Metabolism of Sucrose and Its Five Linkage-isomeric α-D-Glucosyl-d-fructoses by *Klebsiella pneumoniae*

PARTICIPATION AND PROPERTIES OF SUCROSE-6-PHOSPHATE HYDROLASE AND PHOSPHO-α-GLUCOSIDASE*

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*Klebsiella pneumoniae* is presently unique among bacterial species in its ability to metabolize not only sucrose but also its five linkage-isomeric α-D-galactosyl-d-fructoses: trehalulose, turanose, maltulose, leucrose, and palatinose. Growth on the isomeric compounds induced a protein of molecular mass ≈ 50 kDa that was not present in sucrose-grown cells and which we have identified as an NAD<sup>+</sup> and metal ion-dependent 6-phospho-α-glucosidase (AglIB). The *aglB* gene has been cloned and sequenced, and AglIB (M<sub>r</sub> = 49,256) has been purified from a high expression system using the chromogenic p-nitrophenyl α-glucopyranosidase 6-phosphate as substrate. Phospho-α-glucosidase catalyzed the hydrolysis of a wide variety of 6-phospho-α-glucosides including maltose-6'-phosphate, maltitol-6-phosphate, isomaltose-6'-phosphate, and all five 6'-phosphorylated isomers of sucrose (K<sub>m</sub> = 1–5 mM) yet did not hydrolyze sucrose-6-phosphate. By contrast, purified sucrose-6-phosphate hydrolase (M<sub>r</sub> = 53,000) hydrolyzed only sucrose-6-phosphate (K<sub>m</sub> = 80 μM). Differences in molecular shape and lipophilicity potential between sucrose and its isomers may be important determinants for substrate discrimination by the two phosphoglucosyl hydrolases. Phospho-α-glucosidase and sucrose-6-phosphate hydrolase exhibit no significant homology, and by sequence-based alignment, the two enzymes are assigned to Families 4 and 32, respectively, of the glycosyl hydrolase superfamilies. The phospho-α-glucosidase gene (aglIB) lies adjacent to a second gene (aglA), which encodes an EI/C(B) component of the phosphoenolpyruvate-dependent sugar-phosphotransferase system. We suggest that the products of the two genes facilitate the phosphorylative translocation and subsequent hydrolysis of the five α-D-glucosyl-d-fructoses by *K. pneumoniae*.

The discovery in 1964 of the phosphoenolpyruvate-depend*

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank<sup>™</sup> and EBI Data Bank with accession number(s) AF335781.

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** The abbreviations used are: PEP:PTS, phosphoenolpyruvate-dependent sugar-phosphotransferase system; pNP<sub>Glc</sub>, p-nitrophenyl α-D-glucopyranoside; pNP<sub>Glc6P</sub>, p-nitrophenyl α-D-glucopyranoside 6-phosphate; MES, 2(N-morpholino)ethanesulfonic acid; PAGE, polyacrylamide gel electrophoresis; PCR, polymerase chain reaction; IPTG, isopropyl-β-D-thiogalactopyranoside; MD, molecular dynamics; MLP, molecular lipophilicity pattern.

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Phosphoglucohydrolases from K. pneumoniae

Our interest in these issues stemmed from a survey of disaccharide utilization by K. pneumoniae that revealed excellent (and unexpected) growth of this organism on all five isomers of sucrose (12). Furthermore, although organisms grown previously on a particular isomer readily metabolized sucrose and all other isomers, cells of K. pneumoniae grown previously on sucrose fermented only sucrose (12). Comparative analyses of proteins in various cell extracts (by two-dimensional PAGE) revealed high level expression of a specific polypeptide (molecular mass = 50 kDa) during growth on the isomers, but this protein was not induced by growth of the organism on sucrose. These observations provided the first indication that for K. pneumoniae, the initial steps in metabolism of sucrose, and those of its analogs, might be separable and distinct. In the present study we have identified two adjacent genes (aglA and aglB) in K. pneumoniae that encode a membrane-localized transport protein of the PTS (EIICB, or AglA) and a nucleotide (NAD') plus metal-dependent phospho-o-glucosidase (AglB), respectively. Together, these proteins facilitate the phosphorylative translocation and subsequent hydrolysis of the five O-linked isomers of sucrose.

To facilitate the comparison of the properties of sucrose-6-P hydrolase with those of AglB, the genes encoding the two proteins (sucB (7) and aglB, respectively) have been cloned, and both enzymes have been purified from high expression systems. Recently, we prepared trehalulose-6'-P, turanose-6'-P, maltotriose-6'-P, maltose-6'-P, and palatinose-6'-P in substrate quantity (12), and the availability of these novel compounds permitted the determination of the substrate specificities of highly purified AglB and sucrose-6-P hydrolase. Remarkably, sucrose-6-P hydrolase, which by sequence-based alignment is assigned to Family 32 of glycosyl hydrolases, hydrolyzed only sucrose-6-P. In contrast AglB, which belongs to Family 4, catalyzed the cleavage of the five isomeric 6'-phosphoglucofructoses. In this paper, a comparative assessment of conformational, overall shape and polarity features of sucrose-6-P and its isomeric disaccharide-6'-phosphates is given, providing insight into the molecular basis for substrate discrimination by the two phosphoglucohydrolases.

