and digested with trypsin. Peptides were analyzed by nanoelectrospray-ionization tandem mass spectrometry as previously described (20).
**Expression and Purification of Human Magnesium-dependent Phosphatase**—The open reading frame of human MDP-1 (accession number Q86V88) was PCR-amplified using Pwo polymerase, human liver cDNA as the template, a 5′-primer containing the initiator codon (GACATGCGCGGCTACCGAAGGC) in an Ndel site (in bold) and a 3′-primer containing the putative stop codon (GGATCCCCCGGTCTCAAAATTGCTTC) flanked by a BamHI site (in bold). The 540-bp PCR product was subcloned into pBlueScript and checked by sequencing. A Ndel-BamHI fragment was removed from pBlueScript and inserted into pET-3a, which was used to transform E. coli BL21(DE3)pLysS (21). The expression and preparation of bacterial extracts were performed as described previously (15). The extract (25 ml) resulting from a 500-ml culture was diluted 3-fold with buffer B and applied to a 25 cm³ DEAE-Sepharose column, equilibrated with the same buffer, which was then washed with 25 ml of buffer B. The flow-through and washing of this column were loaded onto a Blue-Sepharose column (12 cm³) equilibrated with buffer B. The column was washed with 20 ml of the same buffer and developed with a linear NaCl gradient (0–0.5 M in 60 ml of buffer B). Fractions of 3 ml were collected. The fractions containing MDP-1, as determined by SDS-PAGE analysis, were supplemented with 10% (w/v) glycerol and stored at −70 °C. Protein concentration was estimated by measuring A_{280} assuming that ε = 16,500 M⁻¹ cm⁻¹.

**Assay of Phosphatase Activity**—All enzymatic assays were performed for 10 min at 30 °C in a medium containing 25 mM Hepes, pH 7.1, 25 mM KCl, 1 mM MgCl₂, 0.1 mg/ml BSA, and the indicated concentration of substrate. p-Nitrophenyl-phosphatase activity was measured spectrophotometrically by following A_{405} using ε = 8000 M⁻¹ cm⁻¹ to calculate the concentration of p-nitrophenol. The activities on other unlabeled compounds were determined in 40 µl and stopped by adding 160 µl of 10 mM HCl, and inorganic phosphate was assayed as described in Ref. 22. Activities on §P-labeled compounds were determined in 40 µl of a solution containing 0.1 mM of substrate (16,000 cpm). The reactions were stopped by the addition of 1 mg of BSA (as a carrier) and 200 µl of ice-cold 10% (w/v) trichloroacetic acid. The samples were centrifuged for 10 min at 10,000 × g and [§P]inorganic phosphate was isolated as a phosphomolybdic complex (23), free from phosphate esters, before being counted. Unless otherwise stated, FN6Pase activity is measured using BSA-[§P]FN6P as a substrate (tested at 0.1 of µM phospho groups).

**Phosphorylation by FN3K of the Dephosphorylation Products of MDP-1**—Hen egg lysozyme (60 mg/ml) was incubated at 37 °C with Glu-6-P (10, 20 or 40 mM) or glucose (100 or 200 mM). After 8 days, the samples were purified on Dowex 1-X8 (samples containing Glu-6-P) and/or Bio-gel P2, and the concentration of lysozyme was assayed (24). The samples (1 mg of lysozyme) were incubated in 0.1 ml of a solution containing 25 mM Hepes, pH 7.1, 1 mM MgCl₂, and 40 µg/ml MDP-1. After 1 h, the incubation medium of FN3K was added (1 mM EGTA, 10 µM Mg-ATP, 10⁶ cpm [γ-§P]ATP, and 0.5 milliliters of mouse recombinant FN3K, in a final volume of 0.15 ml). After the indicated times, aliquots (18 µl) were spotted on P81 papers, washed, and counted for radioactivity as described previously (8).

