Isolation of a Glucosamine-specific Kinase, a Unique Enzyme of Vibrio cholerae

Jae Kweon Park, Lai-Xi Wang, and Saul Roseman

From the Department of Biology and the McCelland-Pratt Institute, The Johns Hopkins University, Baltimore, Maryland 21218 and the Institute of Human Virology, University of Maryland Biotechnology Institute, The University of Maryland, Baltimore, Maryland 21201

We showed previously that chitin catabolism by the marine bacterium Vibrio furnissii involves at least three signal transduction systems and many genes, several of which were molecularly cloned, and the corresponding proteins were characterized. The predicted amino acid sequences of these proteins showed a high degree of identity to the corresponding proteins from Vibrio cholerae, whose complete genomic sequence has recently been determined. We have therefore initiated studies with V. cholerae. We report here a novel ATP-dependent glucosamine kinase of V. cholerae encoded by a gene designated gspK. The protein, GspK (31.6 kDa), was purified to apparent homogeneity from recombinant Escherichia coli. The product of the reaction was shown to be GlcN-6-P by matrix-assisted laser desorption/ionization-time of flight (MALDI mass spectrometry) and NMR. The $K_v$ values for GlcN, ATP, and MgCl$_2$ were 0.45, 2.4, and 2.2 mm, respectively, and the $V_{max}$ values were in the range 180–200 nmol/$\mu$g/min (–6 nmol/pmol/min). Kinase activity was not observed with any other sugar, including galactosamine, mannosamine, Glc, GlcNAc, GalNAc, mannose, 2-deoxyglucose, and oligosaccharides of chitosan. The enzyme is also ATP-specific. The kinase can be used to specifically determine micro quantities of GlcN in hydrolysates of glycoconjugates. The physiological function of this enzyme remains to be determined.

We have reported that the chitin catabolism cascade in marine bacterium Vibrio furnissii comprises several signal transduction pathways and many proteins (reviewed in Ref. 1). Among the family Vibrionaceae, Vibrio cholerae is one of the most important with respect to human health and disease. It is a Gram-negative marine bacterium and a human intestinal pathogen that resides in both brackish water and seawater. In the marine environment, V. cholerae is closely associated with copepods, microscopic crustaceans that comprise the most abundant animals on earth. Seasonal blooms in the copepod population in inhabited regions, such as the Ganges delta, coincide with blooms in the chitinivorous V. cholerae and outbreaks of cholera in the human residents (2). The bacteria are said to be protected against stomach acids, the major barrier against human infection, by “burrowing” into the copepod cuticles (3).

Although we have acquired a significant body of information on the chitin catabolic cascade in the bacterium V. furnissii, little is known concerning the pathway in V. cholerae. The complete DNA sequence of the V. cholerae genome has recently been reported (4) and, as we shall show elsewhere, there is a high degree of identity in the predicted amino acid sequences of the proteins we have identified in V. furnissii and their presumptive counterparts in V. cholerae. A number of genes identified previously in V. furnissii appeared to be clustered in the genome of V. cholerae, and since they may constitute a chitin degradation operon, we are currently attempting to identify the functions of the other presumptive genes by subcloning them into Escherichia coli, expressing the proteins, and determining their functions.

As shown under “Experimental Procedures,” we have cloned a gene, designated gspK (0.8 kb), encoding a glucosamine-specific kinase, GspK (31.6 kDa), from the chromosomal DNA of V. cholerae. Furthermore, analysis of the nucleotide sequence of gspK and its putative amino acid sequence indicated that the predicted amino acid sequences of GspK from V. cholerae are 100% identical to a hitherto unrecognized gene in V. furnissii. Although the ubiquitous enzyme, hexokinase, phosphorylates many sugars, including glucosamine and mannosamine (5), and although a GlcNAc-specific ATP-dependent kinase (6) is present in V. furnissii (7), there are apparently no previous reports on a GlcN-specific ATP-dependent kinase.

EXPERIMENTAL PROCEDURES

Materials

The following chemicals, reagents, and materials were purchased from the indicated sources: ADP, ATP, P-enolpyruvate, NADH, Glc, Fru, Man, Gal, GlcN, GlcNAc, mannosamine (ManN), galactosamine (GalN), and other carbohydrates from Sigma unless otherwise indicated; chitosan oligosaccharides (GlcN), $n = 1–3$, and chitin oligosaccharides (GlcN), $n = 1–2$, from Seikagaku America, Inc. (Rockville, MD); glucose oligosaccharides (Glc), $n = 1–5$ and reagents for bacterial media from Difco and J. T. Baker. Reagents for molecular biology were obtained from New England Biolabs (Beverly, MA), Stratagene, and U. S. Biochemical Corp. Pyruvate kinase and lactic acid dehydrogenase for the kinase assay were purchased from Sigma. Other buffers and reagents were of the highest purity commercially available.

