Chitin Catabolism in the Marine Bacterium Vibrio furnissii: Identification, Molecular Cloning, and Characterization of an N,N’-Diacetylchitobiose Phosphorylase*

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RUNNING TITLE

Characterization of an N,N'-diacetylchitobiose phosphorylase
**SUMMARY**

The major product of bacterial chitinases is \(N,N'\)-diacetylchitobiose, or \((\text{GlcNAc})_2\). We have previously demonstrated that \((\text{GlcNAc})_2\) is taken up unchanged by a specific permease in \(V. \text{furnissii}\) (unlike \(E. \text{coli}\)). It is generally held that marine Vibrios further metabolize cytoplasmic \((\text{GlcNAc})_2\) by hydrolyzing it to 2 GlcNAc (i.e., a "chitobiase"). Here we report instead that \(V. \text{furnissii}\) expresses a novel phosphorylase.

The gene, \(chbP\), was cloned into \(E. \text{coli}\); the enzyme, ChbP, was purified to apparent homogeneity, and characterized kinetically. The DNA sequence indicates that \(chbP\) encodes an 89 kDa protein. The enzymatic reaction was characterized as follows:

\[
(\text{GlcNAc})_2 + \text{Pi} \rightleftharpoons \text{GlcNAc-}\alpha\text{-1-P} + \text{GlcNAc}
\]

\[
K'_{eq} = 1.0 \pm 0.2.
\]

The \(K_m\) values for the 4 substrates were in the range 0.3-1 mM. p-Nitrophenyl \((\text{GlcNAc})_2\) was cleaved at 8.5% the rate of \((\text{GlcNAc})_2\), and PNP-GlcNAc was 36% as active as GlcNAc in the reverse direction. All other compounds tested displayed \(\leq 1\%\) of the activity of the indicated substrates including: for phosphorolysis, higher chitin oligosaccharides, \((\text{GlcNAc})_n, n = 3-5, \text{cellobiose, PNP-GlcNAc, and PNP-(GlcNAc)}_3\); for synthesis, \((\text{GlcNAc})_n, n = 2-5; \text{glucose, etc.}\)

\((\text{GlcNAc})_2\) is a major regulator of the chitin catabolic cascade. Conceivably \(\text{GlcNAc-}\alpha\text{-1-P}\) plays a similar, but different role in regulation.
INTRODUCTION

Chitin, a polymer of β-1,4 linked N-acetylglucosamine residues, is one of the most abundant organic compounds in nature, with greater than $10^{11}$ tons produced annually in the marine biosphere alone. This vast quantity of highly insoluble polymer is degraded so rapidly that only traces are found in marine sediments. Work in the 1930's showed that marine bacteria, such as Vibrios, were primarily responsible for this massive turnover (6). The enzymatic hydrolysis of chitin has been studied for almost a century, and early work (7,8) established that two enzymes were required, a chitinase which yielded the disaccharide $N,N'$-diacetylchitobiose, or $(\text{GlcNAc})_2$, and a “chitobiase”, or β-$N$-acetylglucosaminidase, that gave the final product, GlcNAc. This pathway has remained the central concept for chitin catabolism through the 20th century (9), including in marine bacteria (10).

We have reported that chitin catabolism in the marine bacterium Vibrio furnissii is much more complex, involving a minimum of 3 signal transduction systems and many genes and proteins, only some of which have been identified (11-20). In this process, extracellular chitin is partially hydrolyzed by extracellular chitinases, and the oligosaccharides diffuse through a chitoporin (1) into the periplasmic space. The combined action of two unique enzymes in the periplasm, a chitodextrinase (17) and a specific β-$N$-acetylglucosaminidase (18) yield two products, GlcNAc and $(\text{GlcNAc})_2$. The monosaccharide is taken up via the phosphotransferase system (20), while the disaccharide is taken up unchanged (16). The further catabolism of $(\text{GlcNAc})_2$ is the subject of this report.

In our earlier work (12), crude extracts of V. furnissii were shown to hydrolyze p-nitrophenyl-β-GlcNAc, and the enzyme was assumed to be a typical bacterial chitobiase (10). Numerous unsuccessful attempts were made to clone this enzyme. Instead, an atypical periplasmic β-$N$-acetylglucosaminidase was identified (18), which has virtually no activity on $(\text{GlcNAc})_2$ at the pH of sea water.

The present studies were based on the same approach, an attempt to clone the hypothetical chitobiase. We have, instead, cloned, isolated, and characterized a novel enzyme, a specific
(GlcNAc)$_2$ phosphorylase which catalyzes the following reaction:

\[(\text{GlcNAc})_2 + \text{Pi} \leftrightarrow \text{GlcNAc-}\alpha-\text{P} + \text{GlcNAc}\]
EXPERIMENTAL PROCEDURES

Materials-The following chemicals, reagents, and materials were purchased from the indicated sources: chitin, N-acetylglucosamine (GlcNAc), pNP-glycosides from Sigma; chitin oligosaccharides, (GlcNAc)n, n=2-6, were prepared by N-acetylation of chitosan oligosaccharides prepared as described (21), or were purchased from Seikagaku America, Inc.(Rockville, MD); reagents for bacterial media from Difco and J.T. Baker. Radioisotope (32Pi) was purchased from ICN. Reagents for molecular biology were obtained from New England Biolabs (Beverely, MA), Stratagene, and U.S. Biochem. Corp. Other buffers and reagents were of the highest purity available commercially. E.coli strains BL21 (DE3) (Novagen) and XL1-Blue (Stratagene) harboring designated plasmid constructs where indicated were stored as frozen cultures in Luria Broth (LB) with 5% glycerol. Cell densities were measured at 600 nm, where 1 O.D. unit corresponded to 0.5 mg cell protein.

