The Gene glvA of Bacillus subtilis 168 Encodes a Metal-requiring, NAD(H)-dependent 6-Phospho-α-glucosidase

ASSIGNMENT TO FAMILY 4 OF THE GLYCOSYLHYDROLASE SUPERFAMILY*

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The gene glvA (formerly glv-1) from Bacillus subtilis has been cloned and expressed in Escherichia coli. The purified protein GlvA (449 residues, M, 50,513) is a unique 6-phosphoryl-O-α-D-glucopyranosylphosphoglucohydrolase (6-phospho-α-glucosidase) that requires both NAD(H) and divalent metal (Mn2+, Fe2+, Co2+, or Ni2+) for activity. 6-Phospho-α-glucosidase (EC 3.2.1.122) from B. subtilis cross-reacts with polyclonal antibody to maltose 6-phosphate hydrolase from Fusobacterium mortiferum, and the two proteins exhibit amino acid sequence identity of 73%. Estimates for the M, of GlvA determined by SDS-polyacrylamide gel electrophoresis (51,000) and electrospray-mass spectroscopy (50,510) were in excellent agreement with the molecular weight of 50,513 deduced from the amino acid sequence. The sequence of the first 37 residues from the N terminus determined by automated analysis agreed precisely with that predicted by translation of glvA. The chromogenic and fluorogenic substrates, p-nitrophenyl-α-D-glucopyranoside 6-phosphate and 4-methylumbelliferol-α-D-glucopyranosyl-6-phosphate were used for the discontinuous assay and in situ detection of enzyme activity, respectively. Site-directed mutagenesis shows that three acidic residues, Asp41, Glu11, and Glu359 are required for GlvA activity. Asp41 is located at the C terminus of a βββ fold that may constitute the dinucleotide binding domain of the protein. Glu11 and Glu359 may function as the catalytic acid (proton donor) and nucleophile (base), respectively, during hydrolysis of 6-phospho-α-glucoside substrates including maltose 6-phosphate and trehalose 6-phosphate. In metal-free buffer, GlvA exists as an inactive dimer, but in the presence of Mn2+ ion, these species associate to form the NAD(H)-dependent catalytically active tetramer. By comparative sequence alignment with its homologs, the novel 6-phospho-α-glucosidase from B. subtilis can be assigned to the nine-member family 4 of the glycosylhydrolase superfamily.

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† The abbreviations used are: PEP-PTS, phosphoenol pyruvate-dependent sugar phosphotransferase system; PAGE, polyacrylamide gel electrophoresis; GlvA, 6-phospho-α-glucosidase; MalH, maltose 6-phosphate hydrolase; pNP-Glc6P, p-nitrophenyl-α-D-glucopyranoside 6-phosphate; 4MU-Glc6P, 4-methylumbelliferol-α-D-glucopyranoside 6-phosphate; kb, kilobase(s); PCR, polymerase chain reaction; MES, 2[N-morpholino]ethane sulfonic acid; βME, β-mercaptoethanol.

The serendipitous discovery in 1964 (1, 2) of the bacterial phosphoenol pyruvate-dependent sugar phosphotransferase system (PEP-PTS)† by Roseman and colleagues represents a landmark in our understanding of carbohydrate transport by microorganisms (3, 4). Since the initial description in Escherichia coli, this phosphoryl group-transfer system (5, 6) has been established as the primary mechanism for the accumulation of sugars by bacteria from both Gram-negative (7, 8) and Gram-positive genera (9–12). Operationally, the multi-component PEP-PTS (13) comprises two membrane-localized and cytoplasmic enzymes in concert catalyze the simultaneous phosphorylation and vectorial translocation of sugar across the cytoplasmic membrane. Catalytically, each PEP-PTS requires two general components (Enzyme I and HPr) that, allied with sugar-specific proteins (IIA, -B, and -C; for discussion, see Ref. 14), promote the sequential transfer of the high energy, phosphoryl moiety from PEP to the incoming sugar. Prior to catabolism via energy-yielding pathways, the intracellular di- or trisaccharide phosphates must first be hydrolyzed to their constituent hexose 6-phosphate and aglycone moieties. Several phosphoglycosyldrolases (whose genes are frequently encoded within PTS operons) have been purified, cloned, and sequenced. Particularly well characterized are the 6-phospho-β-galactosidases (EC 3.2.1.85; Refs. 15–21) and 6-phospho-β-glucosidases (EC 3.2.1.86; Refs. 22–30) that are included in family 1 of the glycosydrolase superfamily (31, 32).

In 1996, as participants in the Bacillus genome project (33), Sekiguchi and co-workers determined the nucleotide sequence of a 12.4-kb fragment of DNA near the 76° region of the Bacillus subtilis chromosome (34). Following translation of the ten open reading frames within this fragment, a search of the protein data bases revealed that the deduced amino acid sequences of open reading frames glv-1 and glv-2 exhibited similarity to 6-phospho-β-glucosidase and the IIC domain (GlvC) of the arubtin (PEP-PTS) of E. coli, respectively. It seemed likely, as suggested by Yamamoto et al. (34), that the products of glv-1 and glv-2 might participate in the transport and dissolution of β-glucosides in the Gram-positive spore-forming organism. Contemporaneous with the Bacillus studies in Japan, our pro-
gram at the National Institutes of Health was directed toward the purification and characterization of a metal-dependent maltose 6-phosphate hydrolase (MalH) from the anaerobic pathogen *Fusobacterium mortiferum* (35). This novel enzyme, together with an inducible maltose PEP-PTS permits growth of *F. mortiferum* on a wide variety of α-glucosides including maltose, α-methyl glucoside, trehalose, palatinose, and turanose (36). The 6-phospho-α-glucosidase gene (malH) has recently been cloned, sequenced, and expressed in *E. coli* (37). Remarkably, the deduced amino acid sequence of MalH exhibited 73% identity (88% similarity) to that deduced from the nucleotide sequence of *glv-1* in *B. subtilis*. These findings raised doubts concerning the initial classification and catalytic activity of the polypeptide encoded by *glv-1* (34).