1 An extract of Schistosoma mansoni (28), known to contain several sugar kinases, was found to phosphorylate GlcN, as well as other sugars such as Glc. It was partially fractionated, and the kinase activity for GlcN was separated from that for Glc. However, no other sugars were assayed with the GlcN active fraction.

2 The abbreviations used are: P-enolpyruvate, phosphoenolpyruvate; MALDI, matrix-assisted laser desorption/ionization-time of flight; TAPS, (2-hydroxy-1,1-bis(hydroxymethyl)ethyl)amino)-1-propanesulfonic acid.
Glucosamine-specific Kinase from *V. cholerae*

**Growth and Maintenance of Strains**

*V. cholerae* E1 Tor N15691 is the strain used for obtaining the DNA sequences of the two chromosomes (4) and was used in the present work, along with *V. furnisii* 1519, which is similar to 1514, the strain used in our earlier work. Both *Vibrio* strains were grown either in high salt LB (LMB, Luria broth supplemented with an additional 10 g of NaCl/liter) or in minimal media 50 mM HEPES buffer, pH 7.5, containing 50% artificial seawater, 0.5% NH4Cl, 0.01% KH2PO4, and 0.5% DL-lactate (7). *E. coli* strains BL21(DE3) (Novagen) and XL1-Blue (Stratagene) harboring designated plasmid constructs were stored as frozen cultures in LB with 5% glycerol at −80 °C. Typically, *E. coli* strains were grown overnight in LB media plus appropriate antibiotics with vigorous shaking. Fresh medium was inoculated with cells from the overnight culture at a 1:20 dilution, and this culture was grown to mid-exponential phase, usually to a density of OD600 of 0.3–0.4 at 37 °C with vigorous aeration.

**Molecular Analysis and Construction of pBTgskK**

DNA preparations, restriction enzyme digests, ligation, and transformations were performed using standard techniques (8). The glucosamine-specific kinase gene, *gskK*, located at VC0614 in the *V. cholerae* genome was amplified by PCR using synthesized primers based on the gene sequence of the chromosomal DNA of *V. cholerae*. The 5′ PCR primer was designed to contain an Ndel restriction site to facilitate cloning into the start site following a T7 promoter in the overexpression vector pET21a (Novagen, Madison, WI). The primers used to construct the overexpression plasmids were: 5′-AGAAAAATATGCGTTACTG-ACCAA-3′ and 5′-GCAACGATTCTTGAATTACACT-3′. The amplified PCR fragment (2.5 kb) contained both the bgla gene (1.4 kb) described elsewhere in this paper and the gskK gene (0.8 kb). The PCR fragment was transferred into the Ndel (5′ end) and HindIII (3′ end) of pET21a. The 2.5-kb PCR fragment contains *BamHI* and *HindIII* restriction enzyme sites, between which is the DNA sequence for the entire *gskK* (0.8 kb) gene. The fragment was isolated, ligated into pET21a, and transformed into the T7 polymerase-inducible host strain *E. coli* BL21(DE3). Transcription in BL21(DE3) is under control of the T7 promoter, and a start site for the *gskK* gene is 79 bp downstream from the *BamHI* restriction site. Control cells consisted of wild type *E. coli* and of the same strain transformed with the vector pET21a without the DNA insert. Extracts of the controls exhibited no GspK activity.

**Purification of the Glucosamine-specific Kinase (GspK)**

Protein concentration was measured with the Bio-Rad protein assay using bovine serum albumin as the standard.

**Step I—Crude Extracts**—A single colony of *E. coli* strain BL21(DE3) harboring pET2gskK was inoculated into 100 ml of LB medium supplemented with 50 μg/ml ampicillin and grown overnight at 37 °C with vigorous shaking. Two liters of LB medium supplemented with 1 mM isopropyl-l-thio-β-D-galactopyranoside (final concentration) in a 6-liter flask was inoculated with 50 ml of the overnight culture and allowed to grow at 37 °C with aeration until OD600 = 0.3–0.4. The cells were harvested by centrifugation at 4,000 rpm at 4 °C for 10 min. Subsequent steps were performed at 4 °C unless otherwise specified.

The cell pellet (4.1 g) was washed twice with 800 ml of 20 mM Tris chloride buffer containing 0.1 mM NaCl and 1 mM EDTA, pH 7.5, and resuspended in 35 ml of 20 mM Tris chloride buffer with 1 mM EDTA, pH 7.5. The cells were disrupted by two passages through a Wabash French pressure cell. Unlysed cells were removed by centrifugation at 10,000 × *g* for 10 min.

