Structure and function of a novel periplasmic chitooligosaccharide-binding protein from marine *Vibrio* bacteria

Received for publication, November 18, 2017, and in revised form, February 11, 2018. Published, Papers in Press, February 14, 2018, DOI 10.1074/jbc.RA117.001012

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Edited by Wolfgang Peti

Periplasmic solute-binding proteins in bacteria are involved in the active transport of nutrients into the cytoplasm. In marine bacteria of the genus *Vibrio*, a chitooligosaccharide-binding protein (CBP) is thought to be the major solute-binding protein controlling the rate of chitin uptake in these bacteria. However, the molecular mechanism of the CBP involvement in chitin metabolism has not been elucidated. Here, we report the structure and function of a recombinant chitooligosaccharide-binding protein from *Vibrio harveyi*, namely VhCBP, expressed in *Escherichia coli*. Isothermal titration calorimetry revealed that VhCBP strongly binds shorter chitooligosaccharides ((GlcNAc)_n, where *n* = 2, 3, and 4) with affinities that are considerably greater than those for glycoside hydrolase family 18 and 19 chitinases but does not bind longer ones, including insoluble chitin polysaccharides. We also found that VhCBP comprises two domains with flexible linkers and that the domain–domain interface forms the sugar-binding cleft, which is not long extended but forms a small cavity. (GlcNAc)₂ bound to this cavity, apparently triggering a closed conformation of VhCBP. Trp-363 and Trp-513, which stack against the two individual GlcNAc rings, likely make a major contribution to the high affinity of VhCBP for (GlcNAc)₂. The strong chitobiose binding, followed by the conformational change of VhCBP, may facilitate its interaction with an active-transport system in the inner membrane of *Vibrio* species.

Vibrio harveyi is a Gram-negative bioluminescent marine bacterium found in tropical marine waters and is a serious pathogen of aquatic fish and invertebrates, including crusta-

cean and zooplankton (1). Chitin, a β -1,4-linked polysaccharide of GlcNAc, is an important marine biomass, which is efficiently converted by *Vibrios*. Thus, *V. harveyi* has an efficient chitin degradation system, including a chitinase A (2–5) belonging to glycoside hydrolase family 18 (GH18),² a β -N-acetylglucosaminidase (6, 7) belonging to the GH20 family, and a chitinoligosaccharide deacetylase (8, 9) belonging to carbohydrate esterase family 4. GH18 chitinases hydrolyze β -1,4-glycosidic linkages of chitin in an endo-splitting mode, producing chitin oligosaccharides (GlcNAc)_n (*n* = 2, 3, 4, and more), which are further hydrolyzed into GlcNAc monomer in an exo-splitting mode by GH20 β -N-acetylglucosaminidase. The carbohydrate esterase family 4 enzyme hydrolyzes the amide bond of the reducing end residue of (GlcNAc)₂ producing the deacetylated derivative (10). *V. harveyi* also has an efficient uptake system of GlcNAc or (GlcNAc)_n, including an ATP-binding cassette-type (ABC) transporter for GlcNAc or (GlcNAc)₂ (11) and a chitoporin for the transport of chitin oligosaccharides (GlcNAc)_n (*n* = 3, 4, 5, and 6) (12–14). In *Vibrio* species, all proteins described in this study are most likely crucial for efficient degradation/uptake of chitin as nutrients.

On the other hand, it has been recognized that solute-binding proteins (SBPs) localized to the periplasmic space of Gram-negative bacteria are involved in nutrient import as components of ABC transporters (15). The solute binding triggers the association of SBPs with the ABC transporter located in the cytoplasmic membrane. The solute is then released from SBP and actively transported into the cytoplasm using energy provided by ATPase as one of the components of the transporter (15). In some cases, SBPs are also involved in transduction of solute signals into cytoplasm (16). Most bacterial SBPs investigated to date adopt a similar fold composed of two lobes, which are connected by one or more polypeptide chains. In the solute-free conformation, the two lobes are separated; however, the solute binds to the cleft formed between the two domains,

This work was supported by Suranaree University of Technology and by Office of the Higher Education Commission under National Research University Project of Thailand Contract FtR.12/2558 (to W. S. and T. F.), by Thailand Research Fund and Suranaree University of Technology through Basic Research Grant BRG578001 (to W. S.), and by Suranaree University of Technology Grant SUT1-102-58-36-10 (to W. S.). The authors declare that they have no conflicts of interest with the contents of this article.

