Reaction mechanism of chitobiose phosphorylase from *Vibrio proteolyticus*: identification of family 36 glycosyltransferase in *Vibrio*

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A family 36 glycosyltransferase gene was cloned from *Vibrio proteolyticus*. The deduced amino acid sequence showed a high degree of identity with ChBP (chitobiose phosphorylase) from another species, *Vibrio furnissii*. The recombinant enzyme catalysed the reversible phosphorolysis of $(GlcNAc)_2$ (chitobiose) to form 2-acetamide-2-deoxy-α-D-glucose 1-phosphate [GlcNAc-1-P] and GlcNAc, but showed no activity on cellobiose, indicating that the enzyme was ChBP, not cellobiose phosphorylase. In the synthetic reaction, the ChBP was active with α-D-glucose 1-phosphate as the donor substrate as well as GlcNAc-1-P to produce β-D-glucosyl-(1 $\rightarrow$ 4)-2-acetamide-2-deoxy-D-glucose with GlcNAc as the acceptor substrate. The enzyme allowed aryl-β-glycosides of GlcNAc as the acceptor substrate with 10–20 % activities of GlcNAc. Kinetic parameters of $(GlcNAc)_2$ in the phosphorolysis and GlcNAc-1-P in the synthetic reaction were determined as follows: phosphorolysis, $k_0 = 5.5\ s^{-1}$, $K_m = 2.0$ mM; synthetic reaction, $k_0 = 10\ s^{-1}$, $K_m = 14$ mM, respectively. The mechanism of the phosphorolytic reaction followed a sequential Bi Bi mechanism, as frequently observed with cellobiose phosphorylases. Substrate inhibition by GlcNAc was observed in the synthetic reaction. The enzyme was considered a unique biocatalyst for glycosidation.

Key words: cellobiose phosphorylase, chitobiose phosphorylase, glycosyltransferase family 36, phosphorolytic reaction, synthetic reaction, *Vibrio*.

INTRODUCTION

CBP (cellobiose phosphorylase; EC 2.4.1.20) and CDP (cellodextrin phosphorylase; EC 2.4.1.49) have been classified as belonging to GT36 (glycosyltransferase family 36), based on the amino acid sequence identities (CAZY, http://afmb.cnrs-mrs.fr/CAZY/). CBP catalyses the reversible phosphorolysis of cellobiose to form Glc-1-P (α-D-glucose 1-phosphate; structure **2** in Figure 1) and D-glucose, but it does not phosphorolyse cellotriose or higher cello-oligosaccharides. On the other hand, CDP catalyses reversible phosphorolysis of cello-oligosaccharides with a degree of polymerization of three or higher. These enzymes participate in intracellular cellulose catabolism in bacteria. CBP has been found in several bacterial genera, but CDP has been found only in Clostridia. The genes encoding CBP and CDP have been isolated from *Cellvibrio gilvus*, *Clostridium thermocellum* YM4 and *Thermotoga maritima*, and the recombinant enzymes have been well characterized in our laboratory [1–4].

Recently, a gene from *Vibrio furnissii* encoding a sequence homologous to CBP was found neighbouring an N-acetylhexosaminidase gene and the expressed protein was determined to be ChBP (chitobiose phosphorylase), not CBP [5]. This ChBP catalysed reversible phosphorolysis of $(GlcNAc)_2$ (chitobiose; structure **17** in Figure 1) to form GlcNAc-1-P (2-acetamide-2-deoxy-α-D-glucose 1-phosphate; **1**) and GlcNAc. Based on its amino acid sequence, the ChBP was classified as belonging to GT36, similarly to CBP and CDP [6].

The whole genomic DNA sequences of some species of pathogenic *Vibrio* (*V. cholerae*, *V. parahaemolyticus*, and *V. vulnificus*) are available in databases, and open reading frames encoding GT36 enzymes have been found in all of the genomic DNA sequences examined to date (see the Kyoto Encyclopedia of Genes and Genomes; http://www.genome.ad.jp/kegg/kegg2.html) [7,8]. The open reading frames of *V. cholerae* (accession no. AE004146) and *V. vulnificus* (AE016802) were annotated as CBP, whereas that of *V. parahaemolyticus* (AP005081) was annotated as ChBP. Although the genes have not been expressed *in vitro*, based on their amino acid sequences, all of these genes are predicted to encode ChBP. The degrees of identity of the deduced amino acid sequence with CBPs or CDPs are less than 40 %, whereas the identities within *Vibrio* species including *V. furnissii* ChBP are more than 80 %. No genes showing such high levels of identity with ChBP have been found in any other genus. It is also notable that CBP has not been found in any species of *Vibrio*. Thus we assumed that ChBP is common in *Vibrio*, and the hypothesis prompted us to clone the GT36 gene from a non-pathogenic species of *Vibrio*, *V. proteolyticus*.

To confirm this hypothesis, it was necessary to determine the activity of a GT36 protein from *Vibrio* other than *V. furnissii*, because ChBP activity has only been confirmed with the enzyme from this species [5]. It is also important to investigate the kinetic properties of ChBP to determine the reaction mechanism of family 36 glycosyltransferases as well as CBP and CDP. However, there have been no previous reports of detailed kinetic analyses of ChBP. Here we describe the molecular cloning of a family 36 glycosyltransferase from *V. proteolyticus* that was determined to be ChBP. The substrate specificities and kinetic properties of the enzyme are also described.

Abbreviations used: CBP, cellobiose phosphorylase; CDP, cellodextrin phosphorylase; ChBP, chitobiose phosphorylase; GT36, glycosyltransferase family 36; Glc-1-P, α-D-glucose 1-phosphate; $(GlcNAc)_2$, chitobiose; $(GlcNAc)_3$, chitotriose; GlcNAc-1-P, 2-acetamide-2-deoxy-α-D-glucose 1-phosphate; GlcNAc-oxazoline, 2-methyl-(1,2-dideoxy-α-D-glucopyrano)-[2, 1-*d*]-2-oxazoline; TAIL, thermal asymmetric interlaced; Glc-GlcNAc, β-D-glucosyl-(1 $\rightarrow$ 4)-2-acetamide-2-deoxy-D-glucose; GlcNAc-UMB, 4-methylumbelliferyl-2-acetamide-2-deoxy-β-D-glucoside; GlcNAc-PNP, 4-nitrophenyl-2-acetamide-2-deoxy-β-D-glucoside.