Molecular Analysis; Construction and Screening of V. furnissii Genomic Cosmid Library, and Construction of pET:chbP, a Phosphorylase Overexpression Vector-DNA preparations, restriction enzyme digests, ligation, and transformations were performed using standard techniques (22,23). A cosmid library was constructed using bacterial genomic DNA from V. furnissii strain 1514 as described. Library construction, including conditions for partial genomic DNA restriction (using Sau3A) and ligation into the cosmid vector SuperCos1 were performed according to the supplier’s recommendations (Stratagene). The ligation mixture was packaged into λ phage using GigaPack Gold III packaging extract (Stratagene) and transfected into various E. coli strains (typically XL1-Blue) according to the supplier’s recommendations.

The recombinant V. furnissii cosmid library in E. coli strain XL1-Blue was screened by hybridization using a fragment from the previously cloned V. furnissii periplasmic β-N-acetylhexosaminidase (ExoI) as a probe using standard techniques. Briefly, a 3.7 kb ClaI-NcoI fragment corresponding to the cloned exoI gene was labeled ([α32P]-dATP, 3,000 Ci/mmol, Amersham-Pharmacia Biotech) by random priming (Random Primed DNA labeling Kit, Boehringer Mannheim) and used as a probe in colony lifts from the recombinant cosmid library.
A total of 3 positive clones were isolated from a screen of 1,000 recombinants. Restriction analysis of the 3 clones revealed inserts of approximately 25-35 kb, with the clones sharing many similar sized bands when digested by several different restriction enzymes. One clone, designated as pRhexo was chosen for further study.

The nucleotide sequence downstream (3'-end) of *exoI* was determined at the Genetics Core Facility (Johns Hopkins Medical School) using an ABI-373 automated sequencer. DNA and amino acid sequence analyses were performed using the GCG sequence analysis package (Version 7, Genetics Computer Group, Madison, WI). GenBank™ and SWISS-Protein databases were used for nucleotide and amino acid sequence searches using the FASTA program.

The (GlcNAc)₂ phosphorylase gene, *chbP*, was amplified by PCR using primers based on the gene sequence determined from the cosmid clone, pRhexo. The 5' PCR primer was designed containing an *NdeI* restriction site in order 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 plasmid were: (1) 5'-GGAAATTCATATGAAATACGGCTATTTT-3' and (2) 5'-ATTTCGAATTAACCTAAAATCACCGTG-3'. The amplified PCR fragment (2.4 kb) corresponding to *chbP* was first blunt-end cloned into pNotA (5 Prime-3 Prime Inc., Boulder, CO), before being transferred into the *NdeI* (5' end) and *SacI* (3' end) of pET21a. The constructed vector, pET:chbP, was transformed into the T7 polymerase inducible host strain BL21 (DE3) for overexpression of the protein product.

**Enzyme Assays**-Two quantitative assays used were to measure phosphorolytic enzyme activity: (a) Incorporation of ³²Pi into the product, which required separation from the large excess of ³²Pi. (b)The Morgan-Elson reaction was employed for determining *N*-acetylglucosamine. The method is based on heating the sugar at alkaline pH to form a chromophore, which then reacts with p-dimethylaminobenzaldehyde to give a purple color. A positive reaction requires that the GlcNAc C-1 carbonyl group be free. Substitutions along the chain of the GlcNAc greatly influences the reaction, particularly at C-4. For this reason,
(GlcNAc)₂ gives only 3-6% of the color yield obtained with GlcNAc, and higher oligosaccharides are unreactive with this reagent (reviewed in (24)).

Enzyme assays were conducted at 30°C, and in all assays, product formation was proportional to time of incubation and the quantity of enzyme used. Control incubation mixtures lacked enzyme, or contained heat denatured enzyme, or lacked one of the two substrates, (GlcNAc)₂ or ³²Pi. These controls gave the baseline color levels for the Morgan-Elson method.

*Phosphorolysis as measured by incorporation of³²Pi into product*-- Assay mixtures (100 μl) contained the following: 0.01-5.0 mM sugar substrate, 0.01-5.0 mM ³²Pi (500 cpm/nmol), 10 mM MOPS buffer, pH 7.0 containing 50 mM KCl. Reactions were initiated by adding purified enzyme (0.5-2.0 μg). The reactions were stopped and ³²Pi selectively precipitated by treating with 100 μl precipitation mixture (200 mM of sodium tungstate, 200 mM of triethylammonium hydrochloride, and 50 mM procaine hydrochloride) followed by (20 μl) formic acid (25). The mixture was maintained at 4°C for 5 min and the precipitate removed by centrifugation at 14,000 rpm, 10 min at 4°C in a microcentrifuge. The supernatant containing the product, GlcNAc-1-³²P, was quantitated in a Packard Liquid Scintillation Spectrometer (50 μl of supernatant, 3.0 ml UltimaGold-XR).