In a collaborative program, we have addressed and resolved these issues. This communication describes the cloning, expression, and site-directed mutagenesis of *glv-1* (now designated *glvA*) from *B. subtilis*. Purification and characterization of the novel 6-phospho-α-glucosidase (GlvA) encoded by *glvA* was facilitated by the availability of the natural substrate for the enzyme (maltose 6-phosphate) and by chemical synthesis of the chromogenic and fluorogenic analogs pNPGlc6P and 4MUcGlc6P, respectively. In contrast to other phosphoglycosylhydrolases, GlvA from *B. subtilis* exhibits specific requirements for both divalent metal (Mn$^{2+}$, Fe$^{2+}$, Co$^{2+}$, or Ni$^{2+}$) and NAD(H) for activity. Furthermore, by sequence similarity and conservation of functionally important acidic residues, GlvA can be assigned to the nine-member family 4 of the glycosylhydrolase superfamily (31, 37).

**EXPERIMENTAL PROCEDURES**

**Materials and Reagents**

PD-10 gel filtration columns, isoelectric focusing standards, Amphotoline PAG plates (pH 3.5–9.0), DEAE-Sephacel, and phenyl-Sepharose CL-4B were purchased from Amersham Pharmacia Biotech. Utrengel AcA 44 and TrisAcryl DEAE-M were supplied by Sepracor. Enzymes, nucleotides, cofactors, and trehalose 6-phosphate were obtained from Sigma. Trimethylphosphate, phosphorus oxychloride, and cyclohexylamine were obtained from Aldrich. Pressure concentration cells and Diaflo FM-10 ultrafiltration membranes were from Amicon Corp. [U-15]Malose 6-phosphate was prepared enzymatically by PEP-dependent phosphorylation of the disaccharide by the maltose PEP-PTS in permeabilized cells of *F. mortiferum*. The radiolabeled disaccharide phosphate was purified by ion exchange chromatography, Ba$^{2+}$ and ethanol precipitation, and finally by paper chromatography (36). Chemical syntheses of pNPGlc6P, pNpMan6P, pNPGal6P, and 4MUcGlc6P were initiated with the commercially available non-phosphorylated glycosides (Sigma) using the procedure of Wilson and Fox (24). Selective phosphorylation at the C6 hydroxyl group of the nonreducing glucopyranose was achieved by use of a mixture of phosphorus oxychloride in trimethylphosphate containing small amounts of water. The phosphorylated derivatives were obtained as white, crystalline cyclohexylamine salts in 25–30% yield.

**Bacterial Strains, Plasmids, and Culture Conditions**

*B. subtilis* 168 and *E. coli* strains JM109 and XL1-Blue were grown in Luria-Bertani (LB) medium at 37 °C as described previously (38). When required, ampicillin was included in the medium at a final concentration of 50 μg/ml. *E. coli* plasmids pUC119 (Takara Shuzo Co., Kyoto) and a high expression vector pKP1500 (39) were used to construct a plasmid (pKPglv-1) containing *glvA*. Site-directed mutagenesis was carried out by the Pfu polymerase method (QuickChange site-directed mutagenesis kit, Stratagene). The desired mutations and the primers used to effect these changes are described in the text (Table IV).

**Primers and Sequence Analysis**

For the amplification of the gene *glvA* (also *glv-1* (33, 34)), two primers were synthesized: forward primer G1PFE, 5′-GCGCGATCTCATGAGAAAAATCATCCTCAA-3′ (the *glvA* sequence is italicized and the EcoRI site is underlined) and reverse primer GIPBR, 5′-GCGCGATCCCTGATTTGATCAGTTCTTCG-3′ (the sequence complimentary to the downstream region of *glvA* is italicized and the BamHI site is underlined). PCR amplification was performed with the GeneAmp PCR Core kit (Perkin Elmer) using 0.1 μg of *B. subtilis* 168 genomic DNA as template, 10 μl of 10 × reaction buffer, 8 μl of 25 mM MgCl$_2$, 2 μl each of 10 mM dNTP, 30 pmol of each primer, and 0.5 unit of Taq polymerase in a total volume of 100 μl. The annealing temperature was 56 °C. The PCR product was analyzed by agarose gel electrophoresis and subsequently digested with EcoRI and BamHI. The 0.5-kb EcoRI and 0.95-kb EcoRI-BamHI restriction fragments corresponding to the 5′ and 3′ regions of *glvA*, respectively, were purified with GeneClean II kit (BIO 101). The fragments were ligated into the corresponding sites of pUC119 and used for transformation of *E. coli* JM109. Nucleotide sequences of the fragments inserted into the recombinant plasmids (designated pUC119E and pUC119EB, respectively) were determined by the following procedure. Transformed cells were boiled in water, and the sample was transferred to the PCR reaction mixture containing universal forward and reverse primers of pUC plasmids (M13 primers M4 and RV; Takara), Taq polymerase, and dNTP. After amplification, the primers were removed by Microcon (Amicon). Sequences were determined using a Taq Dye Primer Cycle Sequencing kit (Perkin Elmer) and a 373A DNA sequencer from Applied Biosystems. After confirmation of the sequences, the 0.95-kb EcoRI-BamHI fragment from pUC119EB was purified and ligated to the EcoRI and BamHI sites of pK1500 to form pKPGE. Transformants of *E. coli* JM109 containing the recombinant plasmids were boiled in water and transformed with the PCR reaction mixture containing primers of pKPglv-1 and GIPBR, Taq polymerase, and dNTP. After amplification and transformation, those colonies that contained a 1.45-kb DNA restriction fragment were considered to harbor a plasmid (pKPglv-1) containing the insert in the correct orientation. For confirmation, pKPglv-1 plasmid DNA was isolated from the transformant, digested with EcoRI or HindIII, and subjected to agarose gel electrophoresis. The size of the restriction fragments corresponded to those expected from the sequence of *glva*, thereby confirming the presence of the complete gene in pKPglv-1.