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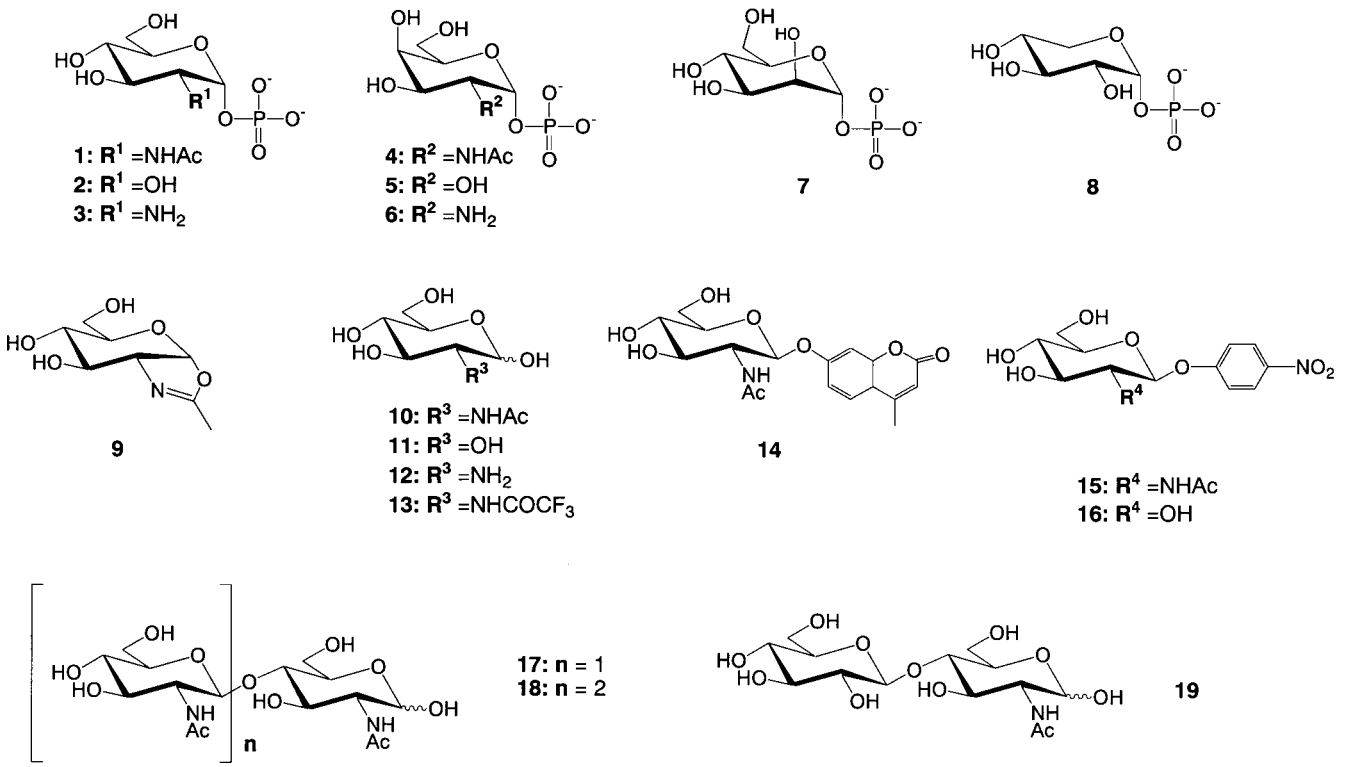

Figure 1 Structures of the substrates used in this study

1, 2-acetamide-2-deoxy-α-D-glucose 1-phosphate (GlcNAc-1-P); **2**, α-D-glucose 1-phosphate (Glc-1-P); **3**, 2-amino-2-deoxy-α-D-glucose 1-phosphate (GlcN-1-P); **4**, 2-acetamide-2-deoxy-α-D-galactose 1-phosphate (GalNAc-1-P); **5**, α-D-galactose 1-phosphate (Gal-1-P); **6**, 2-amino-2-deoxy-α-D-galactose 1-phosphate (GalN-1-P); **7**, α-D-mannose 1-phosphate (Man-1-P); **8**, α-D-xylose 1-phosphate (Xyl-1-P); **9**, GlcNAc-oxazoline; **10**, 2-acetamide-2-deoxy-D-glucose (GlcNAc); **11**, D-glucose (Glc); **12**, 2-amino-2-deoxy-D-glucose (GlcN); **13**, 2-trifluoroacetamide-2-deoxy-D-glucose [GlcN(TFA)]; **14**, 4-methylumbelliferyl-2-acetamide-2-deoxy-β-D-glucoside (GlcNAc-UMB); **15**, 4-nitrophenyl-2-acetamide-2-deoxy-β-D-glucoside (GlcNAc-PNP); **16**, 4-nitrophenyl-β-D-glucoside (Glc-PNP); **17**, chitobiose [$(GlcNAc)_2$]; **18**, chitotriose [$(GlcNAc)_3$]; **19**, β-D-glucosyl-(1 $\rightarrow$ 4)-2-acetamide-2-deoxy-D-glucose (Glc-GlcNAc).

EXPERIMENTAL

Materials

The genomic DNA of *V. proteolyticus* was prepared as reported previously [9]. The following enzymes were obtained commercially from the sources shown: restriction endonucleases (New England BioLabs, Beverly, MA, U.S.A.), FastStart *Taq* DNA polymerase (Roche Diagnostics, Mannheim, Germany) and DNA polymerase from *Thermococcus kodakaraensis* KOD1 (Toyobo, Osaka, Japan).