**Step 2—Streptomycin Sulfate Precipitation**—Nucleic acids were precipitated using streptomycin sulfate (160 μl of 10% stock solution/ml of crude extract), added dropwise with stirring. The mixture was stirred for an additional 1 h and centrifuged at 100,000 × *g* for 30 min.

**Step 3—Ammonium Sulfate Fractionation**—Proteins in the streptomycin sulfate-treated supernatant (40 ml) were precipitated by the dropwise addition of saturated ammonium sulfate solution to a final concentration of 55%. The solution was stirred for an additional 1 h and centrifuged at 150,000 × *g* for 1 h. The ammonium sulfate pellet was resuspended in 30 ml of 20 mM sodium phosphate buffer containing 50 mM NaCl, pH 7.0, and dialyzed against the same buffer.

**Step 4—DEAE-Column Chromatography**—The 55% ammonium sulfate fraction was transferred to a 200 ml DEAE-Sepharose fast-flow column equilibrated with the phosphate/NaCl buffer in Step 3. After the sample was applied, the column was washed with 2.5 ml volumes (500 ml) of buffer, and a linear gradient (800 ml) from 50 mM NaCl to 1.0 M NaCl in the 20 mM phosphate buffer was used. The activity eluted between 0.6 and 0.7 M NaCl, the active fractions were pooled and dialyzed against 20 mM Tris chloride buffer, pH 7.5, containing 0.2 mM NaCl.

**Step 5—Immolized Metal Affinity Column Chromatography**—The pooled sample from Step 4 was transferred to an immolized metal affinity (15-m1 bed volume) column (Amersham Biosciences), which was pretreated with 0.2 μl ZnCl2. The column was washed with 10 volumes of water, treated with 20 mM Tris chloride buffer, pH 7.5, containing 0.2 mM EDTA, and the column was washed with 2 bed volumes of the same buffer and eluted with a gradient from 0 to 0.1 M imidazole in the same buffer. The active fractions were pooled and dialyzed first against 20 mM Tris chloride buffer, pH 7.5, containing 50 mM NaCl and 1 mM EDTA and then against the same buffer containing 1 mM diithiothreitol without EDTA.

**Step 6—Q-Sepharose Column Chromatography**—The active pooled fractions from Step 5 were transferred to a Q-Sepharose column (15-m1 bed volume), which had been equilibrated with 20 mM Tris chloride buffer, pH 7.5, containing 50 mM NaCl. After sample loading, the column was washed with 3 volumes (45 ml) of the same buffer, and a gradient (160 ml) from 50 mM NaCl to 1.0 M NaCl was applied to elute the column. The activity eluted between 0.2 and 0.3 M NaCl. The active fractions were pooled, concentrated, and dialyzed against 20 mM Tris chloride buffer, 10 mM NaCl, pH 7.5. Purity was monitored throughout the fractionation by SDS-PAGE. The purified preparation was stored in small aliquots with diithiothreitol (1.0 mM final concentration) at −20 or −80 °C until used for further study. In the absence of diithiothreitol, insoluble aggregates were occasionally observed.

**Enzyme Assay**

Several methods have been used in this laboratory for measuring the rate of sugar-P synthesized by specific kinases and ATP, or, by the phosphoenolpyruvate:glycose phosphotransferase system. These include the use of [γ-32P]ATP or [32P]enolpyruvate or H- or O-labeled sugar. The labeled hexose-P is separated from the labeled substrates by paper electrophoresis or TLC or small ion-exchange columns. In the present studies, we elected to measure ADP instead by a spectrophotometric two-step assay because of the availability, stability, and sensitivity of a Cary Bio 100 Varian spectrophotometer kindly made available for our use by Dr. Ernesto Freire of this department. This type of assay has been used frequently for measuring sugar kinase activities. In the first step, GlcN, ATP, and Mg2+ were incubated with the enzyme, and the reaction was stopped by heating. In the second step, the quantity of ADP produced by the kinase was determined with P-enolpyruvate, pyruvate kinase, NADH, and lactate dehydrogenase. GlcN was omitted from controls to correct for any ADP formed from contaminating enzymes, such as ATPase.

**Step II—Protein fractions to be assayed** were added to the following (final volume, 100 μl): 25 mM Tris chloride buffer, pH 7.0, 10 mM MgCl2, 10 mM ATP, 5 mM GlcN, HCl, or other potential sugar substrates. The enzyme reaction was initiated by the addition of the protein fraction to be assayed or of 0.1 μg of the purified enzyme (unless otherwise specified). Incubations were conducted at 25 or 37 °C, depending on the activity, for 0–10 min. When apparently inactive substrates were tested, incubations were continued at 0 °C overnight at 25 °C. Reactions were stopped by boiling for 2 min at 100 °C.