**EXPERIMENTAL PROCEDURES**

**Materials**—Carbohydrates were obtained from the following sources: trehalulose from Sudzucker, Mannheim/Ochsenfurt, Germany; maltulose and isomaltose from TCI America; leucrose from Fluka; and palatinose from Wako Chemicals. Sucrose, turanose, and other high purity sugars were purchased from Pfannstiehl Laboratories. Maltitol, lactulose, and isomaltose from Pfanstiehl Laboratories. Maltitol, trehalulose from Su-dzucker, Mannheim/Ochsenfurt, Germany; maltulose-6-P, maltose-6-P, and palatinose-6'-P in substrate were obtained from Sigma. Bis-Tris gels and MES-SDS running buffer (pH 7.3) were used together with Novex Mark 12™ protein standards, and proteins were stained with Coo massie Brilliant Blue R-250. For Western blots, proteins were transferred to nitrocellulose membranes using NuPage transfer buffer and SeeBlue™ prestained standards. The Amersham Pharmacia Bio- tech Multiphor flat-bed electrophoresis unit, precast Ampholine PAG plates (pH range, 3.5–9.5) and broad range standards were used for electrophoresis experiments.

**Analytical Methods**—During purification, the activity of AglB in column fractions was detected by hydrolysis of the chromogenic substrate, pNpO6Glc6P. The specific activity of the enzyme was determined in a discontinuous assay that contained in 2-ml: 0.1 M Tris-HCl buffer (pH 7.5), 1 mM MnCl₂, 0.5 mM NAD⁺, and 1 mM pNpO6Glc6P. After the addition of the enzyme preparation, samples of 0.25 ml were removed at 20 min after addition (over a 2-min period) and immediately injected into 0.75 ml of 0.5 M Na₂CO₃. The A₄₅₀nm of the yellow solution was measured, and rates of pNP formation were calculated by assuming a molar extinction for the p-nitrophenoxide anion e = 18,300 M⁻¹ cm⁻¹. One unit of AglB activity is the amount of enzyme that catalyzes the formation of 1 μmol of pNP min⁻¹. Two-dimensional polyacrylamide gel electrophoresis (PAGE) and protein microsequencing were carried out by Kerckhoff Laboratories, Inc. and by the Protein Chemistry Core Facility, Columbia University, NY, respectively. The mass of AglB was determined by electrospray in an HP1100 mass spectrometer, and the sequence of N-terminal amino acids was determined with an ABI 477A protein sequencer (Applied Biosystems Inc.) with an on-line ABI 120A phenylthiohydantoin analyzer. Protein concentrations were determined by the BCA protein assay kit (Ferriee). The procedure for immunodetection of AglB with polyclonal antibody to MalH from F. mortiferum has been described previously (20).

**Cloning and Characterization of a Region Encoding the aglA and aglB Genes of K. pneumoniae ATCC 23357**—Initially, using the unfinished genome sequence of K. pneumoniae (Washington University Genome Sequencing Center, St. Louis, MO) and our own sequence data from five primer sets designed to amplify, clone, and characterize the DNA fragment encoding genes aglA and aglB of K. pneumoniae ATCC 23357. The five primer sets were constructed as follows: KP1F-KP1R, 5'-GCCAGTTTTTCTCTCTGTGATGACG-3'; KP2F-KP2R, 5'-CTTGTAGGAGTTGTTTATGATGAT-3'. The PCR (the se-quence of N-terminal amino acids was determined with an ABI 477A protein sequencer (Applied Biosystems Inc.) with an on-line ABI 120A phenylthiohydantoin analyzer. Protein concentrations were determined by the BCA protein assay kit (Ferriee). The procedure for immunodetection of AglB with polyclonal antibody to MalH from F. mortiferum has been described previously (20).

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Phosphogluconsyl Hydrolases from *K. pneumoniae*

Cloning of the Sucrose-6-P Hydrolase Gene (serB) from *K. pneumoniae*

The *serB* gene was amplified from *K. pneumoniae* genomic DNA using the low-error-rate FailSafe polymerase (Epicentre). In the forward primer (5'-GGGCGATCGCCTTCTGTACTCGTTGCTATC-3'), base pairs 3119–3137 of the *K. pneumoniae* serYAB operon (GenBank accession number X57401) are bolded, and the NcoI site is underlined. In the reverse primer (5'-GGGCGATCGCCTTCTGTACTCGTTGCTATC-3'), base pairs 4587–4606 of serYAB are bolded, and the SalI site is underlined. The amplicon was digested with NcoI and SalI and ligated into similarly digested pProEX Hta (Life Technologies, Inc.), and the recombinant plasmid(s) was transformed into *E. coli* K12 strain DH5α-E (Life Technologies, Inc.). Ampicillin-resistant transformants were selected, and the *serB* genes of four plasmids containing inserts of approximately the correct size were sequenced. All shared the following differences from the published (7) *serB* coding sequence: 991C→T, 1006G→T, 1249C→T, 1270C→T, 1549C→T, 1552A→G, 1675G→A, 1699T→C, 1738G→A (numbering as in pSerBLong). All of these differences are silent, and one plasmid was chosen and designated pSerBLong.