**Growth of Cells and Preparation of Cell Extract**

*E. coli* JM109 (pKPglv-1) was grown at 37 °C in LB medium containing ampicillin (100 μg/ml). Cells were harvested by centrifugation (10,000 × g for 10 min at 5 °C) and washed by resuspension and centrifugation from 25 mM Tris-HCl buffer (pH 7.5) containing 1 mM EDTA and 1 mM NaN$_3$. The washed cell pellet (∼120 ml) was transferred into 120 ml of TM buffer, and the cells were disrupted at (0 °C) by 2 × 1.5-min periods of sonic oscillation with a Branson model 350 sonifier operating at ~75% of maximum power.

**Purification of 6-Phospho-α-glucosidase**

The enzyme was purified by conventional low speed chromatography, and all procedures were performed at 4 °C in a cold room. Column flow rates were maintained by a P-1 peristaltic pump interfaced with a Frac-100 collector. Protein in column eluents was monitored at 280 nm by a UV-1 optical control unit connected to a single channel chart recorder (all instrumentation from Pharmacia Biotech).

**Step 1: Preparation of High Speed Supernatant Fluid (HSS)—**The sonicated preparation was centrifuged (25,000 × g for 30 min at 5 °C) to remove intact cells and cell debris. The supernatant was collected and centrifuged at 180,000 × g for 2 h at 5 °C. The clarified HSS was transferred to dialysis sacs (molecular weight cut-off, 6000–8000) and dialyzed overnight against 4 liters of TM buffer.

**Step 2: DEAE-TrisAcryl-M (Anion Exchange) Chromatography—**Dialyzed HSS (120 ml) was transferred (0.8 ml/min) to a column (2.6 × 16 cm) of DEAE-TrisAcryl-M previously equilibrated with TM buffer. The column was washed to remove nonadsorbed material, and 6-phospho-α-glucosidase was eluted with 800 ml of a linear, increasing concentration gradient of NaCl (0–300 mM) in TM buffer. Fractions of 10 ml were collected, 10 μl of each fraction was tested for enzyme activity by the formation of a yellow color in microtiter wells containing 100 μl of the standard pNPGlc6P reaction mixture. Fractions 22–27 (inclusive) were pooled and concentrated to 25 ml by pressure filtration (Amicon PM-10 membrane, 35 psi). Ammonium sulfate was then added slowly, and with gentle stirring, to a final concentration of 0.75 M. 3: Phenyl-Sepharose CL-4B (Hydrophobic Chromatography)—The solution from step 2 was transferred (0.4 ml/min) to a 2.6 × 16-cm column of phenyl-Sepharose CL-4B equilibrated with TM buffer containing 0.75 mM (NH$_4$)$_2$SO$_4$. The column was washed with equilibration buffer to remove material that did not bind, and then 600 ml of a decreasing, linear gradient of (NH$_4$)$_2$SO$_4$ (300–0 mOD) in TM buffer was passed through the column. Fractions of 10 ml were collected, and
enzyme was recovered in a broad protein peak comprising fractions 25–45. These fractions were pooled and concentrated to 7.5 ml.

**Step 4: Ultrogel Aa-44 (Molecular Sieve) Chromatography—Approximately 2.5 ml of the preparation from step 3 was applied at a flow rate of 0.15 ml/min to a column (1.6 \times 94 \text{ cm}) of Ultrogel Aa-44 previously equilibrated with TM buffer containing 0.1 M NaCl. Fractions of 2.15 ml were collected, and maximum levels of enzyme activity were found in fractions 49–53, inclusive. These fractions were concentrated to 2 ml, and aliquots were either frozen directly in dry ice or glycerol was added to a final concentration of 10% prior to storage of the enzyme at −20 °C.

**Assay of Enzyme Activity**

The chromogenic analog pNPGel6P was used as substrate in the discontinuous assay for 6-phospho-α-glucosidase activity. The 2.0 ml reaction mixture (at 37 °C) contained, when required, 50 mM Tris-HCl buffer (pH 7.5), 1 mM pNPGel6P, 0.5 mM MnSO4, and 0.1 mM NaN3. After addition of the enzyme preparation, samples of 0.25 ml were removed at intervals of 0.5, 1, 1.5, 2, 2.5, and 3 min and immediately injected into 0.75 ml of 0.5 M Na2CO3 solution. The enzyme activity was measured, and the amount of pNP was calculated by assuming a molar extinction coefficient for the p-nitrophenoxide anion \( \epsilon = 18,300 \text{ M}^{-1}\text{ cm}^{-1} \). One unit of 6-phospho-α-glucosidase activity is the amount of enzyme that catalyzes the formation of 1 μmol of pNP per min at 37 °C.

**Electrophoresis Procedures**

Native gel electrophoresis and SDS-PAGE were carried out in the Novex XCell Mini-Cell system according to manufacturer's instructions. Electrophoresis of proteins under nonreducing (native) conditions was performed in Tris-glycine (4–20%) gels, from Novex, with Tris-glycine (pH 8.3) running buffer. For SDS-PAGE experiments, Novex NuPage (4–12%) Bis-Tris gels and MES-SDS running buffer (pH 7.2) were used together with Novex Mark 12 wide range or Bio-Rad low range molecular weight protein standards. In Western blots, proteins were transferred to nitrocellulose membranes using NuPage transfer buffer (pH 7.2) and standard precast standards. Immunodetection of 6-phospho-α-glucosidase with polyclonal antibody to maltose 6-phosphate hydrolase was as described previously (35).