**Step II—the Quantity of ADP formed in the kinase reaction was measured by determining either (a) the total quantity of NADH oxidized in the coupled assay or (b) the initial rate of NADH oxidation, which was found to be directly proportional to [ADP].** Step II was conducted at 37 °C in jacketed cell holders. Typically, a 10-μl aliquot from Step I was added to 1 ml containing the following mixture: 25 mM Tris chloride buffer, 0.1 mM KCl, pH 7.6, 12.5 mM MgCl2, 0.5 mM P-enolpyruvate, 0.15 mM NADH. The reaction was started by adding 0.5 units each of the coupling enzymes, pyruvate kinase and lactic acid dehydrogenase, and the absorbance was measured continuously for 10–15 min or until no change was observed. The initial absorbance at 340 nm was about 0.90. No change in absorbance was observed with boiled enzyme as the negative control or by omission of either of the coupling enzymes. In the alternate and preferred method, the initial rate of NADH oxidation was measured by determining the slope of the line, i.e., the decrease in initial absorbance as a function of time. Although this reaction is curvilinear over the course of the complete oxidation (10–15 min), the initial rate of oxidation is virtually linear, and a computer linked to the spectrophotometer was used to determine the slopes of these lines.

The slopes were found to be proportional to the quantity of ADP in the reaction mixture in the range of 10–100 nmol per incubation. ADP formation was also proportional to incubation time and the quantity of protein used in the first step of the assay (see Fig. 2a) and was glucosamine-dependent. That is, the formation of ADP in the absence of glucosamine...
was negligible relative to ADP formed in the complete kinase reaction mixture, even when crude extracts were assayed, probably because the kinase constitutes about 5% of the total protein when it is overexpressed.

### Determination of the N-terminal Amino Acid Sequence

The N-terminal amino acid residues of purified recombinant enzyme glucosamine-specific kinase GspK were determined by Dr. Robert Cole using an Applied Biosystems 475A protein sequencer (Amino Acid Sequencing Facility, Department of Biological Chemistry, The Johns Hopkins School of Medicine).

### Effects of pH, Ionic Strength, and Temperature on Enzyme Activity

These parameters were studied with the purified enzyme at 37 °C to determine the optimal conditions for kinetic characterization. Typically, a substrate concentration of 5 mM glucosamine/HCl (GlcN) was used. The following buffer systems were used for the pH studies: sodium citrate buffer, pH 3.0–6.5; imidazole buffer, pH 6.5–7.7; Tris chloride buffer, pH 7.0–9.0; TAPS buffer, pH 7.7–9.0; and glycine-NaOH buffer, pH 8.5–10.0. Where possible, overlapping pH ranges were used with different buffers. The effect of ionic strength on enzyme activity was determined using 25 mM Tris chloride buffer, pH 7.0, supplemented with 0–0.5 M NaCl or KCl.

Two types of temperature studies were conducted: (a) The temperatures of the incubation mixtures were varied between 4 and 60 °C. (b) The thermal stability of the enzyme was measured over the temperature range from 4 to 65 °C using the purified enzyme (0.1–0.5 μg) in 25 μl of 25 mM Tris buffer, pH 8.0, incubating at the desired temperature for 20 min, cooling to room temperature, and then initiating reactions by adding the substrates. Enzyme activity was determined as described above.

### Purification and Identification of GlcN-P

Glucosamine-phosphate (GlcN-P) was purified by ion-exchange column chromatography as described (5). Typically, the following materials (in millimoles) are dissolved in 100 ml of distilled water: β-glucosamine/HCl (5.0), ATP (10.0), and MgCl₂ (5.0). The solution was adjusted to pH 7.5 with 0.5 M NaCl or KCl. The following buffer systems were used for the pH studies: sodium citrate buffer, pH 3.0–5.0; glycine-NaOH buffer, pH 7.5–10.0; and glycine-NaOH buffer, pH 8.5–10.0. Where possible, overlapping pH ranges were used with different buffers. The effect of ionic strength on enzyme activity was determined using 25 mM Tris chloride buffer, pH 7.0, supplemented with 0–0.5 M NaCl or KCl.

Two types of temperature studies were conducted: (a) The temperatures of the incubation mixtures were varied between 4 and 60 °C. (b) The thermal stability of the enzyme was measured over the temperature range from 4 to 65 °C using the purified enzyme (0.1–0.5 μg) in 25 μl of 25 mM Tris buffer, pH 8.0, incubating at the desired temperature for 20 min, cooling to room temperature, and then initiating reactions by adding the substrates. Enzyme activity was determined as described above.