Growth of *E. coli* DH5αE (pSerBLong) and Expression of Sucrose-6-P Hydrolase

The organism was grown in LB medium containing 200 μg/ml ampicillin. At A600nm = 0.5, IPTG was added (1 mM) and growth was continued for ~4 h. Cells were harvested and washed with 25 mM HEPES buffer (pH 7.5) as described earlier. The yield was ~2.9 g wet weight of cells/liter.

Purification of Sucrose-6-P Hydrolase

Briefly, the purification of sucrose-6-P hydrolase was as follows. A high-speed supernatant was prepared, after resuspension and sonication, of 10 g of *E. coli* DH5αE (pSerBLong) resuspended with 20 ml of 25 mM HEPES buffer (pH 7.5). The dialyzed preparation was applied to a column of Tris Acryl M-DEAE, and after washing with the same buffer, sucrose-6-P hydrolase was eluted with an increasing gradient of NaCl (0.0–0.5 M). Fractions with sucrose-6-P hydrolase activity were pooled, concentrated to 8 ml, and then mixed gently with 30 ml of 0.1 M MES buffer (pH 5). Precipitated material was removed by centrifugation, and the clarified solution was applied to a column of phosphocellulose P-11 (Whatman) previously equilibrated with 0.1 M MES buffer (pH 5). Nonadsorbed proteins were removed, and sucrose-6-P hydrolase was eluted with an increasing concentration of potassium phosphate buffer (0.0–0.1 M, pH 7). Active fractions (eluted at ~50 mM P) were pooled and concentrated. Sucrose-6-P hydrolase was purified to homogeneity by passage of this solution through an AcA-44 gel filtration column previously equilibrated with 50 mM HEPES buffer, pH 7.5, containing 0.1 M NaCl. Concentration of active fractions yielded about 22 mg 25% sucrose-6-P hydrolase of specific activity 12.5 units/mg (with 10 mM sucrose as substrate in the assay, see below).

Sucrose-6-P Hydrolase Assay

Sucrose-6-P is the natural substrate for sucrose-6-P hydrolase, but the enzyme also hydrolyzes sucrose when the disaccharide is present at high concentrations. Because of the limited availability of sucrose-6-P, the parent sugar was used as substrate during purification of sucrose-6-P hydrolase, and the glucose-6-P dehydrogenase/hexokinase-NADP+ coupled assay measured glucose formed by sucrose hydrolysis. The 1 ml assay contained: 0.1 mM HEPES buffer (pH 7.5), 1 mM MgCl2, 1 mM MnCl2, 1 mM NAD+, 1 mM NADP+, 1 mM substrate (6-P isomer of sucrose, or phospho-α-glucose), and 2 units of glucose-6-P dehydrogenase/hexokinase. Reactions were initiated by addition of 15 μl of 45 μg/ml of AglB preparation, and the increase in A540 nm was recorded in a Beckman DU 640 spectrophotometer. Initial rates were determined using the kinetics program of the instrument, and a molar extinction coefficient ε = 6,220 μM−1 cm−1 was assumed for calculation of NADPH formed (equivalent to glucose-6-P liberated). In kinetic analyses the concentration range of substrate was usually 0.2–4 mM, and kinetic parameters were determined by Hofstee plots with an Enzyme Kinetics program (dogStar software, Version 1.0c). The products of turanose-6-P hydrolysis (glucose-6-P and fructose) were determined by inclusion of 5 mM ATP and 2 units of phosphoglucom isomerase in the assay.
that equilibrate between different anomeric or ring (pyranoid or furanoid) forms, only the most predominant tautomer was considered, i.e. the 6'-phosphates of β-p-trehalulose, β-p-turanose, β-p-maltulose, β-p-leucrose, β-p-palatinose, β-p-p-maltose, and β-p-p-isomaltose. Each compound was centered in a periodic box (truncated octahedron, box size ~ 33.5 Å) filled with pre-equilibrated TIP3P (transferable intermolecular potential-3) water molecules, yielding (after removal of the solvent molecules that overlap with the solute) simulation systems including 643 (disaccharides) or 641 (disaccharide phosphates) water molecules, respectively. In the latter series, two NH₄⁺ counterions were added at random positions within 6 Å around the glucose-6-CH₂OPO₃⁻ group. After full lattice energy minimization, all boxes were slowly heated from 0 to 300 K within 15 ps of MD simulation and were subsequently equilibrated for an additional 85 ps; the final MD data were sampled using simulations of 1 ns in each case; molecular configurations were saved every 100 fs for analysis purposes. All MD runs were carried for constant pressure (P祢 = 1 atm, isothermal compressibility 4.63 × 10⁻⁵ atm⁻¹, pressure coupling constant τₚ = 5 ps) and constant temperature (T祢 = 300 K, temperature coupling constant τₚ = 5 ps, allowed temperature deviation ΔT = ±10 K) conditions (NPT ensemble (constant number of molecules, constant pressure, and constant temperature)) using the following simulation parameters: time step Δt = 1 fs (leapfrog integrator, all X-H bond lengths were constrained using the SHAKE protocol), dielectric constant ε = 1.0, cut-off distance for long range interactions 12 Å, cut-off of 5 Å for image charges and 13 Å. The following averages were recalculated from the final MD runs (standard deviations are in parentheses): disaccharides: temperature <T祢> = 296(5) K, box size 33.66(8) Å, volume <V祢> = 19060(145) Å³, density <ρ祢> = 1.039(8) g cm⁻³; disaccharide 6-phosphates: temperature <T祢> = 296(5) K, box size 33.59(5) Å, volume <V祢> = 18950(130) Å³, density <ρ祢> = 1.049(8) g cm⁻³. For each MD time series a mean solvent geometry was obtained by three-dimensional fitting of all configurations (heavy atoms only, excluding CH₂OH oxygen atoms); the best-fit models from this procedure were selected as representative molecular geometries in aqueous solution (Fig. 9, 10). For a comparison, the conformation of all glucosyl-6'-CH₂OH groups were set to gauche-trans (gt, torsion angle 0°–C5–C6–O6–ω = 0°). Solvent-accessible surfaces (38) and color-coded molecular lipophilicity patterns (MLPs) (39, 40) were generated using the MOLCAD modeling program (41, 42).