$(GlcNAc)_2$ (chitobiose; **17**) and $(GlcNAc)_3$ (chitotriose; **18**), were purchased from Seikagaku Kogyo Co. (Tokyo, Japan). To remove impurities, $(GlcNAc)_2$ was purified by gel-filtration column chromatography (Toyopearl HW-40C, 25 mm $\times$ 410 mm $\times$ 2; Tosoh, Tokyo, Japan) prior to use. GlcNAc-1-P (**1**) was obtained from Sigma Aldrich Chemicals (St. Louis, MO, U.S.A.). 2-Trifluoroacetamide-2-deoxy-D-glucose (**13**) was purchased from Tokyo Chemical Industry Co. (Tokyo, Japan). GlcNAc-oxazoline {2-methyl-(1,2-dideoxy-α-D-glucopyrano)-[2, 1-*d*]-2-oxazoline; **9**} was synthesized as described previously [10]. Structures and abbreviations of various sugar derivatives used in this study are summarized in Figure 1. Other reagents were of analytical grade and were obtained commercially.

DNA manipulation

Recombinant DNA techniques and agarose gel electrophoresis were performed as described by Sambrook et al. [11]. Plasmid DNA was prepared using a QIAprep Spin Plasmid kit (Qiagen, Hilden, Germany). Digestion by restriction enzymes was carried out in the appropriate buffer at concentrations of 1–10 units/μg of DNA for 0.5–16 h at 37 °C. Completion of the reaction was confirmed by agarose-gel electrophoresis. A QIAEX Agarose Gel Extraction kit (Qiagen) was used for the extraction and purification of DNA from agarose gels.

Nucleotide sequence analysis

The nucleotide sequence was determined by the dideoxynucleotide chain-termination method using an automated DNA sequencer (model 310A; Applied Biosystems, Foster City, CA, U.S.A.) with a dRhodamine Terminator Kit (PerkinElmer, Freemont, CA, U.S.A.). At least three independent clones of each PCR product were sequenced. Sequence data were analysed by using Genetyx Mac software version 11.0 (Genetyx Software Development Co., Tokyo, Japan).

PCR cloning of the *chbp* gene

The PCR cloning strategy is summarized in Figure 2. PCR primers for amplification of an internal sequence of the *chbp* gene were designed based on the multiple alignment of conserved amino acid sequences in GT36s: sense primer, 5′-ATGAAATACGGCTATTTCGAT-3′; antisense primer, 5′-CCABSCHGMDGTDCCBGTNAR-3′. The first PCR (using FastStart *Taq* DNA polymerase) was performed with *V. proteolyticus* genomic DNA as the template under the following conditions: 98 °C for 30 s, 45 °C for 30 s and 72 °C for 1 min (5 cycles), then 98 °C for 30 s,

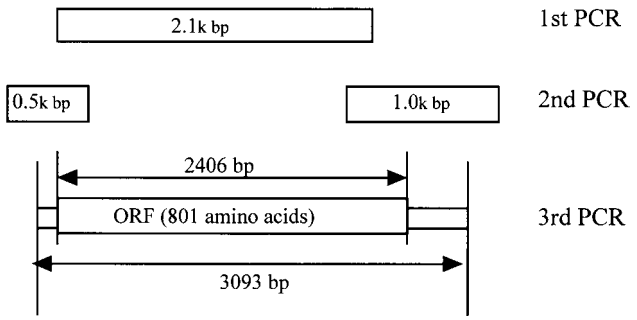

Figure 2 Strategy for PCR cloning of the gene encoding ChBP from *V. proteolyticus*

ORF, open reading frame.

55 °C for 30 s and 72 °C for 1 min (25 cycles), with a final extension step at 72 °C for 10 min. The amplified fragment (2.1 kb) was cloned into pCR®2.1-TOPO® (Invitrogen, San Diego, CA, U.S.A.).

The 5′ and 3′ regions of the ChBP gene were amplified by the TAIL (thermal asymmetric interlaced) PCR method (using FastStart *Taq* DNA polymerase) [12,13]. The specific antisense primers to amplify the 5′ region were: ChBP-5′-TAIL-I, 5′-GTTGTCGGACTGAATGTGGCTGAA-3′; ChBP-5′-TAIL-II, TCCACAAACGAAAACGCACTGATG-3′; and ChBP-5′-TAIL-III, 5′-AATTTTTCGGTGCCCAGATAGTTG-3′. The specific sense primers to amplify the 3′ region were: ChBP-3′-TAIL-I, 5′-TGCTCTCCGGGCTGGCTTCTC-3′; ChBP-3′-TAIL-II, 5′-TCAGGAGCGGGGCGAACAAGC-3′; and ChBP-3′-TAIL-III, 5′-CGTGTACCAGGGCGTGAAAGA-3′. The random degenerate primer and the TAIL-PCR parameters were the same as described previously [12,13]. The amplified fragments were cloned into pCR®2.1-TOPO® and their DNA sequences were determined.

Finally, the whole gene was amplified from the genomic DNA using DNA polymerase from *T. kodakaraensis* KOD1 and two oligonucleotide primers designed with the sequence obtained by TAIL-PCR. The amplified fragments were inserted in a pCR®2.1-TOPO® vector (*chbp*-TOPO) and subjected to DNA sequencing.

Expression of ChBP in *Escherichia coli*

The gene encoding *chbp* was amplified from *chbp*-TOPO by PCR (using *T. kodakaraensis* KOD1 DNA polymerase). The primers used for the reaction were as follows: forward primer, 5′-CATATGAAATACGGCTATTTCGATAATGACAAT-3′ (containing an *Nde*I site, underlined); reverse primer, 5′-CTCGAGACCGAGTACCACAACCTGGTTGTC-3′ (containing an *Xho*I site, underlined). The amplified fragment was subcloned into pCR®2.1-TOPO® (Invitrogen). The plasmid was then digested with *Nde*I and *Xho*I, and the digested fragment was ligated into pET30b (Novagen, Madison, WI, U.S.A.) at the corresponding sites, generating the plasmid pET30b-ChBP, encoding *chbp*, with a six-His sequence added to its C-terminus. Next, the expression vectors containing the *chbp* genes were electroporated into *E. coli* BL21GOLD (DE3), and positive colonies were selected. Resultant transformants were incubated in Luria broth (100 ml) containing 0.05 mg/ml kanamycin at 37 °C until the D_{600} reached a level of 0.6. Isopropyl β-D-thiogalactoside was then added to give a final concentration of 1 mM, and the cultures were incubated for 24 h at 25 °C. The cells (1 g) were harvested and ChBP was extracted in 5 ml of 50 mM Tris/HCl buffer (pH 8.0) using a sonicator (Branson Model 250D Sonifier; Branson, Danbury, CT, U.S.A.).