### Purification and Properties of Recombinant Glucosamine-specific Kinase—The enzyme was purified about 21-fold (Table I) from crude extracts of E. coli BL21(DE3) harboring pET-gspK as described under “Experimental Procedures.” The final product was an apparently homogenous protein (Fig. 1), which migrated at ~30 kDa relative to the markers. The N-terminal sequence of the final preparation was Met-Asn-Tyr-Tyr-Val-Gly-Asp-Ile-Asp-Gly-Gly-Thr. This sequence is identical to the N-terminal amino acid sequence predicted from the nucleotide sequence. The DNA sequence of the gene predicts that the protein, GspK, is 31.6 kDa and contains 294 amino acids.

### Properties of GspK—Enzyme activity was determined as a function of pH, ionic strength, temperature, and other parameters as described under “Experimental Procedures.” In collecting the following results, shown in Fig. 2, assays were conducted so that product formation was proportional to protein concentration and time of incubation.

The pH optimum of the purified enzyme was between pH 7.5–8.5. At the pH optimum, enzyme activity was measured as a function of ionic strength with 0–0.5 M NaCl and KCl. There was only a small effect of salts on the enzyme, the activity being inhibited 11–14% at 0.5 M salt concentrations.

Purified GspK displayed a temperature optimum from 40–42 °C. However, the enzyme is not very stable to heat. When incubated for 0.5 h in the temperature range 4–30 °C, it retained full activity, and it retained at least 91% of its activity at 37 °C. At 65 °C, it lost 100% of its activity. It should be noted, however, that these temperature stability studies were conducted at low enzyme concentrations, and activity may conceivably have been retained at higher concentrations or in the presence of stabilizing substances, such as albumin and the enzyme substrates.

### Effect of Substrate Concentrations on Enzyme Activity—The effects of each substrate concentration on enzymatic activity were systematically investigated. Fig. 3 shows the rate versus substrate concentration of glucosamine, ATP, and Mg²⁺. The kinetic parameters (Kₘ and Vₘₐₓ) of ATP and Mg²⁺ were determined (Fig. 3, A and C) and glucosamine (Fig. 3, B and C) were calculated from Woolf-Augustinsson-Hofstee (ν versus ω/K) plots (11), and were found to be: glucosamine, Kₘ = 0.48 mM, Vₘₐₓ = 196 nmol/min/μg (or 6.1 nmol/min/μmol); ATP, Kₘ = 1.96 mM, Vₘₐₓ = 186 nmol/min/μg (5.8 nmol/min/μmol); Mg²⁺, Kₘ = 1.98 mM, Vₘₐₓ = 186 nmol/min/μg (6.6 nmol/min/μmol). The results are based on the averages of three separate experiments each.

### Substrate Specificity of GspK—The enzyme was relatively specific for ATP, exhibiting about 10% activity with GTP and no detectable activity with ITP, UTP, or CTP.

GspK is specific for glucosamine. The following sugars were inactive (no ADP formation) in assays conducted for as long as 12 h: galactosamine, mannosamine, glucose, 2-deoxyglucose, 2-deoxygalactose, D,L-arabinose, fructose, galactose, mannose,
was used in the negative mode, a peak of 258.04 was obtained, which corresponds to the mass of the fully protonated acid is 259.2 Da. When MALDI-TOF mass spectrometry was performed, the purified enzyme displayed a molecular mass of ~30 kDa. The gel was stained using Coomassie Brilliant Blue. The band marked with the arrow was observed in crude extracts of control cells, and the band was not observed in crude extracts from the ion-exchange resin. E. coli BL21(DE3) transformed with pET21a.

Identification of the Phospho-GlcN—Two methods were used to determine whether GlcN could be detected in the hydrolysates of glycans. Such hydrolysates contain many byproducts, especially of amino acid/sugar interactions (12). Lane M, molecular size standards; Lane 1, purified glucosamine-specific kinase (2 µg); Lane 2, crude extract (25 µg). Samples were derived from pET-glspK and analyzed by SDS-PAGE. The purified enzyme contained a molecular mass of ~30 kDa. The gel was stained using Coomassie Brilliant Blue. The band marked with the arrow was not observed in crude extracts of control cells, and the band was not observed in crude extracts from the ion-exchange resin. E. coli BL21(DE3) transformed with pET21a.

Assay for Glucosamine—It appeared likely that the enzyme could be used for the quantitative determination of glucosamine. The most persistent analytical problem in hydrolysates of glycans is that many of these substances contain both GlcN and GalN. Galactosamine was therefore added at various concentrations to the standards and was found to have no effect. That is, it neither acted as a substrate nor inhibited the activity of the enzyme toward GlcN (data not shown).