*Morgan-Elson Assay* - One product of the phosphorolysis of (GlcNAc)₂ is GlcNAc. To assay for this monosaccharide, the incubation mixtures were increased to twice the volumes described above, 100 μl samples were removed at the indicated times, heated at 100°C for 5 min to stop the reaction, cooled, treated with 100 μl sodium borate, pH 9.2, heated at 100°C for 12 min, cooled to room temperature, and finally treated with 550 μl of the Ehrlich Reagent (26,27).

*Protein Determination and Analysis*—Protein concentrations were measured by the Bio-Rad protein assay, using bovine serum albumin (BSA) as the standard. SDS-PAGE was preformed as described (22,23).

*Overexpression and Purification of the (GlcNAc)₂ Phosphorylase*—

*Step 1. Crude extracts* - A single colony of *E. coli* strain BL21 (DE3) harboring pET:chbP was inoculated into 150 ml of LB medium supplemented with 50 μg / ml ampicillin,
and grown overnight at 37°C with aeration. Two liters each of LB medium supplemented with 3 mM melibiose (final concentration) in two 6-liters flasks were inoculated with 40 ml of the overnight culture and allowed to grow at 37°C with aeration until OD_{600} = 3.0. The cells were harvested by centrifugation at 4000 rpm for 20 min at 4°C. Subsequent steps were performed at 4°C unless otherwise specified.

The cell pellet (8 gm) was washed twice with 800 ml of 50 mM Tris-HCl buffer containing 0.1 M NaCl and 1 mM EDTA, pH 7.0, and finally resuspended in 35 ml, 50 mM Tris-HCl buffer with 1 mM EDTA, pH 7.0. The cells were disrupted by two passages through a Wabash French Press. Unlysed cells were removed by centrifugation at 10,000xg for 20 min.

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

**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 60%. The solution was stirred overnight and centrifuged at 150,000xg for 1 h. The ammonium sulfate pellet was resuspended in 30 ml 10 mM Tris-HCl buffer with 10 mM NaCl, pH 7.0, and dialyzed against the same buffer.

**Step 4. DEAE-column chromatography**-The 60% ammonium sulfate fraction was transferred to a 40-ml DEAE-Sepharose CL-6B column equilibrated in 10 mM Tris-HCl buffer with 10 mM NaCl, pH 7.0 buffer. After sample loading, the column was washed with 2 volumes (80 ml) of buffer, and a gradient (400 ml) from 0.01 M NaCl to 1.0 M NaCl was applied to the column. The activity eluted between 0.4-0.6 M NaCl and active fractions were pooled and dialyzed against 10 mM Tris-HCl buffer, pH 7.5 containing 10 mM NaCl.

**Step 5. FPLC anion exchange (Mono-Q) chromatography**-The pooled sample from Step 4 was transferred to a Mono-Q HR 10/10 (8 ml bed volume) FPLC column (Pharmacia) equilibrated in 10 mM Tris-HCl buffer, pH 7.5, containing 10 mM NaCl. The column was
washed at room temperature with 10 ml of the same buffer, and subsequently eluted with a
gradient (160 ml) from 0.01 to 1.0 M NaCl in the same buffer. Purified (GlcNAc)_2
phosphorylase eluted between 0.4-0.5 M NaCl. Purity was monitored throughout the
fractionation by SDS-PAGE and enzyme activity. The purified enzyme was stored in small
aliquots at –20°C or –80°C.

**N-Terminal Amino Acid Determination** - The N-terminal amino acid residues of purified
recombinant ChiP were determined using an Applied Biosystems 475A protein sequencer
(Amino Acid Sequencing Facility, Dept. of Biological Chemistry, Johns Hopkins School of
Medicine).

**Effects of pH, Ionic Strength, and Temperature on Enzyme Activity** - The effects of these
parameters on enzyme activity were studied with the purified enzyme at 30°C to determine the
optimal conditions for kinetic characterization. Both assay methods (incorporation of ^32^Pi into
product and the Morgan-Elson) were alternately used and always gave complementary results.

**Effects of pH** - Typically, 5 mM substrate concentrations were employed for both
(GlcNAc)_2 and phosphate. The following buffer systems were used to generate a wide pH range:
citrate buffer (pH 3.0-6.5), imidazole buffer (pH 6.5-7.7), 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.

**Effect of Ionic Strength** - The effect of ionic strength on enzyme activity was determined
using buffer 10 mM MOPS, pH 7.0, supplemented with NaCl or KCl at various concentrations,
ranging from 0-2 M.

**Effect of Temperature** - The optimum temperature and thermostability of the enzyme were
investigated using (GlcNAc)_2 as the substrate. The optimum temperature was determined by
incubating reaction mixtures as described above, over a temperature range of 0°C to 75°C.
Reaction mixtures were preincubated at the given temperatures before addition of purified
enzyme (0.5-2.0 µg). The thermostability of the enzyme was measured by addition of purified
enzyme to 100 µl of buffer without substrate, incubating at the desired temperature for 20 min,
cooling to room temperature, and then initiating reactions by the addition of substrate. Formation of GlcNAc-1-P or GlcNAc was determined as described above.