**Analytical Methods**

The concentrations of glucose and Glc6P were determined enzymatically in an NADP+-coupled assay that contained (in 1 ml) 0.1 M potassium phosphate (pH 7) buffer, 1 mM MgCl2, 5 mM ATP, 1 mM NADP+, and a unit each of GlgC dehydrogenase (EC 1.1.1.49) and hexokinase (EC 2.7.1.1). Formation of NADPH was followed in a Beckman DU 70 recording spectrophotometer, and a molar extinction coefficient \( \epsilon = 6,220 \text{ M}^{-1}\text{ cm}^{-1} \) was assumed for calculation of NADPH produced (i.e., equivalent to glucose or Glc6P formed). The Pharmacia Biotech MultiPhor flat-bed electrophoresis unit and precast Ampholine PAG plates (pH range 3.5–9.5) were used for electrofocusing experiments as described previously (40). Protein concentrations were routinely determined by the BCA protein assay kit (Pierce). Chromatographic procedures are described in a previous report (35). The N-terminal amino acid sequence of 6-phospho-α-glucosidase was determined by automated Edman degradation in a 494A Procise sequenator (Applied Biosystems, Inc.) using pulse liquid chemistry. PTH derivatives were identified by on-line high pressure liquid chromatography. The mass of purified 6-phospho-α-glucosidase was determined by electrospray in an HP1100 mass spectrometer. The enzyme sample was dissolved in 0.05% trifluoroacetic acid, injected onto a Zorbax 300SB-C3 narrow bore (2.1 × 30 cm) column and eluted with a gradient of 5% acetic acid to 100% acetonitrile. A mass range from m/z 200–1,500 was scanned every 0.1 s, and the protein mass (50,510) was obtained by deconvolution from the only peak that eluted from the column.

**Analytical Ultracentrifugation**

Analytical ultracentrifugation experiments were conducted at 20 °C in a Beckman Optima model XL-I instrument equipped with a four-place An-Ti rotor. Specific absorption coefficients were determined by a combination of absorbance and interference optics. Absorbance readings on protein samples were measured in a Perkin Elmer model 320 double-beam spectrophotometer at 20 °C. Protein concentrations (mg/ml) were determined on the same solutions using the analytical ultracentrifuge in an interference mode as a differential refractometer (41) and an experimentally determined value of 5.191 ± 0.005 fringes (mg/ml)−1 for the same instrument. A 12-mm cell housing equipped with a double-sector capillary synthetic boundary centerpiece and sapphire

\[ ^2 \text{M. Zolkiewski and A. Ginsburg, unpublished data.} \]
windows was used with initial volumes of 130 μl of protein solution and 410 μl of dialysate buffer. After temperature equilibration at 20 °C at 3000 rpm, the rotor speed was increased to 10,000 rpm to initiate boundary formation and to 20,000 rpm (in 2000-rpm steps) until protein and solvent-side menisci were matched (total time 10 min) and then decelerated to 3000 rpm; 10 interference scans were then recorded at 2-min intervals (42). For 10 scans, the difference between the fringes in the plateau and solvent sides of the boundary (100 data points in both radial positions) was constant and within 0.1% accuracy during the period of data collection. The specific absorption coefficient for 6-phospho-α-glucosidase was determined to be $A_{280\,\text{nm}} = 1.25 \pm 0.01 \text{ ml/mg}$ (average of three independent measurements). This value is similar to that calculated from the amino acid composition of the protein (1.20 ml/mg). Sedimentation velocity experiments were conducted using charcoal-filled double-sector Epon 12-mm centerpieces. Enzyme solution ($A_{280\,\text{nm}} = 1.0$) was loaded on the right (330 μl per channel) with the reference buffer on the left (340 μl per channel). After thermal equilibration at 3000 rpm, the rotor was accelerated to 40,000 rpm, and radial scans were collected at 280 nm (0.003-cm step size, 4-min intervals) with triple averaging in a continuous scan mode. For sedimentation equilibrium experiments, a 12-mm cell equipped with a carbon-filled 6-channel centerpiece and plane quartz windows was used. Enzyme solutions ($A_{280\,\text{nm}}$ from 0.17 to 0.35) were loaded on the right (100 μl per channel) with the reference buffer on the left (110 μl per channel). Radial scans at 10,000 rpm with 13 averages were made at every 0.001-cm steps (step mode) after 18 and 30 h (equilibrium was reached by 16 h). Analysis of ultracentrifugation data was performed as described previously (43) with software from Beckman, Inc. and A. P. Minton (NIDDK, National Institutes of Health). The densities of dialysate buffers (20.0 °C) were determined using an Anton Paar model DMA 58 densitometer. The partial specific volume for the protein ($\gamma = 0.720 \text{ ml/g}$) was calculated from the amino acid composition (44).

RESULTS

Nucleotide Sequence of glvA—The nucleotide sequence of the gene glvA (previously called glv-1 (34)) is presented in Fig. 1. The 1347-base pair open reading frame begins with an ATG initiation codon at nucleotide position 15 and terminates with a TAA stop codon at position 1362. A putative ribosome binding site AAGGAGGT precedes the start codon. Translation of the initiation codon at nucleotide position 15 and terminates with a TAA stop codon at position 1362. A putative ribosome binding site AAGGAGGT precedes the start codon. Translation of the codon sequence of glvA predicts a polypeptide of 449 residues of 51,510. However, the molecular weight of the protein determined by electrospray-MS was 50,510. However, the site AAGGAGGT precedes the start codon. Translation of the codon sequence of glvA predicts a polypeptide of 449 residues of theoretical pI 4.77. The 48.1 μmol% (G+C) base composition of glvA is somewhat higher than the average 43.5 mol% (G+C) content of the B. subtilis chromosome (33).

Expression of GlvA—Cells of E. coli JM109 transformed with plasmid pKP glv-1 (Fig. 2A) produced a polypeptide that cross-reacted strongly with antibody prepared against purified MalH from F. mortiferum (Fig. 2B, lane 2). The estimated $M_\text{r}$ of this immunoreactive protein was of the size (~51 kDa) expected for the product of glvA, and the protein was not detected in a Western blot of an extract prepared from plasmid-free E. coli JM109 (Fig. 2B, lane 1). The cross-reactivity noted in Fig. 2B (lane 2) indicated epitopic and conformational similarity between GlvA and the MalH, and an extract of E. coli JM109 (pKP glv-1) rapidly hydrolyzed pNPGlc6P to form the (yellow) $p$-nitrophenolate anion and Glc6P (specific activity 0.22 nmol of pNPGlc6P hydrolyzed/mg protein/min). Under the same reaction conditions, there was no detectable hydrolysis of the nonphosphorylated analog pNPGlc, and GlvA was tentatively identified as a 6-phospho-α-glucosidase.