**RESULTS**

**Glycation by Glucose and Glucose 6-Phosphate**—Several experiments were performed to compare the rate of glycation by glucose and Glu-6-P. We first measured the rate of incorporation of repurified [U-¹⁴C]glucose (see “Experimental Procedures”) and [U-¹⁴C]Glu-6-P into two distinct proteins (hen egg lysozyme and BSA) in the presence of two different concentrations of the unla-

![Figure 1](https://example.com/figure1.png)

**FIGURE 1.** Glycation of proteins with glucose or glucose 6-phosphate. Hen egg lysozyme (A) or bovine serum albumin (B) were incubated, at neutral pH and 37 °C, with [U-¹⁴C]glucose (squares and triangles) or [U-¹⁴C]Glu-6-P (circles and diamonds) and the indicated concentrations of unlabeled compound. After different times, samples were spotted on 3MM papers and immersed in trichloroacetic acid to precipitate proteins. The radioactivity bound to the paper was used to calculate the glycation level of proteins. Means ± S.E. (n = 3).

beled sugar or sugar phosphate. As shown in Fig. 1, the incorporation of glucose and Glu-6-P was first order with respect to the sugar concentration and nearly proportional with time. It was about 6-fold more rapid with Glu-6-P than with glucose in the case of lysozyme and ~3-fold more rapid in the case of albumin.

To compare the intrinsic reactivity of glucose and Glu-6-P, without interference of the protein environment, we also compared their rate of reaction with N-α-β-Boc-lysine (preferred to lysine to avoid interference by glycation of the α-amine) by measuring the formation of fructoselamine and FL6P derivatives with a specific enzymatic assay. This formation, which was proportional with time over at least 10 days, was evaluated after 2 days. As shown in Fig. 2, it was proportional with the concentration of glycatying agent and was ~8-fold more rapid with Glu-6-P than with glucose.

**Purification of a Phosphatase Acting on Protein-bound Fructosamine 6-Phosphates**—As will be shown later in this paper, FN6Pase is not substrates for FN3K. Glu-6-P-glycation products will therefore not be phosphorylated by FN3K unless the phosphate is removed from the sixth carbon of FN6P. To search for a FN6Pase, we prepared BSA-[§P]FN6P, by incubating glycated BSA with [γ-§P]ATP and E. coli FL6K. Incubation of this substrate with rat tissue extracts disclosed the formation of a radioactive compound that was no longer precipitated with acid and corresponded to [§P]inorganic phosphate. This phosphatase activity was found to be widely distributed in tissues (Fig. 3). Upon chromatography of extracts from skeletal muscle (Fig. 4), brain, and red blood cells (not shown) on DEAE-Sepharose, FN6Pase was eluted as a
MDP-1 Is a Protein-Fructosamine-6-phosphatase

in the presence of 0.5, 1, and 2 mM MgCl₂, respectively (not shown). Unlike protein-serine phosphatases of type I and type IIA (26), FN6Pase was not affected by okadaic acid (tested at 5 μM; not shown). These data agreed with the identification of FN6Pase as a member of the haloacid dehalogenase family.

Expression and Characterization of Recombinant Human MDP-1—To confirm the identity of FN6Pase, human MDP-1 was overexpressed in E. coli and purified to homogeneity by chromatography on DEAE-Sepharose and Blue-Sepharose (not shown). About 14 mg of pure protein was produced from a 500-ml culture. The purified enzyme catalyzed the dephosphorylation of BSA-[³²P]FN6P with a specific activity of 8.3 nmol/min/mg of protein at 0.1 μM of phospho groups.

We next investigated the ability of MDP-1 to dephosphorylate low M₄ FN6Ps and compared it to its action on known substrates. As shown in Table 2, both free FL6P and fructosevaline 6-phosphate were substrates for this enzyme, with Kₘ and Vₘₐₓ values of the same order of magnitude as observed with arabinose 5-phosphate, the best low M₄ substrate yet identified for MDP-1 (27). Catalytic efficiency values (kₐₑₚ/Kₘ) for the two FN6Ps were slightly lower than for arabinose 5-phosphate (a non-physiological compound in animals), but significantly higher than those observed with ribose 5-phosphate, fructose 6-phosphate, and the phosphotyrosine analog p-nitrophenyl phosphate.