RESULTS

Growth of K. pneumoniae on Sucrose and Its Isomers—Recently (12) we reported the growth of K. pneumoniae on sucrose and its five isomeric α-D-glucosyl-D-fructoses (see Fig. 1 for structures). Additionally, we showed that organisms grown previously on a particular isomer readily fermented sucrose as its five linkage isomeric glucosyl-fructoses, and of some related disaccharides (R = H) and their respective monophosphates (R = PO₃⁻), invariably carrying their phosphate ester groups attached to the glucosyl-C-6. For the reducing disaccharides, only the tautomeric form predominating in solution (10, 11) is depicted. The nonreducing sucrose-6-P is the singular substrate for the sucrose-6-P hydrolase, whereas all others are hydrolyzed by the 6-phospho-D-glucosidase described herein.

Phospho-α-glucosidase activity is readily detected by the intensely yellow p-nitrophenolate (pNP) anion released upon hydrolysis of pNPαGlc6P. This chromogenic substrate was rapidly hydrolyzed by extracts of cells grown on the glucosyl-fructoses and other α-glucosides, but essentially no activity was detectable in the extract from sucrose-grown cells (Table I). Western blots performed with antibody raised against phospho-α-glucosidase from F. mortiferum (20) revealed a striking correlation between the amount of induced immunoreactive protein of ~50 kDa (Fig. 3) and the hydrolytic activities of the various extracts (Table I). The protein induced during growth on the five α-D-glucosyl-D-fructoses was thus identified as phospho-α-glucosidase.

Cloning and Sequence Analysis of the Agl Region of K. pneumoniae—Although suggestive, the available data (Figs. 2 and 3 and Table I) did not establish a functional role for phospho-α-glucosidase in dissimilation of the five α-D-glucosyl-D-fructoses by K. pneumoniae. Recently, we demonstrated the PEP-dependent phosphorylation of the five sucrose isomers via the PTS activity of maltose-grown cells of K. pneumoniae, and trehalulose-6'-P, turanose-6'-P, maltulose-6'-P, leucrose-6'-P, and palatinose-6'-P were prepared in 20–50-mg amounts (12). To determine whether these derivatives were hydrolyzed by AglB, it was first necessary to purify this enzyme. To this end, aglB, the gene encoding the phospho-α-glucosidase, and an adjacent upstream gene, aglA, were cloned and sequenced. Fig. 4 shows the ~3.5-kilobase pair nucleotide sequence containing the two genes (aglA and aglB) of the alpha-glucose utilization region of the K. pneumoniae genome. The aglA gene comprises a coding sequence of 1,619 nucleotides commencing with an ATG codon at position 394 and terminating with a TGA (stop) codon at position 2014. This open reading frame encodes a

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2 The major part of the MOLCAD program is included in the SYBYL package of TRIPPOS Associates, St. Louis, MO.
polypeptide of 540 residues (calculated Mr/H11005 58,373) that contains fused C and B domains characteristic of a membrane-localized EII(CB) transport protein of the PTS (46). The aglA gene is preceded by a potential ribosome-binding site (GAGGA) centered 11 nucleotides from the start codon. The aglA stop codon overlaps the Met start codon of aglB. The latter gene extends from nucleotide 2013 and terminates with a TAA codon at position 3333. Translation of aglB predicts a polypeptide of 440 amino acids (calculated Mr/H11005 49,256), in which residues 138–169 display the signature pattern of Family 4 glycosyl hydrolases (47, 48): 3 P X (S/A)(W/T)(L/I/V/M/F)2(Q/N) X 2NP X 4(T/A) X 9,10(K/R) X (L/I/V) X C. From the alignment shown in Fig. 5, it is clear that AglB exhibits homology with phospho-glucosidases from other species including MalH from F. mortiferum (75% identity), GlvA from B. subtilis (72%), and truncated GlvG from E. coli (77%), respectively.