Purification of recombinant ChBP

The cell-free extract was loaded on an Ni^{2+}-nitriloacetate agarose (Qiagen) column (1 cm × 3 cm) and the enzyme was eluted with a stepwise gradient of imidazole (1, 10 mM; 2, 20 mM; 3, 250 mM) in 50 mM Tris/HCl buffer (pH 8.0) containing 0.3 M NaCl. The appropriate fractions were collected and the purity was checked by SDS/PAGE [14]. A 10 kDa protein ladder (Gibco-BRL, Grand Island, NY, U.S.A.) was used as a standard molecular marker for SDS/PAGE. To determine the native molecular mass of the enzyme, the protein was loaded on a gel-filtration column (Superdex 200 HR10/30; Amersham Biosciences, Uppsala, Sweden) pre-equilibrated with 50 mM sodium phosphate buffer, pH 7.0, containing 0.15 M NaCl, at a flow rate of 0.5 ml/min. MW-Marker HPLC (Oriental Yeast Co., Tokyo, Japan) was used as a molecular-mass standard.

Protein concentrations were determined from the absorbance at 280 nm based on theoretical molar absorption coefficients (166095 $M^{-1} \cdot cm^{-1}$) determined from the amino acid compositions of ChBP [15]. The N-terminal amino acid sequence of purified recombinant ChBP was determined using a G1000A protein sequencer (Hewlett Packard, Palo Alto, CA, U.S.A.).

Enzyme assay

The enzyme activity was routinely determined by the phosphorolysis of $(GlcNAc)_2$. The enzymic reaction was carried out in 10 mM Bis-Tris buffer (pH 7.5) containing 5 mM $(GlcNAc)_2$ and 5 mM phosphate at 30 °C. Periodically, a portion of the reaction mixture was boiled for 5 min to inactivate the enzyme. The concentration of GlcNAc was quantified by high-performance ion-exchange chromatography on a CARBOPAC PA1 column (4 mm × 250 mm; Dionex, Sunnyvale, CA, U.S.A.) equipped with a pulsed amperometric detector (DX-3; Dionex). Chromatography was performed with a linear gradient of 0–0.2 M sodium acetate in 0.1 M NaOH for 20 min at a flow rate of 1 ml/min.

The synthetic reaction was carried out in 10 mM Bis-Tris buffer (pH 7.5) containing 5 mM of each sugar phosphate derivative and 5 mM GlcNAc at 30 °C. The amount of phosphate liberated in the reaction was determined by the method of Lowry and Lopez [16]. Unless otherwise noted, the enzymic reaction was routinely measured as described above.

Effects of pH and temperature on enzymic activity

The enzymic activity was measured under the standard conditions of $(GlcNAc)_2$ phosphorolysis while changing the pH in the reaction mixture with 10 mM buffers. The pH stability was determined by incubating the enzyme at 30 °C for 30 min at each pH, followed by measuring the activity under the standard conditions. The buffer systems used were Bis-Tris (pH 5.9–7.7) and Taps (pH 8.0–8.7). The final pH values of the reaction solution were determined after addition of the enzyme and the substrates. The optimum temperature of the activity was measured for 10 min under the standard conditions except for the temperature. The thermostability was also determined by incubating the enzyme at each temperature for 30 min in 15 mM sodium phosphate buffer (pH 7.0), followed by measuring the activity under the standard conditions.

Analysis of enzymic products

The enzymic products were separated by TLC on a silica gel 60 F_{254} plate (5.0 cm × 7.5 cm; Merck, Darmstadt, Germany)

with a solvent system of acetonitrile/water (4:1, v/v). Sugars were detected by baking after dipping the plate in 5% H_2SO_4 in methanol.

Preparation of Glc-GlcNAc (β-D-glucosyl-(1 → 4)-2-acetamide-2-deoxy-D-glucose)

GlcNAc (265 mg, 1.2 mmol) and Glc-1-P (258 mg, 1.0 mmol) were dissolved in 24 ml of 6 mM Bis-Tris buffer (pH 7.5) and mixed with ChBP (5.6 mg, 0.06 μmol). The reaction mixture was incubated for 20 h at 25 °C, followed by deionization with Amberlite MB-3 (Organo, Tokyo, Japan), and concentrated. The disaccharide produced (determined to be Glc-GlcNAc; **19**) was isolated by gel-filtration column chromatography (Toyopearl HW-40C, 25 mm × 410 mm × 2; Tosoh) with water as the solvent. The fraction containing Glc-GlcNAc was lyophilized to a white powder (yielding 163 mg, 0.43 mmol, 43%).

Kinetic analysis

To determine the apparent kinetic parameters, $(GlcNAc)_2$ and Glc-GlcNAc were subjected to phosphorolysis in 10 mM Bis-Tris buffer (pH 7.5) containing 5 mM phosphate at 30 °C. The synthetic reactions from GlcNAc-1-P and Glc-1-P were also performed in 10 mM Bis-Tris buffer (pH 7.5) containing 5 mM GlcNAc at 30 °C. The initial rates were measured as the increase in GlcNAc or phosphate as described above. The kinetic parameters were calculated by regressing the experimental data into each appropriate formula by the curve fit method using GraFit Ver. 4.0 (Erithacus Software, Horley, Surrey, U.K.).

RESULTS

Molecular cloning of *chbp* and sequence comparison with family 36 glycosyltransferases

The strategy for PCR cloning of the gene encoding ChBP is shown in Figure 2. First, a 2.1 kb PCR fragment was obtained using degenerate synthetic primers based on the conserved amino acid sequences of CBP and CDP. Next, fragments at the 5′ and 3′ regions were amplified by TAIL-PCR using a random primer and specific primers based on the internal sequence. Finally, a 3.1 kb fragment containing the open reading frames of ChBP was amplified and cloned. The accession number of the sequence is AB096684.

Partial genes of β-N-acetylhexosaminidase and phosphoglucomutase/phosphomannomutase were found upstream and downstream of the open reading frames, respectively, as observed in other species of *Vibrio* [5,7,8].