**Preparation of Phospho-GlcNAc** - The major problem in preparing the sugar phosphate on a large scale was the separation of excess Pi from the product. Shaking the incubation mixture with a large excess of BaCO₃ removed about 90% of the Pi. A more satisfactory method is described below.

The reaction mixture contained 200 mg (GlcNAc)₂ (471 µmol) in 15 ml of 1.0 M sodium phosphate buffer, pH 7.0. The reaction was initiated by adding 3 mg purified ChbP, incubated at 30°C for 2 h, and stopped by boiling for 5 min. Precipitated material was removed by centrifugation (7,500xg, 10 min) and the volume adjusted to 200 ml with H₂O. Inorganic and total phosphate were measured as described (28).

The solution was treated with a 5-fold molar excess Ca(OH)₂ relative to total Pi in the reaction (5.0 g Ca(OH)₂). The mixture was stirred at 22°C for 1 h, after which the insoluble material was removed by filtration. This treatment resulted in the removal of greater than 99% of the Pi. The supernatant material, containing ~250 µmol of sugar-P, was transferred to a Dowex-1 AGX8 resin column (10 ml, bicarbonate form), the column washed with water, and the phospho-sugar purified by eluting with a 0-1.0 M gradient (200 ml) of triethylammonium bicarbonate, pH 8.0, or a gradient of 0-1 M NaCl, pH 7.8. The NaCl gradient gave a sharper separation between residual Pi and GlcNAc-P. Most of the NaCl was removed from the pooled fractions by dialysis against a 100 dalton cut-off membrane. The product prepared by the NaCl method were used in the kinetic studies described below; the residual NaCl did not affect the enzyme kinetics. Fractions were analyzed for GlcNAc, Pi and organic phosphate, and by TLC. The fractions containing the phospho-sugar (290 µmol) were pooled (45 ml), evaporated (repeatedly to remove triethylammonium bicarbonate when present), and lyophilized. The final products were syrups, and exhibited single bands by thin layer chromatography (TLC) on Silica gel 60 using the following solvent system: chloroform:methanol:water, 55:40:5. When used in the studies described below, the syrup was dissolved in water and the concentration of GlcNAc-
1-P determined by analyzing for organic (and inorganic) P.

**Analytical Methods** - Samples of the purified sugar phosphate were very kindly analyzed by MALDI mass spectroscopy by Ms. S. Ramirez and Dr. R. Kotter (Middle Atlantic Mass Spectrometry Laboratory, Dept. of Pharmacology, JHU School of Medicine). $^1$H and $^{31}$P-NMR spectra were obtained and interpreted by Drs. I. Deras, Lai-Xi Wang, and Y.C. Lee of this department, using a Brucker AMX-300 spectrometer, at 25°C, Dept. of Chemistry, Johns Hopkins University.

**Assay of (GlcNAc)$_2$ Phosphorylase Reverse Reaction** - The reverse reaction was measured by quantitation of Pi liberated from GlcNAc-1-P (donor) in the presence of various carbohydrate acceptors. The initial sugar (total organic) phosphate concentration was determined as described (28). Typical reaction mixtures (100 µl) contained; 0.1-10 mM GlcNAc-1-P, and 0.1-10 mM potential acceptor in 50 mM MES buffer, pH 6.2. Reactions were initiated by the addition of ChbP (0.5-2.0 µg), incubated over a time course (typically 0.5 min - 2 h), and heated at 100°C for 5 min. The initial sugar phosphate (organic phosphate) and inorganic phosphate was measured as described (28). Reaction conditions such as temperature and pH optima were determined as described for the forward reaction.

**Determination of Equilibrium Constant** - The $K'_e$q of the reaction in the forward direction was measured using (GlcNAc)$_2$ and Pi as the substrates, and determining the final concentrations of Pi, GlcNAc and GlcNAc-1-P. Typical reaction mixtures contained: 25 nmol each of (GlcNAc)$_2$ and $^{32}$Pi (specific activity, 500-1000 cpm/nmol) in 50 mM MOPS buffer, pH 7.0. Reactions were initiated by adding purified enzyme (0.5-2.0 µg) and aliquots were taken over a time course of 0 to 24 h. Free $^{32}$Pi and sugar phosphate (GlcNAc-1-$^{32}$P) were separated and quantitated by selective precipitation with Ca(OH)$_2$ as described above. The quenched reaction was maintained at 4°C for 5 min and the precipitate removed by centrifugation (14,000 rpm, 10 min, 4°C) using a tabletop microcentrifuge. The precipitated $^{32}$Pi (pellet) was resuspended in 100 µl of H$_2$O and the mixture was sonicated for 5-10 min, before adding 3.0 ml UltimaGold-XR and counted as described above. GlcNAc production was quantitated by the Morgan-Elson method.
RESULTS