The atomic coordinates and structure factors (code 5YQW) have been deposited in the Protein Data Bank (<http://www.pdb.org/>).

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²The abbreviations used are: GH, glycoside hydrolase; SBP, solute-binding protein; CBP, chitooligosaccharide-binding protein; VhCBP, CBP from *V. harveyi*; VcCBP, CBP from *V. cholerae*; (GlcNAc)_n, β -1,4-linked oligosaccharide of GlcNAc with a polymerization degree of *n*; ABC, ATP-binding cassette; ITC, isothermal titration calorimetry; PDB, Protein Data Bank; RMSD, root mean square deviation.

Figure 1. Amino acid sequence and secondary structure alignments of VhCBP and VcCBP. The secondary structures located in the upper domain are drawn in orange (α -helices) and magenta (β -strands), whereas those located in the lower domain are drawn in blue (α -helices) and cyan (β -strands). Individual secondary structures, α -helices and β -strands, are designated as $\alpha 1$ – $\alpha 21$ and $\beta 1$ – $\beta 21$, respectively, from the N terminus, and correspond to $\alpha 1$ – $\alpha 21$ and $\beta 1$ – $\beta 21$ in Fig. 4A. Amino acid residues involved in $(\text{GlcNAc})_2$ binding in the crystal structure of VhCBP in complex with $(\text{GlcNAc})_2$ are written in red, and are all conserved in both proteins, except Glu-10 of VhCBP. The other conserved amino acids are highlighted by bold type in the sequences. The two sequences share 83% homology.

resulting in closure of the two lobes (15). This closed conformation of SBP has been regarded as important for interaction with the transporter; hence, for the active transport of the solute (17). Similar SBPs also exist in the chitin-catabolizing system of marine *Vibrio* bacteria. In *Vibrio cholera*, an SBP has been identified to bind chito oligosaccharides and thereafter referred to as VcCBP, following its specificity toward these specific sugars. It has been proposed that VcCBP may play essential roles in controlling the rate of chito oligosaccharide transport across phospholipid membranes, as well as acting as a negative regulator of chitin catabolic sensor/kinase that controls expression of proteins involving the chitin degradation cascade (11). However, the exact physiological roles of VcCBP in the chitin metabolism of *Vibrios* remain to be elucidated. Although crystal structures of VcCBP have been registered in Protein Data Bank (PDB) under codes, 1ZTY and 1ZU0, no functional data were reported for this protein. We found a homologue of VcCBP in *V. harveyi* (VhCBP), the amino acid sequence of which is highly homologous (83%) to that of VcCBP, as shown in Fig. 1. Based on the amino acid sequence similarity, these two proteins do not match with any CBM family members in the CAZy database (<http://www.cazy.org>)³ (38) but match with the members in the SBP family 5 (InterPro no. IPR000914), which includes oligopeptide-binding proteins, murein-peptide-bind-

ing proteins, and nickel-binding proteins. The binding targets of the members of this family are diverse, suggesting that the mechanism of solute binding may differ from each other despite significant sequence similarity.

We herein produced a recombinant protein of VhCBP, which was characterized with respect to the crystal structure and chito oligosaccharide-binding properties. The crystal structure revealed the molecular basis of the strong binding of chito oligosaccharide to VhCBP followed by the drastic conformational change, which may be important for the active transport.

Results

Production of recombinant VhCBP protein by *Escherichia coli* expression system

Using *E. coli* strain Origami (DE3) and the pET23a expression plasmid, we successfully produced the recombinant VhCBP protein, which was then purified by Ni^{2+} -affinity chromatography followed by anion exchange chromatography (HiTrap Q) and gel filtration on 16/60 Superdex 200. The profile of the final step of purification is shown in Fig. 2A. SDS-PAGE of the purified VhCBP revealed a single protein band at 61 kDa of molecular mass, which correspond to the calculated molecular mass of VhCBP (61223.41 Da). From 1 liter of culture medium, we obtained 10 mg of the purified VhCBP on average.