Attempts to determine Kₘ and Vₘₐₓ values for BSA-[³²P]FN6P failed, because the rate of the reaction was strictly first order with respect to the concentration of this substrate up to the highest concentrations that we could test (6 μM). To compare the ability of MDP-1 to dephosphorylate low M₄ and protein-bound FN6Ps, we therefore tested all substrates at the same low concentration (0.1 μM). As this concentration is much lower than the Kₘ values, the activity observed with each of the substrates is proportional to the catalytic efficiency of the enzyme for that substrate. As shown in Fig. 6, BSA-FN6P was at least 10 times better than the next best substrate, arabinose 5-phosphate. The order of dephosphorylation of the other substrates was consistent with the catalytic efficiencies calculated from the Kₘ and Vₘₐₓ values in Table 2.

Fig. 6 also shows that protein-bound fructosamine 3-phosphates were not detectably dephosphorylated by MDP-1, which indicates that this enzyme does not antagonize the action of FN3K. No activity of MDP-1 was observed on protein-bound hexitolamine 6-phosphates (obtained by reducing BSA-FN6P) indicating the importance of the ketone function on carbon 2 of the fructose moiety.

As MDP-1 has been proposed to be a protein-tyrosine phosphatase (28), we tested also its action on protein-bound phosphoryrosines, pre-

![Figure 2](image2.png)

**FIGURE 2. Glycation of N-acetyl-3-boc-lysine with glucose or fructose 6-phosphate.** N-acetyl-3-boc-lysine (0.2 nM) was incubated at pH 7.1 and 37 °C, with the indicated concentrations of glucose or Glu-6-P. After 48 h, the n-Boc group was removed, and fructoselysine or glycated products were analyzed by tandem mass spectrometry. This allowed the identification of a major band co-eluting with the authentic sample.

![Figure 3](image3.png)

**FIGURE 3. Total tissue distribution of fructosamine-6-phosphatase.** The enzymatic activity was determined on crude extracts, using BSA-[³²P]FN6P as a substrate and is related to the total amount of protein in the extract. Means ± S.E. (n = 3).

![Figure 4](image4.png)

**FIGURE 4. Purification of fructosamine-6-phosphatase from rat skeletal muscle.** A rat skeletal muscle extract (250 ml) was applied to a DEAE-Sepharose column and a linear NaCl gradient was applied. A₂₈₀ and protein-FN6Pase activity (with BSA-[³²P]FN6P as a substrate) were measured.
pared by phosphorylation of myelin basic protein with the tyrosine kinase \( \text{v-abl} \). As shown in the inset of Fig. 6, this substrate, also tested at a concentration of phospho groups of 0.1 \( \mu \text{M} \), was dephosphorylated 200-fold more slowly than BSA-FN6P.

Dephosphorylation by MDP-1 of Proteins Glycated with Glucose 6-Phosphate—For reasons of convenience, the protein-FN6P substrate that had been used until this stage of our study had been prepared by phosphorylating protein-bound fructosamines with \( \text{E. coli FL6K} \). As it is likely that only the most accessible fructosamines have been phosphorylated in this way, this substrate was presumably not totally comparable to more physiological substrates, produced through the spontaneous reaction of Glu-6-P with proteins, in which less accessible FN6P might also arise. In the experiment shown in Fig. 7, we have compared the ability of MDP-1 to dephosphorylate protein-[\(32\text{P}\)]FN6P and MDP-[\(32\text{P}\)]phosphotyrosine with the indicated concentration of MDP-1. Means \( \pm \text{S.E. (n = 3).} \)

### TABLE 1

| Step          | Volume | Protein | Total activity | Specific activity | Purification | Yield |
|---------------|--------|---------|----------------|------------------|--------------|-------|
| Homogenate    | 200    | 14,520  | 700            | 0.05             | 1            | 100   |
| DEAE-Sepharose| 25.6   | 1.38    | 260            | 1.9              | 41           | 39    |
| Q-Sepharose   | 9.6    | 9.6     | 210            | 22.3             | 483          | 32    |
| Sephacryl S-200 | 3      | 0.3     | 37             | 129              | 2,792        | 6     |

*a One unit of FN6Pase is the amount of enzyme that catalyzes the dephosphorylation of 1 pmol of BSA-[\(32\text{P}\)]FN6P/min in the presence of 0.1 \( \mu \text{M} \) of phospho groups.