Molecular Cloning of chbP-We have previously described the cloning of the periplasmic β-N-acetylmuramidase (ExoI) from *V. furnissii* (18). Sequence analysis of the 3' region of the cloned *exoI* gene indicated that it was followed by another open reading frame whose start codon was 169 bp after the *exoI* stop codon. Further analysis indicated the presence of a truncated gene sequence with homology to cellobiose phosphorylase (discussed below) designated *chbP*, in the original clone. To clone the remaining portion of *chbP*, a *V. furnissii* cosmid library, transfected into *E. coli*, was screened by hybridization to *exoI* (data not shown); yielding the cosmid clone pRhexo. Portions of cosmid pRhexo were sequenced to complete the *chbP* nucleotide sequence (deposited in GenBank with the accession number AF230379). The predicted amino acid sequence of ChbP, based on the nucleotide sequence of *chbP*, is a protein containing 800 amino acids (89 kDa). Sequence analysis of the region (169 bp) between *exoI* and *chbP* did not reveal the presence of any ribosome binding sites, nor was there a TATA region preceding the *chbP* start codon. These observations indicate that perhaps *exoI* and *chbP* are in an operon and are expressed by a polycistronic message. A search of the SWISS-PROT Data Bank identified several proteins with significant similarities to the translated open reading frame of *chbP*. These proteins included several cellobiose phosphorylases from different organisms; *Thermotoga neapolitana* (36% identity) (29), *Clostridium stercoarium* (35%) (30), and *Cellvibrio gilvus* (35%) (31), and a cellodextrin phosphorylase from *Clostridium stercoarium* (36%) (30). The similarities between *chbP* and the cellobiose phosphorylases were spread over the entire protein sequences; apparently, the active sites of these enzymes have not been identified.

The sequence determined from the cosmid clone pRhexo was used to design primers for subcloning *chbP* into the pET21a overexpression vector as described in the Experimental Procedures, yielding pET: *chbP*.

Purification and Properties of the Recombinant Phosphorylase- The enzyme was purified from recombinant *E. coli* BL21 (DE3) cells harboring pET: *chbP* as described in Experimental Procedures (Table I), yielding an apparently homogenous protein (Fig. 1A). The
enzyme was purified 7-fold from the crude extract with a 40% yield. N-Terminal amino acid sequencings of the purified recombinant protein resulted in the following sequence: Met-Lys-Tyr-Gly-Tyr-Phe-Asp-Asn-Asp-Asn, in complete agreement with the N-terminal sequence predicted from the nucleotide sequence.

\((\text{GlcNAc})_2\) phosphorylase activity was measured using two complementary assays, each of which measures one product of the reaction, i.e., GlcNAc-1-P or GlcNAc. GlcNAc-1-P was determined by the incorporation of \(^{32}\text{Pi}\) into the product, and GlcNAc by the Morgan-Elson assay. A time course of GlcNAc-1-P and GlcNAc formation from a mixture of \((\text{GlcNAc})_2\) and \(^{32}\text{Pi}\) is shown in Fig. 1B. Equimolar quantities of GlcNAc and GlcNAc-1-P are formed from the disaccharide. It is important to emphasize that no GlcNAc was formed in the absence of Pi (data not shown), and therefore the enzyme is not a hydrolase or \(\beta\)-N-acetylglucosaminidase.

Enzyme activity was determined as a function of pH, ionic strength, and temperature as described in Experimental Procedures. The pH optimum of the purified recombinant enzyme was between 6.5-7.0. At the pH optimum enzyme activity was measured as a function of ionic strength. NaCl and KCl gave similar results with an optimum at 50 mM, although this was only 20% higher than without any additional salt beyond the dilute buffer. The enzyme retained up to 65% of its activity in 0.5 M salt. The purified enzyme retained greater than 95% of its activity in up to 20 mM EDTA or EGTA, indicating that it had no divalent cation requirement.

Purified ChbP displayed a broad temperature optimum, from 20-37\(^\circ\)C. It was stable for at least 1 h from 4-37\(^\circ\)C. Incubation at 40\(^\circ\)C and 65\(^\circ\)C for 1 h, however, resulted in 50% loss of enzyme activity and complete inactivation respectively, and it was therefore routinely assayed at 30\(^\circ\)C.'

**Effects of Substrate Concentrations on the Phosphorolysis of \((\text{GlcNAc})_2\)** - The enzyme reversibly converts two substrates to two products. The results of kinetic studies on the phosphorolytic cleavage of the disaccharide are presented here, and on the reverse reaction are given below. Fig. 2A shows the effect of varying \((\text{GlcNAc})_2\) in the presence of 5 mM Pi, and the data are plotted by the Woolf-Augustinsson (v versus v/[S]) method in Fig. 2B. The same kind of
experiment was conducted with 5 mM (GlcNAc)$_2$ and varying concentrations of Pi as shown in Fig. 2C, D. The kinetic values obtained from these data were: (GlcNAc)$_2$, $K_m = 0.58$ mM, $V_{max} = 4.6$ nmol/min/µg protein at 30°C; Pi, $K_m = 0.51$ mM, $V_{max} = 4.8$ nmol/min/µg.

Substrate Specificity of Phosphorolytic Activity of ChbP—The specificity of the enzyme was assayed with $^{32}$Pi and a series of potential substrates at 5 mM concentrations.

Only one compound other than (GlcNAc)$_2$ showed detectable phosphorylytic activity, p-nitrophenyl-(GlcNAc)$_2$, which was about 8.5% as active as the disaccharide per se. The following compounds were inactive: (GlcNAc)$_n$, $n = 3-6$, p-nitrophenyl-GlcNAc, p-nitrophenyl-(GlcNAc)$_3$, (GlcNH$_2$)$_2$, cellobiose, trehalose, lactose, maltose, and sucrose. None of the p-nitrophenyl analogues yielded p-nitrophenol.