Purification of GlvA—The activity of GlvA in an extract of E. coli JM109 (pKP glv-1) declined by >90% during overnight dialysis at 4 °C in 25 mM Tris-HCl (pH 7.5) buffer, but there was little loss of activity when the buffer contained 1 mM Mn$^{2+}$. For this reason, Mn$^{2+}$ ion was included in all buffers throughout the purification of the enzyme (Table 1). The four-stage procedure yielded about 50 mg of pure 6-phospho-α-glucosidase from 25 g of cells (wet weight). Although successful in terms of providing a significant amount of purified material, the procedure yielded a final preparation of extremely low activity. Indeed, the specific activity of the purified enzyme (0.36 units/mg) was not significantly greater than that of the original high speed supernatant (0.22 units/mg). The instability of the 6-phospho-α-glucosidase from B. subtilis (see below) was reminiscent of the behavior of MalH during its purification from F. mortiferum (35).

Homogeneity and Size—Analysis of denatured 6-phospho-α-glucosidase by SDS-PAGE (Fig. 3A, lane 4) revealed a single polypeptide of $M_\text{r} \approx 51,000$, and the molecular weight of the protein determined by electrospray-MS was 50,510. However,

**Fig. 2. Structure of the plasmid vector containing glvA and expression of the gene product (GlvA) in E. coli. A, structure of plasmid pHglv-1 and the restriction sites for insertion of the gene glvA. Also shown are ori, origin of replication, and bla, β-lactamase gene. B, expression of GlvA in E. coli JM109 (pKP glv-1) and immunoreaction of the protein with antibody raised against MalH from F. mortiferum. Also shown in the Western blot are extract of E. coli JM109 (lane 1), extract of E. coli JM109 (pKP glv-1) (lane 2), and molecular weight markers (kDa) (lane 3).**
since the enzyme emerged close to the void volume of the AcA-44 gel filtration column (exclusion limit about 200 kDa; Table I, step 4), it seems that in the native state 6-phospho-α-glucosidase exists as a tetrameric species. Enzyme preparations that had been heated (or unheated) in the presence or absence of dithiothreitol revealed a single ~51-kDa polypeptide by SDS-PAGE (Fig. 3B). The absence of higher molecular weight species thus precludes participation of disulfide bonds in formation of the oligomeric structure. The homogeneity of 6-phospho-α-glucosidase was confirmed by the unambiguous data obtained by microsequence analysis of the protein. The first 37 residues from the N terminus agreed perfectly with the data obtained by microsequence analysis of the protein. The estimated pl values of 4.3 and 4.5 for the major (A) and minor (B) polypeptides, respectively, agreed quite well with the pl of 4.77 calculated from the deduced amino acid composition of GlvA.

Requirements for NAD(H) and Me2+ for GlvA Activity—The extensive loss in enzyme activity incurred throughout the purification (Table I) could be attributed to either (a) irreversible inactivation due to conformational changes of the protein or (b) removal of a required cofactor or activator. Preliminary studies showed that incubation of 5-μl (~100 μg) samples of 6-phospho-α-glucosidase with 5, 10, and 15 μl of an extract of plasmid-free E. coli JM109 (~20 mg/ml) enhanced enzyme activity by 8-, 15-, and 19-fold, respectively. The extract of E. coli was then heated (2 min at 100 °C), and precipitated material was removed by centrifugation. The clarified supernatant also activated the enzyme. Finally, a portion of E. coli extract was separated into high and low molecular weight components by passage through a PD-10 gel filtration column. The low molecular weight fraction produced >80% of the total increase in 6-phospho-α-glucosidase activity. Collectively, these findings suggested that a small, relatively heat-stable effector was required for catalytic activity. A study of potential cofactors (Table I) identified NAD+ and, to a lesser degree, NADH as the enzyme activators. With this knowledge, a more thorough examination of the metal requirements of the enzyme was under-

![Fig. 3. Analysis of 6-phospho-α-glucosidase by SDS-PAGE and by isoelectrofocusing. A, SDS-PAGE of samples from each of the four steps of enzyme purification. All samples were heated in the presence of β-mercaptoethanol (βME) prior to electrophoresis: lane 1, HSS; lane 2, DEAE-TrisAcryl M; lane 3, phenyl-Sepharose CL-4B; and lane 4, Ultrogel AcA-44. Inset, electrophoresis under nondenaturing conditions revealed two polypeptides (Fig. 3 inset). Surprisingly, electrophoresis of the ble II) identified NAD+ as the fluorogenic substrate 4MUAAPAD, 3-acetylpyridine adenine dinucleotide; NMN, nicotinamide mononucleotide.

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**Table II**

| Nucleotide and metal ion requirements for activity of GlvA (6-phospho-α-glucosidase) from B. subtilis |
|---------------------------------------------------------------|
| **Assay addition** | **Specific activity** | **Assay addition** | **Specific activity** |
|-------------------|----------------------|-------------------|----------------------|
| units/mg          | units/mg             | units/mg          | units/mg             |
| Control (none)    | 0.19                 | None              | 0.02                 |
| NAD+              | 1.50                 | Mn2+              | 2.16                 |
| NADH              | 0.92                 | Fe2+              | 0.73                 |
| APAD+             | 0.38                 | Ni2+              | 0.46                 |
| NADPH             | 0.30                 | Co2+              | 0.45                 |
| NMNα             | 0.28                 | Sr2+              | 0.27                 |
| ATP               | 0.24                 | Ca2+              | 0.12                 |
| NADPH             | 0.24                 | Mg2+              | 0.08                 |
| FAD               | 0.23                 | Cd2+              | 0.04                 |
| FMN               | 0.23                 | Zn2+              | No detectable activity |

*Assay conditions are described under “Experimental Procedures”; all assays contained 0.5 mM Mn2+. The enzyme was incubated (~5 min) with 1 mM each compound prior to addition to the assay.

APAD, 3-acetylpyridine adenine dinucleotide; NMN, nicotinamide mononucleotide.

Assay conditions are described under “Experimental Procedures.” The enzyme was incubated for 5 min in 50 μl of 50 μM Tris-HCl buffer (pH 7.5) containing 1 mM NAD+ and 2 mM of desired Me2+ prior to addition to the 2-ml assay that contained 50 mM Tris-HCl buffer (pH 7.5), 1 mM pNPGLc6P, and 0.5 mM desired Me2+.