The gene (*chbp*) encoded a protein of 801 amino acid residues with a molecular mass of 90360 Da. The deduced amino acid sequence showed a degree of identity with ChBP of *V. furnissii* (82%) [5]. It also showed high levels of identity with other family 36 glycotransferases found in the genomes of the pathogenic *Vibrio* species *V. parahaemolyticus* (89%) [8], *V. cholerae* (83%) [7] and *V. vulnificus* (83%). The identities with CBP and CDP were as follows: CBP, *Thermotoga neapolitana* (34%) [17], *C. gilvus* (33%) [1], *Thermotoga martima* (34%) [18], *Clostridium stercorarium* (33%) [19], *Cl. thermocellum* (32%) [20] and *Cl. thermocellum* YM-4 (32%) [2]; CDP, *Cl. stercorarium* (35%) [19] and *Cl. thermocellum* YM-4 (39%) [4].

Characterization of basic enzymic property

Recombinant ChBP was expressed in *E. coli* BL21GOLD (DE3) and purified, yielding a 90 kDa protein on SDS/PAGE as shown in Figure 3. The native molecular mass was determined to be 150 kDa, indicating that the enzyme existed as a dimer. The enzyme was purified 26-fold as shown in Table 1. The N-terminal sequence was Met-Lys-Tyr-Gly-Tyr, corresponding to the sequence determined from the starting codon.

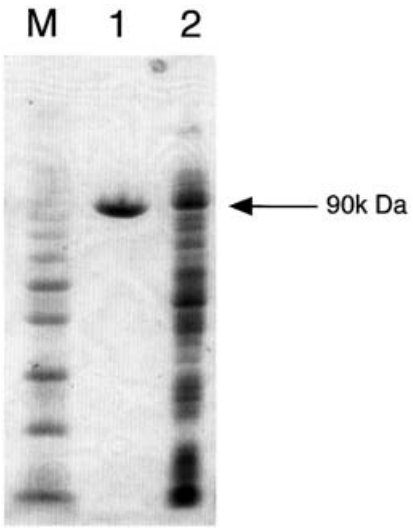

Figure 3 SDS/PAGE of the recombinant ChBP

Lane M, 10 kDa protein ladder (Gibco-BRL); lane 1, purified recombinant ChBP (2.2 μg); lane 2, crude extract from *E. coli* BL21GOLD (DE3) cells containing pET30-ChBP.

Table 1 Purification of the recombinant ChBP from *V. proteolyticus*

Enzymic activity was determined by phosphorolytic reaction of 5 mM $(GlcNAc)_2$ and 5 mM phosphate in 10 mM Bis-Tris buffer (pH 7.5) at 30 °C.

	Protein (mg)	Activity (μmol/min)	Specific activity (μmol/min per mg)
Crude extract	233.0	27.9	0.12
Ni-NTA agarose	6.5	20.0	3.08

First, to determine ChBP activity, reversible phosphorolysis was confirmed by analysis of the enzymic products on TLC. When $(GlcNAc)_2$, phosphate and the ChBP were incubated together, the enzyme produced GlcNAc-1-P and GlcNAc, and the reversible synthetic reaction was also detected. On the other hand, the ChBP showed no phosphorolytic activity on cellobiose. Therefore, the enzyme was identified as a ChBP, and not a cellobiose phosphorylase.

Enzymic properties as a function of pH and temperature were determined with the phosphorolysis of $(GlcNAc)_2$. The pH optimum of the activity was between 7.0 and 7.7 and the enzyme was completely stable between pH 7.0 and 8.1 at 30 °C for 30 min. The enzyme was stable at temperatures up to 35 °C and the optimal temperature was 30 °C. The concentration of Bis-Tris buffer (10 and 50 mM) did not affect the reaction rate, eliminating the possibility of the inhibition by Bis-Tris often observed with carbohydrate related enzymes.

Substrate specificity of synthetic reaction of ChBP

Table 2 summarizes the substrate specificity of the enzyme in the synthetic reaction. GlcNAc-1-P was the best donor substrate in the synthetic reaction. ChBP was also active on Glc-1-P with

Table 2 Substrate specificity in the synthetic reaction catalysed by the purified ChBP

The enzymic activity of the synthetic reaction was determined by quantification of liberated phosphate (described in the Experimental section). Reaction conditions were as follows. Donor specificity: each donor (8 mM), GlcNAc (7 mM) and ChBP (0.1 μM) were incubated for 12 h in 15 mM Bis-Tris buffer (pH 7.5) at 30 °C. For GlcNAc-oxazoline (**9**), the reaction product was analysed by high-performance ion-exchange chromatography. Acceptor specificity: each acceptor (5 mM), GlcNAc-1-P (5.5 mM) and ChBP (0.1 μM) was incubated for 12 h in 15 mM Bis-Tris buffer (pH 7.5) at 30 °C. The concentrations of GlcNAc-UMB, GlcNAc-PNP and Glc-PNP were 0.29, 0.26 and 1.2 mM, respectively, due to their low solubility, and the rates were compared with the reaction rate at 0.3 mM GlcNAc. A dash (–) indicates less than 1 % activity in comparison with GlcNAc-1-P and GlcNAc. Abbreviations are defined in the text and in the Figure 1 legend.

Donor	Specificity	Acceptor	Specificity
GlcNAc-1-P (**1**)	100 %	GlcNAc (**10**)	100 %
Glc-1-P (**2**)	10 %	$(GlcNAc)_2$ (**17**)	–
GlcN-1-P (**3**)	–	$(GlcNAc)_3$ (**18**)	–
GalNAc-1-P (**4**)	–	Glc (**11**)	–
Gal-1-P (**5**)	–	GlcN (**12**)	–
GalN-1-P (**6**)	–	GlcN(TFA) (**13**)	20 %
Man-1-P (**7**)	–	GlcNAc-UMB (**14**)	20 %
Xyl-1-P (**8**)	–	GlcNAc-PNP (**15**)	10 %
GlcNAc-oxazoline (**9**)	–	Glc-PNP (**16**)	–