Characterization of GlcNAc-1-P—To characterize the phosphorylated reaction product, 200 mg of (GlcNAc)$_2$ was used as described in Experimental Procedures, giving 150 mg of purified product isolated as the disodium salt.

The molecular weight of the phosphorylated sugar product was determined by MALDI mass spectroscopy. In the negative ion mode, a peak of 343.5 daltons was observed, and two lower molecular weight fragments. In the positive ion mode, a single ion was detected with a mass of 345.9 daltons. The molecular weight of the disodium salt of GlcNAc-1-P is 345. The negative ion corresponds to loss of one proton from this molecule, while the positive ion corresponds to a gain of one proton.

NMR spectroscopy: $^{31}$P-NMR (D$_2$O, 300 MHz): 1.3072; $^1$H-NMR (D$_2$O, 300 MHz): $\delta$ 5.341 (dd 1 H $J_{1,2} = 3.3$ Hz, $J_{1,p} = 7.3$ Hz, H-1), 3.970-3.866 (m, 3 H, H-5,6a,6b), 3.793 (t, 1H, $J_{2,3} = J_{3,4} = 9.5$ Hz, H-3), 3.754 (ddd, 1 H, $J_{1,2} = 3.3$ Hz, $J_{2,3} = 9.5$ Hz, $J_{2,p} = 1.3$ Hz), 3.471 (t, 1H, $J_{3,4} = J_{4,5} = 9.5$ Hz, H-4), 2.005 (s, 3 H, acetyl).

The single peak observed in the $^{31}$P-NMR corresponds to the value expected for a phosphate ester under these conditions (32,33). Inorganic phosphate exhibits a peak of about 4 ppm (relative to concentrated phosphoric acid) and no such peak was observed.

The $^1$H-NMR spectrum of the compound showed that the anomeric proton, H-1 appeared
at 5.341 ppm as a double doublet \( J_{1,2} = 3.3 \text{ Hz}, J_{1,p} = 7.3 \text{ Hz} \). This assignment is consistent with the observation for glucopyranosyl \( \alpha\)-1-phosphate, in which the H-1 resonates at 5.435 ppm as a double doublet \( J_{1,2} = 3.3 \text{ Hz}, J_{1,p} = 7.3 \text{ Hz} \). The relatively small coupling between H-1 and H-2 is typical for an \( \alpha\)-linked glucopyranoside, and for \( \alpha\)-linked N-acetyl-D-glucosaminopyranoside (34); the methyl glycosides of \( N\)-acetylglucosame exhibit H-1 and H-2 coupling of 3.6 for the \( \alpha\) anomer, and 8.5 for the \( \beta\)-anomer. These results strongly suggest that the 1-phosphate is located at the \( \alpha\)-position in the molecule. Other NMR features of the compound are also in agreement with the assignment.

**Kinetics and Specificity of Phosphorylase Reverse Reaction**—The reaction catalyzed by ChbP was examined in the reverse direction using GlcNAc-1-P prepared as described above. The reaction was monitored by measuring the rate of Pi formation as it is released from the donor compound, GlcNAc-\( \alpha\)-1-P. The kinetics were studied at 30°C using: (1) 5 mM GlcNAc as the acceptor in the presence of various concentrations of GlcNAc-1-P (Figs. 3A and 3B); (2) 5 mM GlcNAc-1-P as the donor and various concentrations of GlcNAc (Fig. 3C and 3D). Under these conditions, the kinetic constants were found to be as follows: GlcNAc, \( K_m = 0.93 \text{ mM}, V_{max} = 0.84 \text{ nmol/min/µg} \); GlcNAc-\( \alpha\)-1-P, \( K_m = 1.0 \text{ mM}, V_{max} = 0.77 \text{ nmol/min/µg} \).

The enzyme displayed a very narrow acceptor specificity; transferring GlcNAc from GlcNAc-1-P to GlcNAc and to PNP-GlcNAc. The latter accepted the GlcNAc residue at about 36% of the rate that it was transferred to GlcNAc per se. The following compounds were inactive as acceptors of the GlcNAc moiety \( i.e., <1\% \) of the rate observed with GlcNAc): (GlcNAc)_n, n = 2-5, PNP-(GlcNAc)_2, PNP-(GlcNAc)_3, PNP-cellulobiose, glucose, and glucosamine.

**Determination of Phosphorylase Reaction Equilibrium Constant**—The equilibrium constant for the phosphorolysis of (GlcNAc)_2 was determined by monitoring reactant and product concentrations until the reaction had arrived at a steady state.