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Figure 2. A, gel filtration of VhCBP using a column of 16/60 Superdex 200. Elution was conducted using 20 mM Tris-HCl buffer, pH 8.0, containing 150 mM NaCl. Individual protein fractions were analyzed by SDS-PAGE (inset), which was performed according to the method of Laemmli (28). The gel was stained with Coomassie Brilliant Blue. B, binding abilities of VhCBP toward colloidal α -chitin and β -chitin. A mixture solution (500 μ l) comprising 5 μ g of protein, 1.0 mg of colloidal chitin, and 20 mM Tris-HCl buffer, pH 8.0, containing 150 mM NaCl was incubated for 30 min at 4 $^{\circ}$ C, and the bound protein fraction was calculated from the protein content of the supernatant determined by the method of Bradford (30). A chitinase A from *V. harveyi* (18) was used as a positive control.

Because prediction of post-translational glycosylation suggested two putative *N*-glycosylation sites (amino acids 367–369, Asn-Asn-Thr and amino acids 493–495, Asn-Thr-Thr) for VhCBP, we carried out digestion of the protein by *N*-glycosidase F to prove the glycosylation status of VhCBP. The results showed no difference in the migration of VhCBP on SDS-PAGE gel before and after the enzyme digestion, indicating that VhCBP is not *N*-glycosylated (data not shown).

Chitooligosaccharide binding to VhCBP

Before testing soluble sugars, GlcNAc to (GlcNAc)₆, we conducted the binding experiments using insoluble chitin. As shown in Fig. 2B, the relative amounts of bound proteins were much lower in VhCBP than in chitinase A from *V. harveyi* (VhChiA), which was reported to have a strong affinity to both colloidal α - and β -chitins (18), as a positive control. VhChiA was bound to both the colloidal α - and β -chitins by 50–55% of the total protein content, whereas the amounts of bound fractions for VhCBP were less than 1%. VhCBP did not exhibit a

significant ability of insoluble chitin binding. Then we tested soluble sugars from GlcNAc to (GlcNAc)₆ for its binding ability to VhCBP using isothermal titration calorimetry (ITC). The thermograms and theoretical fits for the individual titration experiments are shown in Fig. 3 (A–F). Titration of GlcNAc (Fig. 3A) released only a background heat, indicating no significant interaction. The thermograms obtained for (GlcNAc)₂, (GlcNAc)₃, and (GlcNAc)₄ exhibited strong heat releases (Fig. 3, B–D), and their binding isotherms suggested high affinities to VhCBP. The thermodynamic parameters obtained from data fitting are listed in Table 1. The stoichiometries for (GlcNAc)₂, (GlcNAc)₃, and (GlcNAc)₄ were from 0.7 to 0.8, suggesting a simple 1:1 binding mechanism. The binding affinities were almost identical to each other ($\Delta G^{\circ} = -38$ to -40 kJ/mol). The favorable enthalpy changes (ΔH°) for (GlcNAc)₃ and (GlcNAc)₄ (-41.8 and -44.4 kJ/mol) were much lower than that of (GlcNAc)₂ (-91.6 kJ/mol), and the lower enthalpy changes were compensated by the decrease in unfavorable entropy changes (from 51.8 to 3.0 and 6.3 kJ/mol of $-T\Delta S^{\circ}$), resulting in

Figure 3. ITC thermograms (upper panels) and binding isotherms with theoretical fits (lower panels) obtained for the binding of GlcNAc (A), (GlcNAc)₂ (B), (GlcNAc)₃ (C), (GlcNAc)₄ (D), (GlcNAc)₅ (E), and (GlcNAc)₆ (F) to VhCBP. The buffer used was 20 mM Tris-HCl buffer, pH 8.0, containing 150 mM NaCl. Protein concentrations were 80–90 μM. The other experimental details are described in the text. DP, differential power.

Table 1
Thermodynamic parameter for ligand binding to VhCBP determined by ITC

The equilibrium dissociation constants (K_d) were obtained from the thermograms shown in Fig. 3 (B–F). The values represent means $\pm$ S.D. from three independent sets of the experiments.