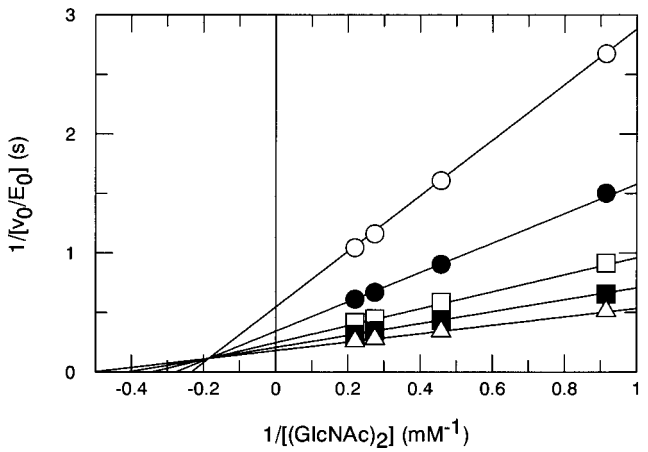

Figure 4 Double-reciprocal plot of the phosphorolytic reaction of $(GlcNAc)_2$ at various concentrations of P_i

Symbols indicate phosphate concentrations: ○, 0.5 mM; ●, 1.0 mM; □, 1.9 mM; ■, 3.0 mM; △, 5.0 mM. Theoretical lines are shown based on the rate equation for a sequential Bi Bi mechanism as described in the Results section. Other conditions are described in the text.

10 % activity as compared with that on GlcNAc-1-P. The enzyme showed no activity on any other sugar-phosphate examined. In addition, the enzyme did not use GlcNAc-oxazoline (**9**) as a donor substrate. The reaction product with Glc-1-P and GlcNAc was prepared as described in the Experimental section. The structure was determined to be that of Glc-GlcNAc (**19**) by ^{1}H- and ^{13}C-NMR (Table 3) electrospray ionization MS, with *m/z*, $[M+Na]^+$, 406.1303; $[M+K]^+$, 422.1044; calculated for $C_{14}H_{25}NO_{11}$, $[M+Na]^+$, 406.1326; $[M+K]^+$, 422.1065.

ChBP utilized GlcNAc as the best acceptor substrate. Chito-oligosaccharides $(GlcNAc)_n$ did not act as acceptors for the synthetic reaction, indicating that the enzyme had no chito-dextrin phosphorylase activity. However, the enzyme was active on aryl-β-glycosides of GlcNAc [GlcNAc-PNP (4-nitrophenyl-2-acetamide-2-deoxy-β-D-glucoside; **15**) and GlcNAc-UMB (4-methylumbelliferyl-2-acetamide-2-deoxy-β-D-glucoside; **14**)], producing aryl-β-chitobiosides. ChBP was also active on the trifluoroacetamide derivative of GlcNAc.

Kinetic properties of ChBP

To determine the reaction mechanism of ChBP, a double-reciprocal plot of $(GlcNAc)_2$ phosphorolysis was performed by changing the concentrations of phosphate (Figure 4). The lines of double-reciprocal plots for the phosphorolytic reaction crossed at a certain point on the second quadrant, indicating that the enzymic reaction followed a sequential Bi Bi mechanism. The kinetic parameters of the sequential Bi Bi mechanism were calculated

Table 3 Assignments of ^{1}H- and ^{13}C-NMR chemical shifts of Glc-GlcNAc (19)

Measurements were taken in ^2H_2O. The ^{1}H- and ^{13}C-NMR chemical shifts were determined from two-dimensional spectra of double-quantum-filtered COSY, HMBC (heteronuclear multiple-bond coherence) and heteronuclear single quantum coherence. I, reducing end; II, non-reducing-end residue. Asterisks (*, **, ***) indicate the signals where inter-ring HMBC cross-peaks were observed.

	C1	C2	C3	C4	C5	$C6_x$	$C6_y$	C=O	CH_3
α									
I									
^{1}H	5.111 (d, $J_{1,2}=2.8$)	3.789–3.805 (m)		3.597–3.651* (m)	3.864–3.884 (m)	3.789–3.805 (m)		–	1.950 (s)
^{13}C	90.903	54.179	69.567	79.501**	70.63	60.289	–	175.098	22.258
II									
^{1}H	4.442** (d, $J_{1,2}=7.9$)	3.228 (dd, $J_{2,3}=9.4$)	3.421 (dd, $J_{3,4}=9.2$)	3.315 (dd, $J_{4,5}=9.7$)	3.403 (ddd, $J_{5,6y}=5.8$)	3.814 (dd, $J_{5,6x}=2.1$)	3.638 (dd, $J_{6,6}=12.4$)	–	–
^{13}C	102.948*	73.54	75.853	69.81	76.352	60.981	–	–	–
β									
I									
^{1}H	4.442 (d, $J_{1,2}=7.9$)		3.597–3.651 (m, $J_{4,5}=9.2$)		3.501 (ddd, $J_{5,6y}=5.6$)	3.872 (dd, $J_{5,6x}=2.2$)	3.741 (dd, $J_{6,6}=12.3$)	–	1.948 (s)
^{13}C	95.216	56.708	72.764	79.085***	75.203	60.418	–	174.836	22.550
II									
^{1}H	4.438*** (d, $J_{1,2}=8.0$)	3.215 (dd, $J_{2,3}=9.4$)	3.415 (dd, $J_{3,4}=9.2$)	3.323 (dd, $J_{4,5}=9.8$)	3.395 (ddd, $J_{5,6y}=5.8$)	3.811 (dd, $J_{5,6x}=2.1$)	3.142 (dd, $J_{6,6}=12.4$)	–	–
^{13}C	102.948	73.54	75.853	69.81	76.352	60.981	–	–	–

Table 4 Kinetic parameters of the reversible phosphorolytic reaction by the ChBP

In phosphorolysis, phosphate concentration was fixed at 5.0 mM. In the synthetic reaction, GlcNAc concentration was fixed at 5 mM. The enzymic reaction was carried out in 10 mM Bis-Tris buffer (pH 7.5) at 30 °C. Other conditions are described in the text.