Three substrates were measured, \( ^{32}\text{Pi}, \text{GlcNAc-1-}[^{32}\text{P}] \), and GlcNAc. Fig. 4 gives one set of results obtained at 30°C. Under the conditions described in the legend, reactants and products
were at equilibrium within 1 h, but to assure that equilibrium had actually been attained, the reaction was continued for an additional 24 h with the addition of 2- to 4-fold fresh enzyme at 3 and 12 h. There was no detectable change in the concentrations of the components of the mixture. The calculated value for the apparent equilibrium constant, $K'_{eq} = 1.0 \pm 0.2$. 
DISCUSSION

Chitin degradation in the marine bacteria *V. furnissii* is a complex process involving several signal transduction systems and many different classes of enzymes, some with overlapping substrate specificities. These classes include: (1) chitinases, which hydrolyze the insoluble chitin polymer to soluble oligosaccharides, (2) chitodextrinases, which display little if any activity against chitin, but readily hydrolyze soluble higher chito-oligosacharides, yielding mainly di- and trisaccharides, and (3) β-GlcNAcidases or chitobiases which hydrolyze the disaccharide to GlcNAc. Although there are some exceptions, these enzymes are often compartmentalized in accordance with their substrate specificities. Thus, chitinases are typically extracellular, chitodextrinases are periplasmic, and β-GlcNAcidases or *N,N'*-diacetylchitobiases are cytoplasmic. Although many cytoplasmic *N,N'*-diacetylchitobiases have been described, we have thus far been unable to isolate such an enzyme as part of the *V. furnissii* enzyme repertoire (see Introduction). Earlier attempts led to the molecular cloning of the periplasmic β-GlcNAcidase

In the present studies, we attempted again to clone the hypothesized cytoplasmic β-GlcNAcidase, but again isolated a unique enzyme, a phosphorylase. The enzyme catalyzes the reaction shown in Fig. 5. The phosphorolysis takes place with inversion of anomeric configuration, i.e., from the β- to the α-glycosidic bond. Other bacterial disaccharide phosphorylases have been characterized. In the classic work of Abeles and co-workers (35,36), sucrose phosphorylase was shown to convert sucrose (α-Glc) to Glc-α-1-P and fructose. Anomeric configuration is retained by a double displacement mechanism with Glc covalently bound to the enzyme as the intermediate. Other known phosphorylases are all of the single displacement type and are reviewed by Kitaoka, *et al* (37). Single displacement results in inversion of configuration and the known examples are (all phosphorylases) : cellobiose, β → α; maltose, α → β; trehalose, α → β; laminaribiose, β → α. Thus, the enzyme described here falls within the major group of phosphorylases, catalyzing the phosphorolysis of *N,N'*-diacetylchitobiase, resulting in the inversion of anomeric configuration (β → α).
The apparent $K_{eq}$ of the reaction = 1.0 ± 0.2, at pH 7.0, 30°C. By comparison, phosphorolysis of cellobiose yielded a value of 0.32 at pH 7.0, 37°C (37). The $K'_{eq}$ values are of course derived by writing the total concentrations of each species at equilibrium, without taking into account dissociation of the phosphate and sugar phosphates. That is, the three-fold difference in $K'_{eq}$ between cellobiose and (GlcNAc)$_2$ phosphorolysis could conceivably result from slight differences in the pK$_a$ values of the two sugar phosphates.

The substrate specificity of the enzyme was remarkable, unlike cellobiose phosphorylases where celloextrins are good substrates of the enzyme (38), in the phosphorolytic reaction, the oligosaccharides (GlcNAc)$_n$, $n = 3-5$ were less than 1% as active as the disaccharide, if they were active at all. Other compounds were also inactive with one exception, PNP-(GlcNAc)$_2$ , which exhibited about 10% of the activity of the underivatized disaccharide. PNP-GlcNAc and PNP-(GlcNAc)$_3$ were totally inactive. Since PNP-GlcNAc does not yield PNP, the phosphorylase is unlikely to be mistaken for chitobiases or β-GlcNAcidases in crude extracts.

The enzyme was equally specific in the reverse direction, again in contrast to cellobiose phosphorylases. With $\alpha$-GlcNAc-1-P as the GlcNAc donor, only PNP-GlcNAc was an active acceptor, about 36% as active as GlcNAc per se. There was little to no activity with a wide variety of other potential acceptors (see Results).

A potentially important physiological function of the phosphorylase should be noted. The phosphorylase conserves the energy of the glycosidic bond, unlike a hydrolase. Phosphorolysis appears to be the first metabolic step following transport of (GlcNAc)$_2$ unchanged across the cytoplasmic membrane. This conservation of energy could be important to the cell, especially under anaerobic conditions, where only 2 ATP are gained per mole glucose or GlcNAc utilized through the Embden-Meyerhof pathway.

Finally, the $chbP$ gene appears to be in an operon immediately following the previously characterized $V. furnissii$ periplasmic β-GlcNAcidase, or $exoI$ (18). The $chbP$ coding region start site (ATG) is 169 bp after the termination codon (TAA) of the β-GlcNAcidase gene and does not appear to possess any Shine Delgarno-like, -10/-35 regions or any ribosome binding site. Studies
are in progress to characterize this putative operon.²
FOOTNOTES

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1 The abbreviations used are: GlcNAc, N-acetyl-D-glucosamine; HEPES, 4-(2-hydroxyethyl)-1-piperazine-ethansulfonic acid; chitin oligosaccharides, (GlcNAc)n, n=2-6 are β-1,4 linked oligomers of GlcNAc; PNP-, is p-nitrophenyl as in PNP-GlcNAc; the PNP-glycosides are all of the β-anomeric configuration.