**TABLE II**

| **Assay addition** | **Specific activity** | **Assay addition** | **Specific activity** |
|-------------------|----------------------|-------------------|----------------------|
| units/mg          | units/mg             | units/mg          | units/mg             |
| Control (none)    | 0.19                 | None              | 0.02                 |
| NAD+              | 1.50                 | Mn2+              | 2.16                 |
| NADH              | 0.92                 | Fe2+              | 0.73                 |
| APAD+             | 0.38                 | Ni2+              | 0.46                 |
| NADPH             | 0.30                 | Co2+              | 0.45                 |
| NMNα             | 0.28                 | Sr2+              | 0.27                 |
| ATP               | 0.24                 | Ca2+              | 0.12                 |
| NADPH             | 0.24                 | Mg2+              | 0.08                 |
| FAD               | 0.23                 | Cd2+              | 0.04                 |
| FMN               | 0.23                 | Zn2+              | No detectable activity |

*Assay conditions are described under “Experimental Procedures”; all assays contained 0.5 mM Mn2+. The enzyme was incubated (~5 min) with 1 mM each compound prior to addition to the assay.

APAD, 3-acetylpyridine adenine dinucleotide; NMN, nicotinamide mononucleotide.

Assay conditions are described under “Experimental Procedures.” The enzyme was incubated for 5 min in 50 μl of 50 μM Tris-HCl buffer (pH 7.5) containing 1 mM NAD+ and 2 mM of desired Me2+ prior to addition to the 2-ml assay that contained 50 mM Tris-HCl buffer (pH 7.5), 1 mM pNPGLc6P, and 0.5 mM desired Me2+.

for these experiments, 6-phospho-α-glucosidase was first exhaustively dialyzed against 25 mM Tris-HCl (pH 7.5) buffer to remove endogenous Mn2+ and other metal ion contaminants. Enzyme activity was then measured in the standard assay containing NAD+, pNPGLc6P, and desired Me2+ ion. In the presence of NAD+ alone, hydrolysis of substrate was barely discernible (Table II). However, addition of Co2+, Ni2+, Fe2+, or Mn2+ to the assay elicited a 20–100-fold increase in enzyme activity. These data established that both NAD(H) and Me2+ were required for activity of GlvA. Inclusion of EDTA (2 mM) in these assays resulted in complete inactivation of the enzyme (data not shown).

**Properties of 6-Phospho-α-glucosidase—**Enzyme activity was greatest between 35 and 38 °C in buffer of pH range 7.5–8.0. Although 50 mM Tris-HCl (pH 7.5) was used routinely as the assay buffer, comparable activities (at pH 7.5 and same molarity) were measured in MES, Bicine, Tricine, HEPES, orimidazole buffers (data not shown). In the presence of 0.1 mM NAD+ and 1 mM Mn2+, it was found that the enzyme exhibited Michaelis-Menten saturation kinetics with pNPGLc6 as substrate (Km = 0.09 mM, Vmax = 2.2 μmol hydrolyzed/mg protein/min). The Km values for NAD+ and NADH were estimated to be
FIG. 4. Activation of 6-phospho-α-glucosidase by NAD+ and identification of the products of hydrolysis of [U-14C]maltose 6-phosphate. The autoradiograms show the time course and the products of hydrolysis of [U-14C]maltose in reaction mixtures (A, lacking NAD+ and B, supplemented with NAD+). The reaction mixtures (120 μl at 37 °C) contained 50 mM Tris·HCl buffer (pH 7.5), ~1 mM [U-14C]maltose 6-phosphate (specific activity 1 μCi/μmol), 1 mM MnSO4, 200 μg of enzyme, and, when required, 0.2 mM NAD+. At the times indicated, 15-μl samples were withdrawn from each mixture and immediately frozen in dry-ice to stop the reactions. The samples were then heated (simultaneously) in boiling water for 3 min. After cooling, the precipitated protein was removed by centrifugation, and 10 μl of each clarified supernatant liquid was applied to Whatman 3 MM chromatography paper. Descending chromatography (18 h) was performed in a solvent containing n-butanol/glacial acetic acid/water in proportions 5:2:3 (v/v). Reaction products were detected by autoradiography. Identities of X and Y are presently unknown. Note: complete hydrolysis of substrate within 2 min in the presence of NAD+.

3.7 × 10⁻⁵ M and 2.0 × 10⁻⁴ M, respectively. Perhaps NAD+ is the preferred nucleotide for in vivo activation of the enzyme. The Kₐ for Mn²⁺ was determined to be 0.31 mM. Although pNPαGlc6P was rapidly hydrolyzed by 6-phospho-α-glucosidase, there was no detectable cleavage of nonphosphorylated nitrophenyl-glycosides including pNPαGlc, pNPα-galactopyranoside, and pNPα-mannopyranoside. Enzyme discrimination with respect to the spatial orientation of hydroxyl groups in the glucopyranosyl moiety of pNPαGlc6P is evident from the fact that neither the C-2 nor the C-4 epimer (pNPαMan6P and pNPαGal6P, respectively) is a substrate for 6-phospho-α-glucosidase. Maltose 6-phosphate and trehalose 6-phosphate are substrates for GlvA from *B. subtilis*, and hydrolysis of these O-α (1→4)- and O-α (1→1')-linked disaccharide phosphates yielded equimolar amounts of Glc6P and glucose (data not shown). The rate of cleavage of [U-¹⁴C]maltose 6-phosphate was increased ~10-fold in the presence of NAD+, and chromatographic analysis confirmed formation of [¹⁴C]-labeled Glc6P and glucose as hydrolysis products (Fig. 4). Unexpectedly, the autoradiogram also revealed the presence of two other compounds (X and Y) in the reaction mixture, but the two compounds have not yet been identified. A fluorogenic analog of maltose 6-phosphate (4MUαGlc6P) was also hydrolyzed by 6-phospho-α-glucosidase to form Glc6P and the intensely fluorescent 4-methylumbelliflereone (Fig. 5). These extremely sensitive microtiter assays provide visual confirmation for the requirements of phosphorylation of the substrate and divalent metal and dinucleotide specificity for GlvA-catalyzed hydrolysis.