Purification of Phospho-glucosidase (AglB) from E. coli TOP(pAP-16)—Cells of E. coli TOP(pAP-16) produced high levels of an IPTG-inducible protein with an estimated Mr/H11005 50 kDa as expected for the full-length polypeptide encoded by aglB (Fig. 6A, lane 1). This protein cross-reacted with phospho-glucosidase antibody (Fig. 6B, lane 1), and the cell extract catalyzed the immediate hydrolysis of pNP-Glc6P. AglB was purified by conventional low-pressure chromatography, and to stabilize the enzyme, 0.1 mM NAD

<sup>a</sup> μmol pNP-Glc6P hydrolyzed min<sup>−1</sup> mg protein<sup>−1</sup>.
<sup>b</sup> Compounds in boldface are sucrose isomers.
<sup>c</sup> Values in parentheses = rate as % activity in maltulose extract.
<sup>d</sup> ND, no detectable activity.

**Fig. 2.** Analysis by two-dimensional PAGE of proteins in extracts prepared from cells of *K. pneumoniae* grown on various disaccharides. The white circles indicate the induced ~50-kDa phospho-α-glucosidase (AglB) in organisms grown previously on either maltose, palatinose, or maltulose. This protein was not detectable (white arrow) in the extract prepared from sucrose-grown cells of *K. pneumoniae*. Approximately 50 μg of protein was applied per gel, and polypeptides were visualized by silver staining. Prior to electrophoresis, tropomyosin (1 μg) was added to each sample as an IEF internal standard. This protein (black arrowhead) migrates as a doubles with the lower polypeptide spot ~33 kDa and pI = 5.2.

**Fig. 3.** Western blot showing the sugar-specific induction and cross-reactivity of the ~50-kDa protein (AglB) with antibody raised against purified MalH (phospho-α-glucosidase) from *F. mortiferum* (20). Extracts were prepared from cells of *K. pneumoniae* grown on the indicated sugars, and approximately 15 μg of protein was applied per lane. Note the absence of immunoreactive protein in sucrose-grown cells. Stds., standards.

### Table 1

| Growth sugar      | Rate of pNPαGlc6P hydrolysis<sup>a</sup> |
|-------------------|------------------------------------------|
| Maltulose (α,1-4)<sup>b</sup> | 0.256 (100)<sup>c</sup> |
| Palatinose (α,1-6) | 0.214 (84) |
| Leucrose (α,1-5) | 0.202 (79) |
| Trehalulose (α,1-1) | 0.160 (63) |
| Methyl-α-glucoside | 0.119 (46) |
| Turanose (α,1-3) | 0.096 (38) |
| Maltitol | 0.073 (29) |
| Maltose | 0.031 (12) |
| Sucrose | 0.002 (<1) |
| Cellulose | 0.002 (<1) |
| Galactose | ND<sup>d</sup> |
| Trehalose | ND |
| Glucose | ND |

<sup>a</sup> Values in parentheses = rate as % activity in maltulose extract.
<sup>b</sup> Compounds in boldface are sucrose isomers.
<sup>c</sup> μmol pNP-Glc6P hydrolyzed min<sup>−1</sup> mg protein<sup>−1</sup>.
<sup>d</sup> ND, no detectable activity.
the purification of AglB was monitored by enzymatic assay (Table II), SDS-PAGE (Fig. 6A), and immunoblot methods (Fig. 6B). Approximately 70 mg of electrophoretically pure enzyme was obtained from 38 g wet weight of cells. Although purified in reasonably active form, AglB was progressively inactivated throughout the purification, and the specific activity of the final preparation (4.2 units/mg) was only 3-fold higher than that of the original dialyzed cell extract (1.2 units/mg).

**Properties of AglB**—The molecular weight of AglB determined by electrospray/MS (Mr 49,254) was within two units of the theoretical weight average Mr of 49,256 deduced from the amino acid sequence encoded by aglB. However, in the final stage of purification, AglB emerged from the AcA-44 gel filtration column in a volume suggestive of a protein of molecular mass ~100 kDa. Cross-linking studies also revealed the formation of a similarly sized product after incubation of the enzyme with various homo-bifunctional imidoesters (Fig. 6C, lanes 2–4). It appears likely that in solution AglB exists as a catalytically active homodimer. Analytical electrofocusing revealed two species (Fig. 6D, lane 2) having estimated pI values of 5.4 and 5.6 that agreed fairly well with the theoretical pI (5.69) deduced from the amino acid composition of AglB.