2 The sequence of the cloned *V. furnissii* genomic DNA, including *chbP*, has been deposited in GenBank with the Accession Number: AF230379.
### Table I

**Purification of recombinant (GlcNAc)\(_2\) phosphorylase**

<table>
<thead>
<tr>
<th>Step</th>
<th>Total protein</th>
<th>Total activity(^a)</th>
<th>Specific activity(^b)</th>
<th>Purification factor</th>
</tr>
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<td></td>
<td>mg</td>
<td>units</td>
<td>units/(\mu)g</td>
<td>-fold</td>
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<td>1. Crude extract</td>
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<td>2. Streptomycin</td>
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<td>56</td>
<td>4.21</td>
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</tbody>
</table>

\(^a\)(GlcNAc)\(_2\) phosphorolysis (nmol/min); 30°C, 10 mM MOPS, pH 7.0, 50 mM KCl, 5 mM Pi.

\(^b\)(GlcNAc)\(_2\) phosphorolysis (nmol/\(\mu\)g/min).
Figure Legends

Fig. 1. **Characterization of the purified recombinant (GlcNAc)₂ phosphorylase (ChbP).** A. SDS-PAGE of purified protein. Lane 1, molecular weight standards; Lane 2, purified ChbP (3 µg); Lane 3, crude extract of transformed cells (8 µg). Samples were derived from the *E. coli* transformant harboring the overexpression construct, pET:chbP, and were analyzed by SDS-PAGE (12% polyacrylamide gel). The purified enzyme displayed a molecular mass of approximately 89 kDa. B. Activity as a function of time of incubation. The enzyme was purified and assayed as described in “Experimental Procedures”. Morgan-Elson assay for free GlcNAc (○); incorporation of ³²Pi into the other reaction product, GlcNAc-1-P (●).

Fig. 2. **Effect of substrate concentrations on the phosphorolysis reaction.** Enzyme activity was measured at 30°C by incorporation of ³²Pi into product as described in the Experimental Procedures. A. The initial rate of GlcNAc-1-P formation is plotted versus (GlcNAc)₂ concentration using 5 mM Pi. B. Woolf-Augustinsson Plot of the data shown in A. The rate ν (nmol/min/µg protein) of GlcNAc-1-P formation is plotted versus ν/[S]. This plot gave an apparent $V_{\text{max}} = 4.6$ nmol/min/µg, and a $K_m$ for (GlcNAc)₂ = 0.58. C. The initial rate of GlcNAc-1-P formation is plotted versus Pi concentration using 5 mM (GlcNAc)₂. D. Woolf-Augustinsson Plot of the data shown in C. The rate ν (nmol/min/µg protein) of GlcNAc-1-P formation is plotted versus ν/[S]. This plot gave an apparent $V_{\text{max}} = 4.8$ nmol/min/µg, and a $K_m$ for Pi = 0.51.

Fig. 3. **Effect of GlcNAc and GlcNAc-1-P concentrations on the reverse reaction, synthesis of (GlcNAc)₂.** Activity was measured at 30°C by the liberation of Pi from GlcNAc-1-P (donor) during glycosyltransfer to GlcNAc (acceptor) as described in the Experimental Procedures. A. The initial rate of Pi formation is plotted versus GlcNAc concentration using 5 mM GlcNAc-1-P. B. Woolf-Augustinsson Plot of the data in A. The rate ν (nmol/min/µg protein) of Pi formation is plotted versus ν/[S]. This plot resulted in a calculated apparent $V_{\text{max}} = 0.84$ nmol/min/µg, and a $K_m$ for GlcNAc = 0.93 mM. C. The initial rate of Pi formation is plotted versus GlcNAc-1-Pi concentration using 5 mM GlcNAc. D. Woolf-Augustinsson Plot of the data in C. The rate ν (nmol/min/µg protein) of Pi formation is plotted versus ν/[S]. This plot gave an apparent $V_{\text{max}} = 0.77$ nmol/min/µg, and a $K_m$ for GlcNAc-1-P = 1.0 mM.

Fig. 4. **Determination of the apparent equilibrium constant of the reaction.** The reaction mixture for each time point contained (100 µl): 25 nmol (GlcNAc)₂ and 25 nmol ³²Pi (specific activity:. 500-1000
24 cpm/nmol) in 50 mM MOPS buffer, pH 7.0. The reaction was initiated by the addition of 1.0 µg purified ChbP. Aliquots taken over the indicated time course were analyzed for Pi (▲), GlcNAc-1-P(○), and GlcNAc (●) as described in Experimental Procedures. The equilibrium constant (K_{eq}) calculated from three such experiments was 1.0 ± 0.2. In order to assure that equilibrium had actually been attained, the reaction was continued for an additional 24 h with the addition of 2- to 4-fold fresh enzyme at 3 and 12 h. There was no detectable change from the end values of each component presented in the figure.

Fig. 5. **Reaction catalyzed by ChbP.** The β-anomeric configuration in (GlcNAc)_2 is converted to the α-anomer in the product, GlcNAc-1-P.
Reference List


   Ref Type: Serial (Book,Monograph)

   Ref Type: Pamphlet


Ref Type: Serial (Book,Monograph)


\[
\text{K}_{eq} = 1.0
\]

\[
\text{Pi} \quad \text{GlcNAc} + \text{NHAc}
\]