**Analytical Ultracentrifugation**—Sedimentation velocity analysis was used to characterize 6-phospho-α-glucosidase in solutions containing different concentrations of Mn²⁺ ion. Time derivative analysis showed that in the absence of Mn²⁺, the enzyme exists as a single species (S₂₀,ₐw = 6.3 S) (Fig. 6A). The apo-enzyme showed a single component of .Messaging: The apo-enzyme showed a single component of M₉ = 105,000 in sedimentation equilibrium experiments (Table III). This value approximates that calculated for a dimer from the amino acid sequence of GlvA (M₉ = 101,026). In the presence of Mn²⁺, the major sedimenting component had a sedimentation coefficient of ~9.3 S, which is consistent with a tetrameric form. Small fractions of monomer (~4 S) were present also (Fig. 6, B and C). Weight average molecular weight determinations of the enzyme in buffers containing Mn²⁺ (Table III) were lower than expected for a homotetramer (M₉ = 202,052). Increasing the concentration of Mn²⁺ from 1 to 5 mM did not lead to complete conversion of dimer to tetramer (Table III). Indeed, in the presence of 1 and 5 mM Mn²⁺, the best fits of data sets were obtained when molecular weights for two components were constrained at 100,000 and 200,000, respectively, using a model for nonassociating species. These analyses yielded estimates of ~25 and ~75% of dimer and tetramer, respectively. Thus, some dimer seems to be incompetent in associating to tetramer. Attempts to fit the sedimentation equilibrium to either self-associating or nonassociating models involving 1-2-4, 2-3-4, and 1-4 (where 1 denotes the M₉ = 50,513 monomer) gave unsatisfactory fits.

**Site-directed Mutagenesis of GlvA**—Comparative alignment of the amino acid sequences of GlvA from *B. subtilis*, MalH of *F. mortiferum*, and other members of family 4 illustrates the extensive homology of these glycosyl hydrolases (Fig. 7). Significantly, three acidic residues at sequence positions 41, 111, and 359 of GlvA are conserved in all members of family 4, and site-directed mutagenesis revealed that these residues are essential for activity of GlvA. In experiments summarized in Table IV, residues Asp41, Glu111, and Glu359 of GlvA were changed to either Gly or to the corresponding homolog (Glu or Asp). *E. coli* XL1-Blue was transformed with plasmids encoding *glvA* with the desired mutation, and Western blots verified the production by all transformants of the desired protein. Data not shown). However, the level of GlvA activity in cell extracts of the transformants (Table IV) was ~1% that of *E. coli* XL1-Blue (pKPglv-1) and was not significantly higher than that of an extract prepared from plasmid-free *E. coli* XL1-Blue (approximately 3 nmol of pNPαGlc6P hydrolyzed/min/mg protein).

**DISCUSSION**

The gene *glvA* (formerly *glv-1* (34)) of *B. subtilis* encodes a novel 6-phospho-α-glucosidase. To our knowledge, this is the
Solutes are given by \( s^* \) (maltose 6-phosphate) is hydrolyzed by 6-phospho-
cosides via a maltose (PEP-PTS) in this Gram-positive orga-
nism. Metal ions were added to the vertical wells; potential sub-
strates and dinucleotides were included in horizontal wells.

After 5 min of incubation, the plate was photographed under UV light (360 nm) using a yellow filter. Metal ions were
prerequisites for hydrolysis include (a) an O-\( \alpha \) glycosidic link-
age, (b) –OH groups in the equatorial configuration at C-2 and
C-4, and (c) phosphorylation at the C-6 position of the nonre-
ducing ring. By contrast, the enzyme is remarkably tolerant with respect to charge and size of the aglycone substituent (e.g.,
gluco-para-nitrophenyl, and umbelliferyl groups). Both maltose 6-phosphate (6-O-phosphoryl-\( \alpha \)-glucopyranosyl(4–
4)-\( \beta \)-glucopyranose) and trehalose 6-phosphate (6-O-phosphor-
yl-\( \alpha \)-D-glucopyranosyl-1–1’-\( \beta \)-D-glucopyranosyl) are hydrolyzed
by GlvA to form Glc6P and glucose. Interestingly, genes \( \text{treA} \) of
\( \text{B. subtilis} \) (46–48) and \( \text{treC} \) of \( \text{E. coli} \) (49) encode an enzyme, trehalose 6-phosphate:phosphoglucohydrolase (EC 3.2.1.93),
that catalyzes the hydrolysis of trehalose 6-phosphate to give
these same reaction products. Surprisingly, the latter enzyme
shows little similarity to the 6-phospho-\( \alpha \)-glucosidase we
describe in this report, and by sequence alignment, trehalose 6-phosphate hydrolase is included in family 15 of the
glycosidases.