### TABLE 2

| Substrate | \(K_m\) (mM) | \(V_{max}\) (\(\mu\text{mol/min/mg of protein}\)) | \(k_{cat}/K_m\) (s\(^{-1}\)) |
|-----------|--------------|-----------------------------------------------|-------------------------------|
| p-Nitrophenylphosphate | 23.5 | 19.6 | 0.3 |
| Fructose 6-phosphate | 15.7 | 8.6 | 0.2 |
| Ribose 5-phosphate | 17.5 | 46.4 | 0.9 |
| Arabinose 5-phosphate | 6.8 | 93.1 | 4.6 |
| Fructoselysine 6-phosphate | 5.7 | 45.9 | 2.7 |
| Fructosevaline 6-phosphate | 7.8 | 40.8 | 1.8 |

FIGURE 6. Comparison of the activity of MDP-1 on substrates tested at a concentration much lower than \(K_m\). The activity of human recombinant MDP-1 was measured with the indicated \(32\text{P}\)-labeled compounds (tested at 0.1 \(\mu\text{M}\) of phospho groups). In the inset, time course of dephosphorylation of BSA-[\(32\text{P}\)]FN6P and MBP-[\(32\text{P}\)]phosphotyrosine with the indicated concentration of MDP-1. Means \( \pm \text{S.E. (n = 3).} \)

E. coli FL6K. As it is likely that only the most accessible fructosamines have been phosphorylated in this way, this substrate was presumably not totally comparable to more physiological substrates, produced through the spontaneous reaction of Glu-6-P with proteins, in which less accessible FN6P might also arise. In the experiment shown in Fig. 7, we have compared the ability of MDP-1 to dephosphorylate protein-[\(32\text{P}\)]FN6P substrates prepared either by phosphorylating glycated BSA with FL6K and [\(\gamma-32\text{P}\)]ATP or by allowing lysozyme or BSA to react with [\(32\text{P}\)]Glu-6-P. The first type of substrate was readily and completely dephosphorylated, to an extent of \(\sim 75\%\) in the case of lysozyme and \(50\%\) in the case of albumin, even though much higher levels of MDP-1 were used.
MDP-1 Is a Protein-Fructosamine-6-phosphatase

FORMATION OF A SUBSTRATE FOR FN3K FOLLOWING GLYCATION BY GLUT-6-P AND DEPHOSPHORYLATION BY MDP-1—We also verified that MDP-1 produced a substrate for FN3K when acting on protein glycated with Glu-6-P. To this end we incubated lysozyme with different concentrations of glucose or Glu-6-P for 8 days at 37 °C, then treated the preparations with MDP-1 and submitted them to phosphorylation by FN3K and radiolabeled ATP. As shown in Fig. 8, lysozyme glycated with Glu-6-P was not detectably more phosphorylated by FN3K than control lysozyme (which presumably contains a small amount of fructosamines), indicating that protein-FN6Ps are not substrates for FN3K. When lysozyme glycated with Glu-6-P was treated with MDP-1, it was converted to a substrate for FN3K and was phosphorylated by this enzyme almost to the same extent as lysozyme incubated with a 5-fold higher glucose concentration. No effect of MDP-1 treatment on subsequent phosphorylation by FN3K was observed with lysozyme glycated with glucose.

DISCUSSION

Glucose 6-Phosphate, a Significant Glycating Agent—Glucose, as the main reducing sugar present in tissues, is usually considered as the principal glycating agent in vivo. However, this hexose is intrinsically less reactive than other physiological aldoses or aldose phosphates. In the present work we studied the rate of glycation by Glu-6-P, one of the most abundant reducing sugars in tissues after glucose (29). Results based on the incorporation of radioactivity into protein, on the formation of N-α-t-Boc-fructoselysine or fructoselysine 6-phosphate (as determined with a specific enzymatic assay) and on the formation of a protein substrate for FN3K (see Fig. 8) all concur to indicate that Glu-6-P reacts more rapidly with proteins than glucose. With N-α-t-Boc-lysine, the difference amounted to 8-fold, whereas with two proteins, lysozyme and albumin, it amounted to 6- and 3-fold, respectively. A 3-fold difference was previously reported for lens crystallins (6) and a ≥ 20-fold difference in the case of hemoglobin (4, 5). Thus, a higher reactivity of Glu-6-P than of glucose in the glycation reaction appears to be the rule. However, the magnitude of the difference in reactivity depends on the “glycatable” substrate used, presumably because of charge effects. For instance, the higher difference observed with lysozyme as compared with albumin is possibly because of the fact that the first protein is intrinsically more basic (pl = 9.3) than the latter (pl = 5.8). The much higher reactivity of Glu-6-P (compared with glucose) observed with human hemoglobin is likely because of the fact that the main glycation sites in this protein (the N-terminal valines of the β-chains) are surrounded by a cluster of positive charges (His-β-2, His-β-143, and Lys-β-82, which normally form the binding site for 2,3-bisphosphoglycerate).
**MDP-1 Is a Protein-Fructosamine-6-phosphatase**