Finally, it was important to determine whether GlcN could be detected in the hydrolysates of glycans. Such hydrolysates contain many byproducts, especially of amino acid/sugar interactions (the so-called “browning reaction”), and sometimes these interfere with the commonly employed colorimetric methods for GlcN. Two different specimens of seagull egg white, containing multiple glycoproteins, were therefore hydrolyzed with 4 M HCl for 6 h at 100 °C, and the acid was removed in vacuo. The residues were dissolved and divided into aliquots. One set of aliquots was analyzed by borate ion-exchange chromatography (Dionex), and the other was analyzed by the kinase procedure. Within experimental error, both methods gave the same results (data not shown). The egg white hydrolysates and ion-exchange chromatograms were kindly performed by Drs. Noriko Suzuki and Y. C. Lee of this department. These results indicate that the kinase can be used to specifically quantitate GlcN in mixtures of sugars, amino acids, etc.

Identification of the Phospho-GlcN—Two methods were used to characterize the product eluted from the ion-exchange resin, MALDI-MS and NMR. The ion-exchange method of purification yields the free acid (12); GlcN-P is a zwitterion. Therefore, the expected molecular mass of the fully protonated acid is 259.2 Da. When MALDI was used in the negative mode, a peak of 258.04 was obtained, indicating substitution at this position.
DISCUSSION

The properties of a specific glucosamine kinase are described in this report. Neither GalN added to the incubation nor a hydrolysate of crude seagull egg white interfered with the assay, and the recovery of GlcN from the egg white proteins was excellent (by comparison with an independent method). The standard assay conditions used in this report can determine 10 nmol of GlcN but are easily modified for much greater sensitivity. For instance, the first step in the coupled assay, in which the ADP is generated, comprises a total volume of 0.1 ml, and in the second step, in which the ADP is measured, the volume is 1.0 ml. In the standard assay, only 10 of 100 of the first incubation are added to the second, and this can be increased. Secondly, each of the incubation volumes could be reduced 5-fold or more, which would give a 25-fold increase in sensitivity.

The identification of a glucosamine-specific kinase in *V. cholerae* leads to an important question. What function does it serve? The enzyme is cytoplasmic, but what is the origin of free GlcN in the cytoplasm?

**Fig. 5** presents the problem. To briefly summarize: (a) Chitin oligosaccharides, (GlcNAc)

![Proton NMR spectra of product of kinase reaction (A) and standard D-glucosamine 6-phosphate (B). NMR spectra were collected from samples dissolved in D$_2$O as described under “Experimental Procedures.”](https://www.jbc.org/)

**Fig. 3**. Effects of ATP, Mg$^{2+}$, and glucosamine concentrations on kinase activity. Activity was measured with the coupling assay. Initial rates ($v$) were determined at each of indicated concentrations. *A* and *B*, effects of concentrations of ATP (▲), Mg$^{2+}$ (○), GlcN (□). *C*, Woof-Augustinsson-Hofstee plot. The rate $v$ (nmol/min/µg protein) is plotted versus $v/[S]$. The results represent the averages of four separate experiments. Calculated kinetic constants are given under “Results.”

mine 10 nmol of GlcN but are easily modified for much greater sensitivity. For instance, the first step in the coupled assay, in which the ADP is generated, comprises a total volume of 0.1 ml, and in the second step, in which the ADP is measured, the volume is 1.0 ml. In the standard assay, only 10 of 100 µl of the first incubation are added to the second, and this can be increased. Secondly, each of the incubation volumes could be reduced 5-fold or more, which would give a 25-fold increase in sensitivity.

The identification of a glucosamine-specific kinase in *V. cholerae* leads to an important question. What function does it serve? The enzyme is cytoplasmic, but what is the origin of free GlcN in the cytoplasm?