**Cofactor, Metal Ion Requirements, and Substrate Specificity of AglB**—Phospho-glucosidases MalH and GlvA from F. mortiferum (20, 44) and B. subtilis (45), respectively, exhibit requirements for nucleotide (NAD+) and divalent metal ion (Mn2+, Co2+, or Ni2+) for activity. AglB exhibited similar requirements and, in the absence of these cofactors, was unable to hydrolyze pNP-Glc6P (Table III). Inclusion of NAD+ in the assay elicited substrate cleavage, but enzyme activity increased 3–6-fold upon further addition of Mn2+, Co2+, or Ni2+. Other divalent metal ions tested, including Mg2+, Ca2+, and Zn2+, were either without effect or were inhibitory. The activity...
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Residues of the polypeptide SLPSRLPAILQAVMQGQPQAL-ADSHYPQ. Sucrose-6-P hydrolase catalyzed the hydrolysis of sucrose and sucrose-6-P at comparable rates ($V_{\text{max, sucrose}} = 31.2 \pm 1.1$; $V_{\text{max, S6P}} = 40.4 \pm 2.3$ $\mu$mol hydrolyzed min$^{-1}$). However, the affinity of the enzyme for the phosphorylated disaccharide ($K_{\text{m, S6P}} = 85.3 \pm 15.1$ $\mu$M) was $>200$-fold greater than for sucrose ($K_{\text{m, sucrose}} = 20.3 \pm 1.9$ $\mu$M). There was no detectable hydrolysis (at 1 $\mu$M) of any of the phosphorylated isomers of sucrose, and sucrose-6-P hydrolase failed to hydrolyze other phospho-$\alpha$-glucosides including maltose-$6'$-$P$ and trehalose-$6'$-$P$.

Conformational Analysis of Sucrose-6-P and Disaccharide-$6'$-phosphates—Insight into the remarkable discrimination of sucrose-6-P hydrolase and phospho-$\alpha$-glucosidase for their substrates was provided by conformational analysis of these phosphorylated compounds using molecular dynamics simulations. Sucrose and sucrose-6-P differ from other disaccharides not only by the fact that they are nonreducing (the two sugar units are linked through their anemic centers) but by the predetermined orientation of the glucose and fructose portions toward each other. In the solid state, the two sugars are conformationally fixed by two intramolecular hydrogen bonds (Fig. 8 and Refs. 26, 27, 50, 51). On dissolution of the disaccharide in water, these bonds are replaced by an $H_2O$ molecule bridging the fructosyl-$O$-1 and glucosyl-$O$-2 through hydrogen bonding (Fig. 8, center, and Ref. 52), to yield an overall conformational close to that in the crystalline state. The molecular geometry of sucrose-6-P in water, which emerges from a nanosecond molecular dynamics simulation in a truncated octahedron box containing 641 water molecules, again is very similar to that of sucrose in the crystal and in aqueous solution (Fig. 8, right), so that a water bridge of the Glc-2-O--H$_2$O--O-1-Fru is likewise to be inferred. A comparison of the molecular geometry of sucrose-6-P in water with the geometries of the nine disaccharide-$6'$-phosphates reveals their distinctly different molecular shapes.

Unlike sucrose-6-P, which by virtue of the intramolecular water bridge between glucose and fructose assumes a remarkably compact conformation in solution, the nine disaccharide-$6'$-phosphates lack any interaction of this type and hence invariably adopt a more extended, longish molecular geometry. These differences in molecular shape are emphasized by juxtaposition of the solvent-accessible surface of sucrose-6-P (Fig. 9, top) with those of the nine disaccharide-$6'$-phosphates shown superimposed in Fig. 9 (bottom).

DISCUSSION

Transport and Hydrolysis of Sucrose and Its Isomers by K. pneumoniae—Circumstantial evidence indicated that the transport and dissimilation of the five $\alpha$-linked isomers of sucrose by K. pneumoniae occurred by a route different from the PTS-sucrose-6-P hydrolase route used for sucrose itself (12). For example, sucrose-grown cells failed to metabolize any of the isomers, and the PEP:PTS activity of cells grown on a particular isomer (e.g., palatinose) catalyzed the phosphorylation of all other isomers. Importantly, growth of K. pneumoniae on the five $\alpha$-glucosyl-$d$-fructoses induced a high level expression of a polypeptide (molecular mass $\sim 50$ kDa) that was not present in organisms grown on sucrose. In this study, the gene (aggB) that encodes the induced protein has been cloned and sequenced, and the protein itself (AggB) has been identified as an $\alpha$-NAD$^+$- and metal-dependent phospho-$\alpha$-glucosidase. The gene aggB lies adjacent to a second gene, aggA, which encodes an EII(CB) component of the PEP:PTS (46). It is our contention that together, AggA and AggB facilitate the phosphorylative translocation and subsequent cleavage of phosphorylated isomers of sucrose (and related $\alpha$-glucosides) by K. pneumoniae.

Properties of sucrose-6-P hydrolase and Phospho-$\alpha$-glucosi-
Phosphogluconsyl Hydrolases from K. pneumoniae

FIG. 6. Determination of the M₉, pI, and structural composition of AglB by analytical PAGE. A, purification and M₉ estimate of AglB. Samples from each stage of purification were denatured, resolved by SDS-PAGE, and stained with Coomassie Brilliant Blue R-250. Lane 1, high-speed supernatant; lane 2, Triton X-100; lane 3, phenyl-Sepharose CL-4B; and lane 4, Ultragel AcA-44. B, Western blot of a duplicate gel of panel A showing cross-reaction of AglB with MaH antibody. Stds, standards. C, cross-linking of AglB subunits to the dimeric state by treatment with: lane 1, no agent; lane 2, dimethylpimelimidate; lane 3, dimethylpimelimidate; and lane 4, dimethylsuberimidate. D, determination of the pI of AglB by analytical electrofocusing (lane 2).