The intracellular glucose concentration, which, in liver and human erythrocytes, is about the same as that in the plasma, is much lower in skeletal muscle and heart (12–14), two tissues in which the FN3K specific activity is among the highest ones (10). It is difficult to have a precise estimate of the intracellular glucose concentration in skeletal muscle, because this concentration is determined as the difference between total tissue glucose and extracellular glucose, and the latter represents by far the major pool. A recent NMR study indicates a value that is not different from 0 mM in normoglycemic subjects and of only 0.1 mM in subjects in which the blood glucose is clamped at \( \text{\sim} 10 \text{mM} \). By contrast, the Glu-6-P concentration in muscle and heart ranges from 0.1 to 0.3 mM (11, 30–32), although much higher concentrations (up to 0.6 mM) have been observed, for instance in contracting muscle (33). Thus, taking into account its higher glycating power, Glu-6-P is a more significant glycating agent than glucose in skeletal muscle and probably also in heart.

**Identification of a Protein-Fructosamine-6-phosphatase**—The considerations made above indicate that it could make sense to have a protein-repair mechanism allowing the removal of FN6Ps. As the latter are not substrates for FN3K, we looked for a phosphatase that would convert them to fructosamines. We found such an enzyme in several tissues, purified it substantially, and identified it as MDP-1. This identification was confirmed by showing that recombinant MDP-1 acted as a protein-FN6Pase, with properties similar to those of the purified muscle enzyme.

MDP-1 was initially identified as a phosphatase that co-purified with carbonic anhydrase III (27). The identity of the physiological substrate for this enzyme remained unknown. Until the present work, its best known substrate was arabinose 5-phosphate, a non-physiological compound in vertebrates. MDP-1 also acts, with lower catalytic efficiencies, on physiological phosphate esters such as ribose 5-phosphate, 2-deoxyribose 5-phosphate, and fructose 6-phosphate, but the \( K_m \) values of MDP-1 for these substrates are more than 1000-fold higher than their tissue concentrations. Other ribose 5-phosphate derivatives such as nucleotides were found to be even poorer substrates (27).

Recent findings led to the proposal that MDP-1 is a new type of protein-tyrosine phosphatase. MDP-1 hydrolyzes free phosphotyrosine and \( p \)-nitrophenolphosphate (although with lower catalytic efficiencies than arabinose 5-phosphate). Furthermore, the crystal structure of MDP-1 shows that, unlike other members of the haloacid dehalogenase family (which generally act on low \( M_i \) substrates), it lacks the "lid" that closes the catalytic cleft on the substrate, suggesting that the physiological substrates of MDP-1 are macromolecules (28). Accordingly, MDP-1 was shown to act on protein-phosphotyrosine present in HeLa cell extracts (25). However, no quantification was given for this reaction, which, furthermore, was studied on a denatured substrate. Our results show that MDP-1 acts very poorly on phosphotyrosines present in myelin basic protein. In addition, prior data obtained by Selengut and Levine (27) indicated that MDP-1 acted at much lower (10–140 times) rates on phosphotyrosine-containing peptides than on free phosphotyrosine, whereas the reverse was true for PTP 1B, a \( \beta \)-glycerophosphate, nucleoside 2’- or 3’-monophosphates or phosphoinositides (27). The spontaneous removal of fructosamine 3-phosphates from proteins leads to their conversion to 3-deoxyglucosone and involves elimination of the phosphate group (a good leaving group) from the third carbon (9). Dephosphorylation of fructosamine 3-phosphates by MDP-1 (or any other phosphatase) would therefore prevent this spontaneous reaction from taking place. The specificity of MDP-1 and its ability to form a substrate for FN3K from proteins glycated with Glu-6-P make this phosphatase well suited to team up with FN3K for the removal of FN6Ps from proteins.