**Fig. 5** presents the problem. To briefly summarize: (a) Chitin oligosaccharides, (GlcNAc)$_n$, enter the periplasmic space via a specific porin (13) designated 1 in the figure. The smaller sizes of monosaccharides and (GlcNAc)$_2$ allow them to penetrate the cell envelope through constitutive porins labeled c in the figure. (b) In the periplasmic space, (GlcNAc)$_n$ oligomers are converted by two unique enzymes, 2 and 3, a chitodextrinase and a β-N-acetylgalactosaminidase (9, 14), to two products, GlcNAc and (GlcNAc)$_2$. (c) The monosaccharide, GlcNAc, is taken up by the phosphoenolpyruvate:glycose phosphotransferase system (15, 16), specifically by Enzyme II$^{Nag}$. The gene has been cloned.
from *V. furnissii*, and the protein has been characterized (17). The overall reaction of the transport process is: P-enolpyruvate$_{in}$ + GlcNAc$_{out}$ → GlcNAc-6-P$_{in}$ + pyruvate$_{in}$ (d) The further metabolism of GlcNAc-6-P involves two steps, 8, a deacetylase, and 9, a deaminase (18–20), yielding fructose-6-P, NH$_3$, and acetate. The *nag* regulon of *E. coli*, containing the relevant structural and regulatory genes, has been extensively studied by Plumbridge (21–23). (e) There are two other possible sources for the key intermediate, GlcNAc-6-P. In *V. furnissii*, (GlcNAc)$_2$ generated both outside the cell by chitinases and in the periplasm from higher oligosaccharides is taken up unchanged by a specific transporter (24) labeled 4. In the cell, the disaccharide is cleaved by 5, a specific phosphorylase (10), yielding GlcNAc-1-P and GlcNAc. We presume that the GlcNAc-1-P is converted to the 6-P by a specific mutase, 6, known to occur in Neurospora (25) and other cell types (26, 27). (f) The third source of GlcNAc-6-P is a GlcNAc-specific ATP-dependent kinase (6) that is found in *V. furnissii* (7). The free GlcNAc generated from the disaccharide by the phosphorylase (5) is converted to GlcNAc-6-P by this kinase, 7.

The final reaction shown in Fig. 5 is labeled 10, the specific glucosamine kinase described in this report. However, in viewing the known pathway in the figure, there is no obvious source for the cytoplasmic GlcN. What is its origin, extracellular GlcN or periplasmic GlcN, or is GlcN generated in the cytoplasm? To determine whether extracellular GlcN can be utilized, wild type *V. cholerae*, *V. furnissii*, and *E. coli* cells were tested on MacConkey fermentation plates with GlcN, GlcNAc, and Glc. The three cell types fermented each of the sugars. In a second set of experiments, the three strains were tested in synthetic media, each containing one of the sugars. All of the cell types fermented each of the sugars, yielding fructose-6-P, NH$_3$, and acetate. The *nag* regulon of *E. coli*, containing the relevant structural and regulatory genes, has been extensively studied by Plumbridge (21–23). (e) There are two other possible sources for the key intermediate, GlcNAc-6-P. In *V. furnissii*, (GlcNAc)$_2$ generated both outside the cell by chitinases and in the periplasm from higher oligosaccharides is taken up unchanged by a specific transporter (24) labeled 4. In the cell, the disaccharide is cleaved by 5, a specific phosphorylase (10), yielding GlcNAc-1-P and GlcNAc. We presume that the GlcNAc-1-P is converted to the 6-P by a specific mutase, 6, known to occur in Neurospora (25) and other cell types (26, 27). (f) The third source of GlcNAc-6-P is a GlcNAc-specific ATP-dependent kinase (6) that is found in *V. furnissii* (7). The free GlcNAc generated from the disaccharide by the phosphorylase (5) is converted to GlcNAc-6-P by this kinase, 7.

If extracellular GlcN is catabolized via cytoplasmic GapK, then it must enter the cell unchanged. To our knowledge, no such transport system has been reported. In many obligate and facultative anaerobes that utilize GlcN, the phosphotransferase is the transporter (16), yielding internal GlcN-6-P, which would obviate the need for GapK. One obvious extension of the present studies is to determine how GlcN enters the *V. cholerae* cell. In sum, the function(s) of this unique *V. cholerae* kinase remain to be determined, or more precisely, if the kinase does function in this organism, what is the origin of intracellular GlcN?