TABLE II
Summary of the purification of AglB (phospho-alpha-glucosidase) from E. coli TOP 10 (pAP-16)

| Purification step                  | Total protein (mg) | Total activity (units) | Specific activity (units/mg) | Purification fold | Yield (%) |
|-----------------------------------|--------------------|-----------------------|-----------------------------|-------------------|-----------|
| Dialyzed high-speed supernatant   | 2304 ± 40          | 2815 ± 20             | 1.22 ± 0.05                 | 1 ± 0              | 100 ± 4   |
| TrisAcryl-M-DEAE                  | 618 ± 10           | 1743 ± 10             | 2.82 ± 0.10                 | 2.3 ± 0.1          | 62 ± 2    |
| Phenyl-Sepharose CL-4B            | 174 ± 10           | 715 ± 10              | 4.12 ± 0.10                 | 3.4 ± 0.1          | 25 ± 1    |
| AcA-44                            | 72 ± 10            | 299 ± 10              | 4.15 ± 0.10                 | 3.4 ± 0.1          | 11 ± 0    |

a Units expressed as μmol of pNP/Glc6P hydrolyzed min⁻¹.

TABLE III
NAD⁺ and metal ion requirements for activity of AglB (phospho-alpha-glucosidase)

The purified enzyme had been dialyzed against 25 mM Tris-HCl buffer (pH 7.5).

| Addition to assay | Specific activity |
|------------------|------------------|
| No additions     | ND               |
| + NAD⁺           | 0.35             |
| + Mg²⁺           | 0.01             |
| + NAD⁺ + Mg²⁺    | 0.32             |
| + Ni²⁺           | 0.11             |
| + NAD⁺ + Ni²⁺    | 0.59             |
| + Co²⁺           | 0.26             |
| + NAD⁺ + Co²⁺    | 1.01             |
| + Mn²⁺           | 0.25             |
| + NAD⁺ + Mn²⁺    | 1.35             |

a The 2-ml assay solution contained 50 mM Tris-HCl buffer (pH 7.5). When required, appropriate divalent metal ion (1 mM) and NAD⁺ (0.5 mM) were included. Phospho-alpha-glucosidase (60 μg) was added, and after 2 min of preincubation at 25 °C, pNP/Glc6P was added to a final concentration of 1 mM. Hydrolysis of substrate (i.e. formation of pNP) was followed as described under “Experimental Procedures.”
b Expressed as μmol of pNP/Glc6P hydrolyzed min⁻¹ mg enzyme⁻¹.
ND, no detectable activity.

dase (AglB)—In some of their properties, sucrose-6-P hydrolase and AglB show similarity. For example they are of comparable (monomer) size, they are exacting for the glucose-6-P moiety of their substrates, and both exhibit poor or no affinity for non-phosphorylated disaccharides. However, in their amino acid sequences, cofactor requirements, and assignments to different families of the glycosyl hydrolase superfamily, sucrose-6-P hydrolase and AglB are quite different. The amino acid sequence of sucrose-6-P hydrolase (deduced from the scrB gene (7)) has essentially no homology with that of AglB, and by the amino acid-based sequence classification of Henrissat (47), AglB and sucrose-6-P hydrolase are assigned to Families 4 and 32, respectively, of the glycosyl hydrolase superfamily (48).3 Sucrose-6-P hydrolase has no cofactor requirements, whereas AglB is dependent upon both NAD⁺ and divalent metal ion (Mn²⁺, Ni²⁺, or Co²⁺) for catalytic activity (Table III). Indeed, these cofactor requirements for AglB were predicted by virtue of the extraordinarily high sequence identity between the putative polypeptide encoded by aglB and those of the Family 4 phospho-alpha-glucosidases shown in the multiple alignment in Fig. 5. The role(s) for NAD⁺ and metal ion have not been established.
and it is presently unclear whether these cofactors play catalytic and/or structural roles in AglB and related enzymes of Family 4 (44, 45, 53, 54). Results obtained from site-directed mutagenesis of the phospho-/H9251-glucosidase (GlvA) in B. subtilis (45) suggest that residues close to the N terminus comprise the NAD-/H11001-binding domain (see Fig. 5). Glycosyltransferases comprise a superfamily of Mn2+/H11001-dependent enzymes (55) that use UDP-glucose, UDP-galactose, and related compounds as substrates for modification (via glycosylation) of a wide variety of biological molecules in both prokaryotes and eukaryotes. Most, if not all, members of this large family contain a conserved motif D(X)D that participates in the substrate recognition/catalytic process by interaction of the aspartyl residues with the ribose moiety of the nucleotide or via coordination with Mn2+ ion (56). Interestingly, this motif is also present in AglB and in other phospho-/H9251-glucosidases, and the conserved DND residues lie adjacent to the putative NAD^-binding domain of these enzymes (Fig. 5). Furthermore, site-directed substitution at the first aspartic residue of this motif (D41G and D41E) in GlvA results in loss of hydrolytic activity (45). These findings plus the fact that the D(X)D motif is conserved in other members of Family 4 (see Fig. 4 in Ref. 45) may indicate a role for the two acidic residues in Me2+ ion-binding in AglB and related glycosyl hydrolases.