When the sequences of two or more enzymes can be aligned over an entire domain, they are assigned to the same family,
and on this basis some 50 families of glycosidases have now
been described (31, 32). Family 1 includes phospho-\( \beta \)-
galactosidases (EC 3.2.1.86), phospho-\( \beta \)-galactosidases (EC
3.2.1.85), \( \beta \)-galactosidases (EC 3.2.1.21), and \( \beta \)-galactosidases
(EC 3.2.1.23). Elegant studies by Withers and co-workers (50–
52) have shown that for \( \beta \)-galactosidase from \( \text{Agrobacterium}
faecalis} \) two glutamyl residues (Glu\(^{170} \) and Glu\(^{388} \)) function as
acid catalyst (\( \text{AH} \)) and nucleophile/base (\( \text{A}^- \)), respectively. The
Fig. 7. Multiple alignment of the proteins comprising family 4 of the glycosyl hydrolases. Fully conserved residues are highlighted (black background), and residues conserved in six out of the nine members are shown with gray background. Numbers to the left denote residue positions; numbers above the sequences refer to alignment positions and not to any one of the aligned proteins. Sequences were aligned by CLUSTAL W 1.6 (59). The pairwise gap-opening penalty was 35, and the pairwise gap-extension penalty was 0.75. The multiple alignment gap-opening penalty was 15, and the multiple alignment gap-extension penalty was 0.3. The BLOSUM 30 similarity matrix was used. Dark shading indicates residues that are identical in >50% of the sequences. Light shading indicates residues that are identical in >50% of the sequences. The abbreviations used, references to published sequences, and data bank accession numbers are as follows: Bacsu-glvA (34), 6-phospho-α-glucosidase, B. subtilis (GenBank D50543); Fusmo-glvH (37), maltose β-glucoside hydrolase, F. mortiferum (GenBank U81185); E. coli-glgG (60, 61), truncated 6-phospho-α-glucosidase, E. coli (SwissProt P31450); E. coli-cef (26), β-phospho-β-glucosidase, E. coli (SwissProt P17411); Bacsu-cef (62), putative 6-phospho-β-glucosidase (lhH), B. subtilis (SwissProt P46320); E. coli-Agal (63), α-galactosidase, E. coli (SwissProt P46320); Bacsu-Agal, putative α-galactosidase, B. subtilis (SwissProt P39130); Therm-Aglu (64), α-glucosidase, Thermotoga maritima (EMBL AJ001069).
two acidic residues are spatially poised above and below the plane of the glucopyranosyl ring, and hydrolysis of substrate proceeds via three stages involving (a) protonation of the glycosidic oxygen, (a) formation of a transition-state oxocarbonium ion, and (c) base-assisted departure of the aglycone (53–55). The catalytic roles of Glu160 and Glu359 at the active site of 6-phospho-β-galactosidase (another member of family 1) has been established by crystallographic analysis and by site-directed inactivation of the enzyme with mechanism-based inhibitors (21, 56). Comparative alignment of amino acid sequences shows that the two catalytic glutamyl residues are conserved in the motifs NE(P/I) and ENG, which are present (and separated by about 200 residues) in all members of this large family (27, 28, 57, 58).

Family 4 presently comprises a heterogeneous group of enzymes that includes α-galactosidase, α-glucosidase, putative 6-phospho-β-galactosidase, and 6-phospho-α-glucosidase (Fig. 7). The nine members of this family are all of bacterial origin, and, except for the truncated 6-phospho-α-glucosidase of E. coli, the polypeptides are of comparable length (average 446 amino acids). Inspection of Fig. 7 reveals two acidic residues that are conserved in the motifs GQ(E/D)T(39) and VE(39) protonation of the glycosidic oxygen, (a) formation of a transition-state oxocarbonium ion, and (c) base-assisted departure of the aglycone (53–55). Although these results are evidence for a structural role for Mn2+, they do not preclude a catalytic function for this (and other) divalent metal ion(s).

The role of dinucleotide in activation of 6-phospho-α-glucosidase cannot be deduced from our studies. The requirement for NAD(H) is clearly specific, and NAD(P)H, which differs only in the presence of a phosphoryl moiety at the 2′-OH of the adenine ribose ring, is not an acceptable substitute (Table II, Fig. 5). We have obtained no evidence for oxido/reduction of either NAD+ or NADH, and it seems unlikely that the dinucleotide serves as a reactant during substrate cleavage. Both NAD+ and NADH bind strongly (but noncovalently) to 6-phospho-α-glucosidase, and computer-based analysis of the sequence of GlvA with its homologs reveals a putative dinucleotide-binding motif in all members of family 4 (Fig. 7). This domain comprises a ββ unit containing a glycine-rich turn or loop between the first β strand and the dinucleotide binding helix (69, 70). Both βA and βB strands of this structural element consist almost entirely of hydrophobic amino acids, and the two glycine residues that follow βA (Gly132 and Gly141 in GlvA) are conserved in the sequences of all nine proteins. Although the third glycine residue in the GXXGXXG fingerprint region (69, 70) does not seem to be present, Gly20 could assume this role in the B. subtilis enzyme. Indeed, the number of amino acids between the second and third glycyl residues of this triplet has been found to vary depending on the length of the loop between βA and αA (71). It is also noteworthy that Ser21 follows the second Gly residue in GlvA, and this pattern is found in all family 4 members. We suggest that this positionally conserved serine residue may hydrogen bond to the nicotinamide carboxamide and nicotinamide phosphate oxygen atoms in a manner similar to the conserved asparagine residue of the glutamate dehydrogenase family (70). Further evidence in favor of the proposed nucleotide binding domain is the presence of a conserved aspartic acid residue (Asp41 for GlvA) at the end of the βB strand in family 4 members. In many NAD+ and FAD-dependent enzymes, an acidic residue at this position in the ββ fold is known to hydrogen bond to the 2′ and 3′ hydroxyl groups of the adenine ribose. For GlvA we find that site-directed mutagenesis of Asp41 yields proteins (D41G and D41E) that are catalytically inactive (Table IV). These findings suggest a role for this conserved aspartyl residue in the binding of NAD(H) to 6-phospho-α-glucosidase.

In 1971, Burstein and Kepes described both the instability of α-galactosidase from E. coli (65) and the unexpected require-

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**Table IV**

| Mutation | Primer sequence | Activity a |
|----------|-----------------|------------|
| None     |                 | 482        |
| D41G     | G CTG AAG CTG TAT GGT AAT GAT AAG GAG AGA CAG G     | 4          |
| D41E     | G CTG AAG CTG TAT GAG AAT GAT AAG GAG AGA CAG G     | 3          |
| E111G    | GGA GTT GTC GGC CAG GGG ACG TGG CAG GCG            | 1          |
| E111D    | GGA GTT GTC GGC CAG GAT ACG TGG CAG GCG            | 3          |
| E359G    | C CCG ACT GGG ATT GTG GGG GTG CCA TGC ATC GTC GGC  | 5          |
| E359D    | C CCG ACT GGG ATT GTG GGG GTG CCA TGC ATC GTC GGC  | 5          |

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a Base substitutions are shown underlined.

b Activity expressed as nmol of pNP from GlvA by chelation with EDTA causes dissociation of the active tetramer to the inactive dimeric form of the enzyme.

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