Substrate	k_0 (s^{-1})	K_m (mM)	k_0/K_m ($s^{-1} \cdot mM^{-1}$)
Phosphorolysis			
$(GlcNAc)_2$	5.5 ± 0.1	2.0 ± 0.1	2.8 ± 0.1
Glc-GlcNAc	3.3 ± 0.2	69 ± 6	0.047 ± 0.001
Synthesis			
GlcNAc-1-P	10 ± 1	14 ± 2	0.70 ± 0.04
Glc-1-P	0.48 ± 0.04	13 ± 0.3	0.037 ± 0.006

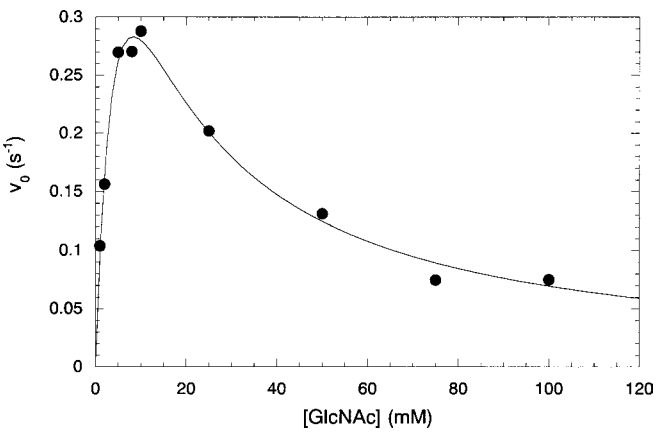

Figure 5 Plot of the synthetic reaction with Glc-1-P and GlcNAc as the substrates

The theoretical curve is based on the uncompetitive substrate inhibition model described in the Results section. Glc-1-P concentration was fixed at 12.0 mM. Other conditions are described in the text.

with eqn (1) [21]:

$$v = k_{cat}[E]_0[A][B]/(K_{iA}K_{mB} + K_{mB}[A] + K_{mA}[B] + [A][B]) \quad (1)$$

where A is $(GlcNAc)_2$ and B is P_i. The theoretical curve generated with eqn (1) agreed well with the experimental data. The kinetic parameters in eqn (1) were calculated to be $k_{cat} = 7.2 \pm 0.3\ s^{-1}$, $K_{mA} = 1.0 \pm 0.2$ mM, $K_{mB} = 1.5 \pm 0.2$ mM and $K_{iA} = 5.4 \pm 0.8$ mM.

Apparent kinetic parameters of the disaccharides [$(GlcNAc)_2$ and Glc-GlcNAc] and sugar phosphates (GlcNAc-1-P and Glc-1-P) in the synthetic reaction with the other substrates at fixed concentrations (5 mM phosphate and 5 mM GlcNAc, respectively) are summarized in Table 4. The k_0 value obtained in the phosphorolysis of Glc-GlcNAc was not significantly different from that of $(GlcNAc)_2$, whereas the K_m value was 35-fold higher than that of $(GlcNAc)_2$. On the other hand, the K_m value for Glc-1-P was not significantly different from that of GlcNAc-1-P but the k_0 was 20-fold lower.

The initial rates of the synthetic reaction were plotted against the concentration of GlcNAc, and a significant substrate inhibition was observed at higher concentrations of GlcNAc as shown in Figure 5. The rate at 100 mM GlcNAc was 26 % of that at 10 mM GlcNAc. The curve in Figure 5 could be regressed by a substrate inhibition model as given in the following formula [22]:

$$v = k_0[E][S]/\{K_m + [S] + ([S]^2/K_i)\} \quad (2)$$

Table 5 Comparison of the properties of ChBP and CBP

ChBP indicates ChBP from *V. proteolyticus* (this study). CBP is from *C. gilvus* [30]. ++, Active; +, less active; −, not active.

	ChBP	CBP
Acceptor	GlcNAc (++) Glc (−)	Glc (++) GlcNAc (−)
Donor	GlcNAc-1-P (++) Glc-1-P (+)	Glc-1-P (++) GlcNAc-1-P (−)
Mechanism	Sequential	Sequential
Competitive substrate inhibition	Yes	Yes

The apparent kinetic parameters were calculated to be $k_0 = 0.72 \pm 0.11\ s^{-1}$, $K_m = 6.4 \pm 1.5$ mM and $K_i = 10.8 \pm 2.4$ mM.

DISCUSSION

ChBP (EC 2.4.1.–) belongs to the GT36 along with CBP (EC 2.4.1.20) and CDP (EC 2.4.1.49), based on the amino acid sequence [6]. These enzymes catalyse reversible phosphorolysis of corresponding oligosaccharides, such as $(GlcNAc)_2$, cellobiose and cello-oligosaccharides, resulting in inversion of the anomeric position of the products. In this study, we determined that a GT36 gene of *V. proteolyticus* encoded ChBP. The basic enzymic properties of *V. proteolyticus* ChBP, such as the effects of pH and temperature, were similar to those of the enzyme of *V. furnissii*, the only ChBP characterized to date [5]. However, this study provided several new functional insights into ChBP (Table 5): (i) ChBP utilized Glc-1-P as a donor in the synthetic reaction; (ii) the reaction proceeded by a sequential Bi Bi mechanism; and (iii) substrate inhibition was observed in the reverse phosphorolysis using GlcNAc as the acceptor. The enzymic properties are discussed in further detail by comparison with other GT36 enzymes.

Sequence comparison

To date, ChBP has been found only in *Vibrio* and not in any other genus. On the other hand, CBP and CDP were found in a variety of genera, such as *Cellvibrio*, *Clostridium*, *Ruminococcus*, *Cellulomonas* and *Thermotoga* [3,19,23–26]. As described in the Results section, the deduced amino acid sequences of GT36 genes from *V. parahaemolyticus*, *V. cholerae* and *V. vulnificus* show very high levels of identity with the ChBP from *V. proteolyticus* (this study) and *V. furnissii* [5,7,8]. Thus these results support the conclusion that these genes from *V. parahaemolyticus*, *V. cholerae* and *V. vulnificus* encode ChBP, but not CBP or CDP.

Comparison of the whole amino acid sequences of ChBP, CBP and CDP belonging to the GT36 revealed several conserved regions, as shown in Figure 6. Despite the high levels of amino acid sequence identity in all five ChBP amino acid sequences (from *V. proteolyticus*, *V. parahaemolyticus*, *V. furnissii*, *V. cholerae* and *V. vulnificus*), a non-conserved region was also found (Figure 7). The enzymes may be divided into two subclasses, subclass I (*V. proteolyticus* and *V. parahaemolyticus*) and subclass II (*V. furnissii*, *V. cholerae* and *V. vulnificus*), based on their amino acid sequences in this non-conserved region.