Substrate Discrimination by Sucrose-6-P Hydrolase and 6-Phospho-/H9251-glucosidase—Sucrose-6-P and its five phosphorylated linkage isomers have recently been prepared and characterized by 1H and 13C NMR spectroscopy (12). The availability of these derivatives in substrate amount permitted specificity and kinetic analyses to be carried out with highly purified sucrose-6-P hydrolase and AglB. These studies establish unequivocally that sucrose-6-P hydrolase hydrolyzes only sucrose-6-P to form glucose-6-P and fructose. The specificity of sucrose-6-P hydrolase for its single substrate (sucrose 6-phosphate) is noteworthy because it suggests that their reciprocal molecular recognition (as a prerequisite to fission of the intersaccharidic linkage to glucose-6-P and fructose) is unique, not even tolerating minor changes in the linkup of the two sugars, as for example those realized in the five isomeric glucosyl-fructoses.

In contrast, the 6-phospho-/H9251-glucosidase (AglB), which is in-
duced by growth of *K. pneumoniae* on the five glucosyl-fructoses, appears to be less specific and is tolerant of a considerable variation in both the structure and size of the O-linked aglycone. Indeed, the NAD$^+$ and metal ion-dependent phospho-$\alpha$-glucosidase hydrolyzed not only the 6-$\alpha$-phosphoglucosyl-fructoses but also the phosphorylated derivatives of related $\alpha$-linked disaccharides such as maltose-6-$\alpha$-P, isomaltose-6-$\alpha$-P, and maltotol-6-P. Remarkably, AglB was unable to hydrolyze sucrose-6-P. Explanations for enzyme specificity and substrate discrimination must reside in the molecular geometries and polarities of the individual disaccharide phosphates and/or in the three-dimensional structures of the two enzymes. Presently, only a structural model based on threading methods has been proposed for those enzymes (including sucrose-6-P hydrolase) that by sequence-based alignment are assigned to Family 32 of glycosyl hydrolases (57). Moreover, only a preliminary x-ray analysis has been reported for one enzyme member (GlvA from *B. subtilis*, (58)) of Family 4, to which AglB is assigned. Thus, we were led to probe the substrates with respect to structure, molecular shape, and polarity for clues to understanding the specificity of the two phosphoglucosyl hydrolases. From the markedly different molecular geometries of the phosphorylated disaccharides in solution (Figs. 8–10), one might reasonably assume that shape recognition (by the respective binding domains) may be an important determinant of enzyme specificity. Another and conceivably more significant contribution to substrate discrimination may originate from differences in the distribution of hydrophobic and hydrophilic regions over the contact surfaces of the disaccharide phosphates. In eliciting the sweetness response, for example, sucrose is believed to dock onto the taste bud receptor protein via its hydrophobic region (59), which, on the basis of the calculated MLP profiles, encompasses the entire outer surface side of the fructose moiety (51, 59). The same docking procedure is expected for sucrose-6-phosphate at the active site of sucrose-6-P hydrolase, inasmuch as the MLP profile of sucrose-6-phosphate (Fig. 10, top center) is essentially the same, i.e., a pronounced hydrophilic 6-phosphoglucosyl part (blue areas) facing a distinctly hydrophobic (yellow) fructose portion. Fig. 10 shows the MLPs of sucrose-6-P and its five isomeric 6-$\alpha$-phosphoglucosyl-fructoses in the fully closed (upper portion) and in the front-side-opened form with ball-and-stick model inserts (lower portion). The MLP patterns of the five 6-$\alpha$-phosphoglucosyl-fructoses, albeit having essentially identical hydrophilic (blue) glucose-6-P halves, clearly differ from sucrose-6-P with respect to the shape, intensity, and distribution of their hydrophobic (yellow) surface domains (Fig. 10). These may perhaps be the major factors that prevent docking of the isomeric phosphates at the sucrose-6-P binding site of sucrose-6-P hydrolase and, conversely, that preclude binding of sucrose-6-P to the active site of phospho-$\alpha$-glucosidase.

**Conclusion**—This study and our earlier paper (12) are the first reports of bacterial growth on the five isomers of sucrose. However, genetic units similar to the Agl region of *K. pneumoniae*...
moniae are present in the genomes of B. subtilis, F. mortiferum, and E. coli (Fig. 11). The phospho-
alpha-glucosidase(s) of these species are clearly homologous (Fig. 5), and the PTS trans-
porter (AglA) has extensive homology with GlvC of B. subtilis, MalB of F. mortiferum, and Glv(CB) of E. coli. The gene organ-
ization is similar in the three Gram-negative species, but for B. subtilis (Gram-positive) the gene order is reversed and a gene glvR, which encodes a regulatory protein, separates the phospho-
alpha-glucosidase and PTS genes (60, 61). Our recent find-
ing that F. mortiferum can also grow on the sucrose isomers \(^4\) suggests that genes homologous to aglA and aglB may be prerequisites for bacterial growth on these compounds. Paren-
thenetically, it may be noted that neither of these genes has been found during sequencing of the S. mutans genome, and these deficiencies may explain the inability of this organism to metabolize the sucrose isomers.

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