Ligands	n (stoichiometry)	K_d	ΔH°	ΔG°	$-T\Delta S^\circ$
		<i>nM</i>	<i>kJ/mol</i>	<i>kJ/mol</i>	<i>kJ/mol</i>
(GlcNAc) ₂	0.7	31 $\pm$ 8.3	−91.6	−39.8	51.8
(GlcNAc) ₃	0.8	48 $\pm$ 10.3	−41.8	−38.8	3.0
(GlcNAc) ₄	0.7	66 $\pm$ 17.9	−44.4	−38.1	6.3

similar affinities of (GlcNAc)_{2–4}. (GlcNAc)₅ and (GlcNAc)₆ also released no significant heat, underlying no binding affinity of VhCBP toward longer-chain chitooligosaccharides. The ITC results suggested that two units of GlcNAc are necessary and sufficient for interaction with VhCBP.

Crystal structure of VhCBP in complex with (GlcNAc)₂

We successfully solved the crystal structure of VhCBP in complex with (GlcNAc)₂ at 1.4 Å resolution but failed to produce X-ray-diffraction quality crystals of the unliganded VhCBP. The crystallographic and refinement statistics are listed in Table 2. Fig. 4A shows the overall structure of VhCBP in complex with (GlcNAc)₂, which adopts a two-domain conformation. The individual domains are designated as the upper

domain (colored in *magenta*) and the lower domain (colored in *cyan*) in this report. (GlcNAc)₂ (colored in *wine red*) was observed in the very narrow cleft between the two domains. In the upper domain (Fig. 1; amino acids 1–241, α 1– α 8, and β 1– β 11; amino acids 488–530, α 21, and β 17), a cluster of several α -helices and loop structures is supported from both sides by two major antiparallel β -sheets (β 1– β 8– β 9– β 10 and β 4– β 5– β 6– β 7) and two minor antiparallel β -sheets (β 2– β 3 and β 11– β 17), forming a hat-like fold, which is decorated by two additional α -helices (α 7 and α 8). The electron density of the C-terminal hexahistidine residues was not observed in this structure. In the lower domain (Fig. 1; amino acids 242–487, α 9– α 20, β 12– β 16), a three-stranded antiparallel β -sheet (β 12– β 15– β 16) and a two-stranded parallel β -sheet (β 13– β 14) are surrounded by 12 α -helices. A metal ion (colored in *orange*) was observed in the central part of the lower domain as shown in Fig. 4A. This was successfully modeled as Ni²⁺, but not as Mg²⁺ or Mn²⁺ as found in the crystal structure of VcCBP (PDB code 1ZU0). The residual Ni²⁺ was likely present during the VhCBP preparation using Ni²⁺-agarose affinity chromatography (see “Experimental Procedures”). Two flexible linkers (colored in *blue*) located between β 11 and β 12 (Tyr-241, Pro-242, and Pro-243) and between β 16 and β 17 (Tyr-488 and Met-489) connect the two domains as a hinge that forms the sugar-binding cleft,

Table 2**Data collection and refinement statistics**

Statistics for the highest-resolution shell are shown in parentheses.

Beamline	I04-1
Wavelength	0.92819
Resolution range (Å)	46.80–1.36 (1.40–1.36)
Space group	P 31 2 1
Unit cell	54.68 54.68 306.45 90.00 90.00 120.00
Total reflections	1105833 (55875)
Unique reflections	115984 (8241)
Multiplicity	9.5 (6.8)
Completeness (%)	99.8 (97.7)
Mean $I/\sigma(I)$	7.6 (1.2)
Wilson B-factor	13.92
R_{merge}	0.132 (1.659)
R_{meas}	0.139 (1.807)
R_{pim}	0.044 (0.697)
$CC_{1/2}$	0.995 (0.067)
Reflections used in refinement	112,276
Reflections used for R_{free}	5458
R_{work}	0.1706
R_{free}	0.2027
Number of non-hydrogen atoms	4466
Macromolecules	4369
Ligands	52
Solvent	472
Protein residues	532
Root-mean-square deviation (RMSD)	
Bonds (Å)	0.010
Angles (°)	1.055
Ramachandran	
Favored (%)	96.98
Allowed (%)	3.02
Outliers (%)	0.00
Rotamer outliers (%)	1.48
Clashscore	1.84
Average B-factor (Å ²)	21.376
Macromolecules	20.477
Ligands	34.056
Solvent	34.532
Number of translation–libration–screw (TLS) groups	10