**Accessibility of Protein-bound Fructosamine-6-Phosphates to MDP-1**—A striking observation was the finding that MDP-1 readily and completely dephosphorylated protein substrates obtained by enzymatic phosphorylation of fructosamines (with FL6K), whereas it acted more slowly, and incompletely, on substrates obtained by incubation of proteins with Glu-6-P. This is certainly a consequence of the difference in accessibility of MDP-1 to the FN6Ps that are produced depending on the labeling method. It is indeed likely that only the most accessible fructosamine residues are phosphorylated by FL6K, because the physiological function of this enzyme is to phosphorylate free fructoselysine (15). By contrast, the spontaneous reaction of Glu-6-P with the amino groups of a protein may take place at a variety of sites, some of which may be accessible to low \( M_i \) compounds (in the present case, Glu-6-P), but not to larger molecules such as enzymes.

Similarly, the deglycating enzyme FN3K has access to some of the fructosamines that are formed when hemoglobin reacts with glucose (e.g., those on Lys-\( \alpha \)-16, Lys-\( \beta \)-17, Lys-\( \alpha \)-139), but not to others (Lys-\( \alpha \)-61, Val-\( \beta \)-1, Lys-\( \beta \)-66) (35). Conceivably, incompleteness of repair may be a feature of protein-repair mechanisms in general because they involve the action of enzymes on folded proteins.

Remarkably, MDP-1, at a concentration of 1 \( \mu \)g/ml, removed 50% of the phosphate present in lysozyme labeled with \( ^{32} \text{P} \)Glu-6-P in less than 1 h at 37°C and up to 75% at a 50-fold higher concentration of enzyme. The fact that different plateaus were observed with the two concentrations of enzyme indicates the presence of substrates with different degrees of accessibility. As the physiological concentration of MDP-1 in muscle is of the order of 1.5 \( \mu \)g/g (calculated by comparing the FN6Pase specific activity of purified MDP-1 with that of crude muscle extracts), it is likely that it is able to dephosphorylate a large share (50% or more) of the FN6P that are formed on intracellular proteins.

**Physiological Role of MDP-1**—Our reasons to believe that a physiological substrate for MDP-1 is protein-FN6P is that the latter is its best physiological substrate. Although the presence of FN6Ps has not yet been demonstrated in cells, their existence is the logical consequence of the fact that proteins spontaneously react with Glu-6-P. This reaction must therefore take place in vivo, and FN6Ps are expected to be elevated in pathological conditions, such as glycogen storage disease type I (glucose-6-phosphatase deficiency) and type VII (muscle phosphofructokinase deficiency, Tarui disease), where the Glu-6-P concentration is elevated (36). The fact that fructosamines are present in tissues where the glucose concentration is negligible and that their concentration is increased as a result of FN3K deficiency provides indirect evidence for the occurrence of a sequence of
events consisting of 1) glycation by Glu-6-P and 2) dephosphorylation by a phosphatase. Further studies using specific inhibitors of MDP-1 or gene inactivation are warranted to test this model.

Acknowledgments—We thank Dr. E. Wiame for providing fructosamine assay enzymes and A. Preumont for the kind gift of FN3K.