REFERENCES

1. Keyhani, N. O., and Roseman, S. (1999) Biochem. Biophys. Res. Commun. 365, 108–122
2. Nalin, D. R. (1976) Lancet 2, 858
3. Nalin, D. R., Daya, V., Reid, A., Levine, M. M., and Cisneros, L. (1979) Infect. Immun. 25, 768–770
4. Heidelburg, J. F., Eisen, J. A., Nelson, W. C., Clayton, R. A., Gwinn, M. L., Doughan, R. J., Haft, D. H., Hickey, E. K., Peterson, J. D., Umayam, L., Gill, S. R., Nelson, K. E., Read, T. D., Tettelin, H., Richardson, D., Ermolaeva, M. D., vanBaa, J., Bass, S., Qin, H., Dragei, I., Sellers, P., McDonald, L., Utterback, T., Fleishmann, R. D., Nierman, W. C., and White, O. (2000) *Nature* 406, 477–483
5. Jourdain, G. W., and Roseman, S. (1962) Biochemical Preparations 9, 44–47
6. Asensio, C., and Ruiz-Argam, M. (1966) Methods Enzymol. 9, 421–425
7. Bierhoff, B. L., Yu, C., Lee, Y. C., and Roseman, S. (1991) *J. Biol. Chem.* 266, 24276–24286
8. Ausubel, F. M., Brent, R., Kingston, R. E., Moore, D. D., Seidman, J. G., Smith, J. A., and Struhl, K. (1990) Current Protocols in Molecular Biology, Vol. 1–3, John Wiley & Sons, Inc., New York
9. Keyhani, N. O., and Roseman, S. (1996) *J. Biol. Chem.* 271, 33425–33432
10. Park, J. K., Keyhani, N. O., and Roseman, S. (2000) *J. Biol. Chem.* 275, 33077–33083
11. Segel, I. H. (1976) Biochemical Calculations, 2nd Ed, pp. 236–264, John Wiley & Sons, Inc., New York
12. Distler, J., Merrick, J. M., and Roseman, S. (1958) *J. Biol. Chem.* 230, 497–509
13. Keyhani, N. O., Li, X., and Roseman, S. (2000) *J. Biol. Chem.* 275, 33068–33076
14. Keyhani, N. O., and Roseman, S. (1996) *J. Biol. Chem.* 271, 33414–33424
15. Kundig, W., Ghosh, S., and Roseman, S. (1984) *Proc. Natl. Acad. Sci. U. S. A.* 81, 1067–1074
16. Postma, P. W., Lengeler, J. W., and Jacobson, G. R. (1993) *Microbiol. Rev.* 57, 543–584
17. Bouma, C. L., and Roseman, S. (1996) *J. Biol. Chem.* 271, 33457–33467
18. Roseman, S. (1957) *J. Biol. Chem.* 226, 115–124
19. Distler, J., Merrick, J. M., and Roseman, S. (1958) *J. Biol. Chem.* 230, 33409–33413
20. Bierhoff, B. L., Yu, C., Lee, Y. C., and Roseman, S. (1991) *J. Biol. Chem.* 266, 125–133
21. Comb, D. G., and Roseman, S. (1958) *J. Biol. Chem.* 232, 867–872
22. Plumbridge, J. (1989) *Mol. Microbiol.* 3, 505–515
23. Plumbridge, J. (1991) *Mol. Microbiol.* 5, 2053–2062
24. Plumbridge, J. (2001) *Nucleic Acids Res.* 29, 1–9
25. Keyhani, N. O., Wang, L.-X., Lee, Y. C., and Roseman, S. (1996) *J. Biol. Chem.* 271, 33409–33413
26. Reisson, J. L. (1956) *J. Biol. Chem.* 219, 753–767
27. Fernandez-Sorensen, A., and Carlson, D. M. (1971) *J. Biol. Chem.* 246, 3485–3493
28. Carlson, D. M. (1966) Methods Enzymol. 3, 179–182
29. Budu, E., Ruppender, H., and Mackinnon, J. (1954) *Proc. Natl. Acad. Sci. U. S. A.* 40, 773–777
30. Bouma, C. L., and Roseman, S. (1996) *J. Biol. Chem.* 271, 33468–33475

*3* If GlcN is generated intracellularly, there are at least two possibilities: (a) Native chitin is not fully N-acetylated, i.e. it contains from 10 to 20% free amino groups. These residues would yield free intracellular GlcN if the hydrolases, transporter, and phosphorylase described in Fig. 5, or similar enzymes, are active with substrates such as GlcNAc-GlcN. (b) The preferred substrate for the deacetylase, Reaction 8, is GlcNAc-6-P. But the *E. coli* enzyme is also active with free GlcNAc (18), which would yield GlcN. However, the *E. coli* enzyme displayed an inordinately high Km for this substrate, 0.12 μM. Conceivably the *V. cholerae* deacetylase (Reaction 8) may be physiologically active with GlcNAc generated, for instance, by the phosphorylase (Reaction 5).

*4* These experiments were kindly performed by Dr. Xibing Li.

*5* The major pathway for the uptake of GlcN by the phosphotransferase in *E. coli* is the relatively non-specific IIMan complex of proteins, which is why it is shown in the plasma membrane in Fig. 5. This complex has been cloned and characterized from *V. furnissii* and is much more specific than the IIMan complex of *E. coli* (29). For instance, *V. furnissii* IIMan does not phosphorylate GlcNAc but is yet to be tested with GlcN.
Isolation of a Glucosamine-specific Kinase, a Unique Enzyme of *Vibrio cholerae*

Jae Kweon Park, Lai-Xi Wang and Saul Roseman

*J. Biol. Chem.* 2002, 277:15573-15578.  
doi: 10.1074/jbc.M107953200 originally published online February 15, 2002

Access the most updated version of this article at doi: 10.1074/jbc.M107953200

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 27 references, 18 of which can be accessed free at http://www.jbc.org/content/277/18/15573.full.html#ref-list-1