Substrate specificity

The donor-binding site, subsite −1, of the ChBP recognized not only GlcNAc-1-P but also Glc-1-P as shown in Table 2. On the other hand, it did not utilize GlcNAc-oxazoline as a

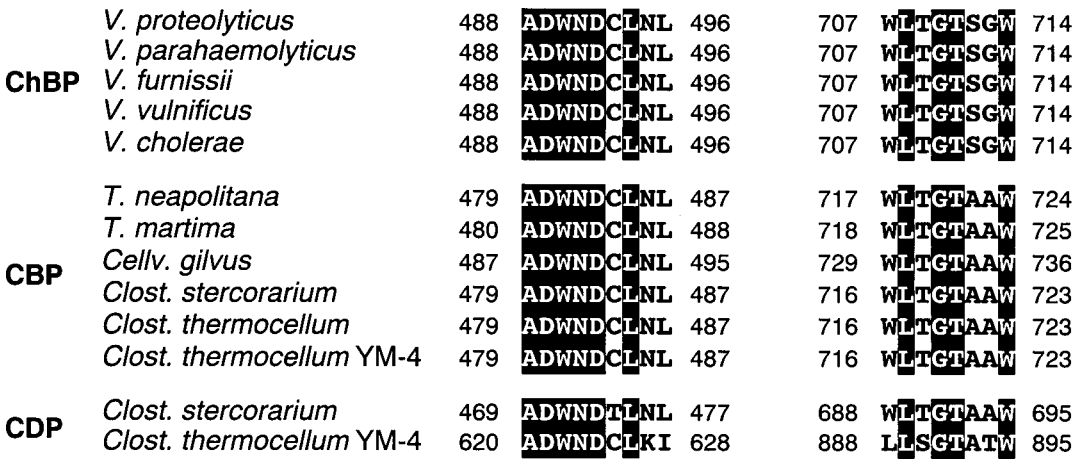

Figure 6 Conserved regions in the amino acid sequences of ChBP, CBP and CDP

Identical amino acids are shown on a black background. Accession nos: ChBP, *V. proteolyticus* (AB096684), *V. parahaemolyticus* (AP005081), *V. furnissii* (AF230379), *V. vulnificus* (AE016802), *V. cholerae* (AE004146); CBP, *T. neapolitana* (AF039487), *T. maritima* (AE001822), *C. gilvus* (AB010707), *Cl. stercorarium* (U56424), *Cl. thermocellum* (AB013109), *Cl. thermocellum* YM-4 (AY072794); CDP, *Cl. stercorarium* (U60580), *Cl. thermocellum* YM-4 (AB061316).

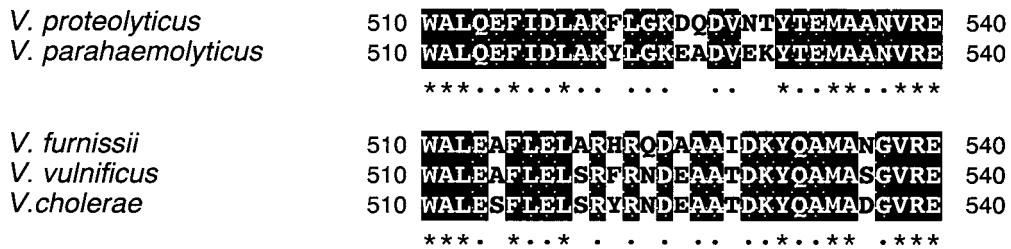

Figure 7 A non-conserved region in the amino acid sequences of ChBP from various *Vibrio* species

Asterisks indicate strictly conserved amino acid residues in all ChBPs. Dots indicate conserved amino acids in subclasses I (*V. proteolyticus* and *V. parahaemolyticus*) and II (*V. furnissii*, *V. vulnificus* and *V. cholerae*).

donor substrate. This specificity was different from that of N-acetylhexosaminidases belonging to GH20 (glycoside hydrolase family 20), the nucleophile residue of which was not found [27]. A model of 'substrate-assisted catalysis' has been proposed to explain the hydrolytic reaction without the nucleophile residue [27]. In the model, the carbonyl oxygen of the N-acetyl group of the donor substrate acts as the nucleophile to form an oxazoline-like intermediate [27]. This model is also supported by the observation that the N-acetylhexosaminidase utilizes GlcNAc-oxazoline in the hydrolytic reaction. Judging from its substrate specificity, it is clear that the mechanism of action of ChBP is different from substrate-assisted catalysis.

Subsite $+1$, the acceptor-binding site, recognized GlcNAc and its aryl-β-glycosides [GlcNAc-PNP (**15**) and GlcNAc-UMB (**14**)], but did not recognize glucose derivatives. These results indicate that subsite $+1$ strictly recognizes the C2 functional group of the sugar. In the synthetic reaction, ChBP produced $(GlcNAc)_2$-UMB (4-methylumbelliferyl-di-*N*-acetyl-β-chitobioside) from GlcNAc-1-P and GlcNAc-UMB with a yield of 47 % (results not shown), suggesting that the ChBP might be a versatile biocatalyst for glycosidation of GlcNAc and its derivatives.

Kinetic properties

Kinetic analysis of $(GlcNAc)_2$ phosphorolysis indicated that the reaction catalysed by the ChBP proceeded via a sequential Bi Bi mechanism as shown in Figure 4. This agreed with the mechanism of action of CBP, another member of GT36 [2,6,28,29]. Thus a sequential Bi Bi mechanism of action may be common to GT36 enzymes.

As shown in Figure 5, at higher concentrations GlcNAc has an inhibitory effect on the synthetic reaction. Such substrate inhibition by the acceptor has been reported in the reactions of several CBP and a laminaribiose phosphorylase [2,6,29–31]. These enzymes reversibly phosphorolyse glucobioses to form Glc-1-P and D-glucose. The inhibition was caused by competition of Glc and Glc-1-P at the Glc-1-P binding site (subsite -1) [6]. In the case of ChBP, the mechanism of substrate inhibition can be explained as follows: GlcNAc binds to subsite -1 competitively because GlcNAc is considered an analogue of GlcNAc-1-P. Substrate inhibition may be common to homo-disaccharide phosphorolytic enzymes.

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