where (GlcNAc)₂ was bound. However, the bound sugar was invisible in the surface model of the structure (Fig. 5A), indicating that (GlcNAc)₂ was buried inside and completely hidden by the surrounding amino acids. The complex structure of VhCBP was very similar to that of the unpublished VcCBP in complex with (GlcNAc)₂ (PDB code 1ZU0) with an RMSD of 0.46 Å (Fig. 4B). On the other hand, the crystal structure of an unliganded form of VcCBP was also registered in the PDB (code 1ZTY) and considerably differed from that of liganded VhCBP (RMSD = 7.05 Å), as shown in Fig. 4C. The cleft between the upper (colored in yellow) and lower (colored in brown) domains was widely opened in the unliganded form of VcCBP, as shown in the surface model of the structure (Fig. 5B). (GlcNAc)₂ binding to the hinge region of VhCBP appeared to strongly affect the protein conformation and to narrow the cleft between the two domains through a domain motion.

Binding mode of (GlcNAc)₂

Fig. 6A shows a close-up view of the (GlcNAc)₂ binding site of VhCBP. Clear electron density for the two sugar units was found at the interface between the upper and lower domains. (GlcNAc)₂ was sandwiched between two tryptophan side chains by face-to-face stacking interactions; one is from the upper domain (Trp-513), and the other is from the lower domain (Trp-363). In addition to the stacking interactions, a number of hydrogen bonds are formed between VhCBP and the

Figure 4. Crystal structure of VhCBP in complex with (GlcNAc)₂. A, stereo view of the main chain conformation shown by ribbon model. The upper domain is colored in magenta, whereas the lower domain is colored in cyan. Two linkers connecting the two domains (Tyr-241, Pro-242, and Pro-243; Tyr-488 and Met-489) are colored in blue. Secondary structure elements are designated as a1–a21 for α -helices and b1–b17 for β -strands, from the N terminus, corresponding to α 1– α 21 and β 1– β 17 in Fig. 1, respectively. The bound (GlcNAc)₂ is represented by stick model colored in wine red. The N and C termini are indicated by N and C, respectively. A metal ion located in the lower domain was modeled as Ni²⁺ and is represented by an orange sphere. B, superimposition of the α trace of the structure of VhCBP-(GlcNAc)₂ complex with that of the VcCBP-(GlcNAc)₂ complex (PDB code 1ZU0), in which the upper domain is colored in yellow, whereas the lower domain is colored in brown. C, superimposition of the α trace of the structure of VhCBP-(GlcNAc)₂ complex with that of the unliganded VcCBP (PDB code 1ZTY). The color system is the same as in B.

bound sugar (Fig. 6B). Five direct hydrogen bonds are observed with the non-reducing end GlcNAc. The hydroxyl oxygens of C3 and C4 interact with the main chain nitrogen of Phe-222 and side chain nitrogen of Asn-204, respectively. The hydroxyl oxygen of C6 also interacts with the side chain carboxylate of Asp-365. The acetamido nitrogen and oxygen interact with the side chain carboxylate of Glu-10 and the indole nitrogen of Trp-513, respectively. On the other hand, three hydrogen bonds appeared to be directly formed with the reducing end GlcNAc. The acetamido oxygen forms a hydrogen bond with the guanidyl nitrogen of Arg-436, and the C3 hydroxyl oxygen also forms a hydrogen bond with the side chain nitrogen of Asn-409. The C6 hydroxyl oxygen interacts with the side chain oxygen of Glu-10. All interacting amino acids described here are conserved in VcCBP (Fig. 1) except Glu-10. In addition to these direct hydrogen bonds, several water-mediated hydrogen

Figure 5. A, surface model of the structure of *Vh*CBP in complex with (GlcNAc)₂. The bound (GlcNAc)₂ is invisible, because it is completely hidden by surrounding amino acids derived from both upper and lower domains. The color system is the same as in Fig. 4A. The binding cleft between the upper and lower domains is closed upon (GlcNAc)₂ binding, as shown in the side view (right panel). B, surface model of the structure of unliganded *Vc*CBP. The color system is the same as in Fig. 4B. The binding cleft between the upper and lower domains is opened, as shown in the side view (right panel).

bonds are also formed with the bound (GlcNAc)₂, as shown in Fig. 6B.