REFERENCES

1. Bunn, H. F., and Higgins, P. J. (1981) Science 213, 222–224
2. Sharma, S. D., Pandey, B. N., Mishra, K. P., and Sivakami, S. (2002) J. Biochem. Mol. Biol. Biophys. 6, 233–242
3. Sandwick, R., Johanson, M., and Breuer, E. (2005) Ann. N. Y. Acad. Sci. 1043, 85–96
4. Stevens, V. J., Vlassara, H., Abati, A., and Cerami, A. (1977) J. Biol. Chem. 252, 2998–3002
5. Haney, D. N., and Bunn, H. F. (1976) Proc. Natl. Acad. Sci. U. S. A. 73, 3534–3538
6. Stevens, V. J., Rouzer, C. A., Monnier, V. M., and Cerami, A. (1978) Proc. Natl. Acad. Sci. U. S. A. 75, 2918–2922
7. Delpierre, G., Collard, F., Fortpied, J., and Van Schaftingen, E. (2002) Biochem. J. 365, 801–808
8. Delpierre, G., Rider, M. H., Collard, F., Stroobant, V., Vanstapel, F., Santos, H., and Van Schaftingen, E. (2000) Diabetes 49, 1027–1034
9. Szewrogold, B. S., Howell, S., and Beiswenger, P. J. (2001) Diabetes 50, 2139–2147
10. Delplanque, J., Delpierre, G., Opperdoes, F. R., and Van Schaftingen, E. (2004) J. Biol. Chem. 279, 46606–46613
11. Roussel, R., Carlier, P. G., Robert, J. J., Velho, G., and Bloch, G. (1998) Proc. Natl. Acad. Sci. U. S. A. 95, 1313–1318
12. Katz, A., Nyomba, B. L., and Bogardus, C. (1988) Am. J. Physiol. 255, E942–E945
13. Ziel, F. H., Venkatesan, N., and Davidson, M. B. (1988) Diabetes 37, 885–890
14. Randle, P. J., Newsholme, E. A., and Garland, P. B. (1964) Biochem. J. 93, 652–665
15. Wiame, E., Delpierre, G., Collard, F., and Van Schaftingen, E. (2002) J. Biol. Chem. 277, 42523–42529
16. Wiame, E., Duquenne, A., Delpierre, G., and Van Schaftingen, E. (2004) FEBS Lett. 577, 469–472
17. Higgins, P. J., and Bunn, H. F. (1981) J. Biol. Chem. 256, 5204–5208
18. Trueb, B., Holenstein, C. G., Fischer, R. W., and Winterhalter, K. H. (1980) J. Biol. Chem. 255, 6717–6720
19. Bradford, M. M. (1976) Anal. Biochem. 72, 248–254
20. Achour, Y., Noel, G., Vertommen, D., Rider, M. H., Veiga-Da-Cunha, M., and Van Schaftingen, E. (2004) Biochem. J. 381, 35–42
21. Studier, F. W., and Moffatt, B. A. (1986) J. Mol. Biol. 189, 113–130
22. Selengut, J. D., and Levine, R. L. (2000) Biochemistry 39, 8315–8324
23. Peisach, E., Selengut, J. D., Dunaway-Mariano, D., and Allen, K. N. (2004) Biochemistry 43, 12770–12779
24. Williamson, D. H., and Bronson, J. T. (1974) in Enzymic Methods (Bergmeyer, H. U., ed) pp. 2266–2302, Academic Press, New York and London
25. Newsholme, E. A., and Randle, P. J. (1964) Biochem. J. 93, 641–651
26. Rothman, D. L., Shulman, R. G., and Shulman, G. I. (1992) J. Clin. Invest. 89, 1069–1075
27. Kashihara, Y., Sato, K., Tsuichiya, N., Thomas, S., Fell, D. A., Veech, R. L., and Passonneau, J. V. (1994) J. Biol. Chem. 269, 25502–25514
28. Price, T. B., Laurent, D., Petersen, K. F., Rothman, D. L., and Shulman, G. I. (2000) J. Appl. Physiol. 88, 698–704
29. Price, T. B., Laurent, D., Petersen, K. F., Rothman, D. L., and Shulman, G. I. (2000) J. Appl. Physiol. 88, 698–704
30. Tonks, N. K., Diltz, C. D., and Fischer, E. H. (1988) J. Biol. Chem. 263, 6722–6730
31. Delpierre, G., Vertommen, D., Communi, D., Rider, M. H., and Van Schaftingen, E. (2004) J. Biol. Chem. 279, 27613–27620
32. Chen, Y. T. (2001) in The Metabolic and Molecular Bases of Inherited Disease, 8th Edition (Scrivner, C. R., Beaudet, A. L., Sly, W. S., and Valle, D., eds) pp. 1521–1551, McGraw-Hill, New York