Discussion

When the amino acid sequence of *Vh*CBP was analyzed by the Pfam protein families database (<http://pfam.xfam.org>),³ the protein was found to belong to the SBP family 5. When the amino acid sequence of *Vh*CBP was analyzed, we found two more proteins possessing an ability to interact with (GlcNAc)_n. The first is periplasmic (GlcNAc)₂-binding protein. This protein is thought to interact with the corresponding ABC transporter from *Vibrio* sp. JCM19052. The amino acid sequence of the protein is 99% homologous to that of *Vh*CBP. The second is the chitooligosaccharide-binding protein from *V. cholera* (*Vc*CBP). The X-ray structure of *Vc*CBP has already been registered in the Protein Data Bank. These proteins may play important roles in the active transport of the solutes derived from chitin and the related compounds. However, most sugar-binding proteins, such as maltose- and galactose-binding proteins, are distributed to SBP families 1 and 2 (InterPro no. IPR000914), (19). Maltose-binding proteins belonging to the SBP family 1 and galactose-binding proteins belonging to the SBP family 2 are smaller in size (396–438 residues and 296–306 residues, respectively) than those of *Vh*CBP and *Vc*CBP belonging to the SBP family 5. The dissociation constants of these binding proteins toward the carbohydrates were reported

to be $\sim 1.0 \mu\text{M}$ (19). Nevertheless, no functional data have been reported for these chitooligosaccharide-binding proteins. We first described herein the structural and functional details of the chitooligosaccharide-binding protein from *V. harveyi* (*Vh*CBP), which is highly homologous to that from *Vibrio cholerae* (*Vc*CBP).

Binding experiments for *Vh*CBP revealed that the protein did not bind insoluble chitin polysaccharide but bind chitooligosaccharides with the polymerization degrees from 2 to 4 (Figs. 2B and 3 and Table 1) with K_d values of 31–66 nM, 15–30 higher affinities than those of maltose- and galactose-binding proteins (19). ITC analysis of (GlcNAc)_n binding were thoroughly conducted for GH18 chitinase B from *Serratia marcescens* and a GH19 chitinase from *Bryum coronatum* (20, 21), both of which have a long-extended binding cleft for (GlcNAc)_n. In both cases, the longer the chain length of (GlcNAc)_n, the higher the favorable free energy changes of binding (ΔG°). The ΔG° values of (GlcNAc)₃ to (GlcNAc)₆ were -20 , -31 , -35 , and -38 kJ/mol for the GH18 enzyme, whereas the values were -21 , -28 , -33 , and -36 kJ/mol for the GH19 enzyme, respectively. For *Vh*CBP, however, the ΔG° values (-38 to -40 kJ/mol) were much higher than those of the chitinases, and were not clearly dependent on the degree of polymerization of (GlcNAc)_n ($n = 2, 3$, and 4). State of the (GlcNAc)_n binding site for *Vh*CBP appears to be different from those for the chitinases. As shown in Figs. 4–6, the (GlcNAc)₂-binding site of *Vh*CBP is unlikely long-extended but forms a small cavity, which can accommodate only a few sugar moieties. This may be the reason why the affinity toward (GlcNAc)₂ is somewhat higher than those toward (GlcNAc)₃ and (GlcNAc)₄. The third and fourth GlcNAc residues may interfere with the binding to *Vh*CBP to some extent.

In the crystal structure of *Vh*CBP in complex with (GlcNAc)₂, five hydrogen bonds are formed with the non-reducing end GlcNAc, whereas three bonds are formed with the reducing end GlcNAc (Fig. 6B). It appeared that the non-reducing end GlcNAc is more strongly recognized by *Vh*CBP. More importantly, stacking CH- π interactions with the individual sugar residues are formed by two tryptophan residues: Trp-363 from the lower domain and Trp-513 from the upper domain, respectively (Fig. 6A). In the unliganded structure of *Vc*CBP, the corresponding tryptophan residues are located apart from each other (17.2 Å). In the liganded structure of *Vc*CBP and also *Vh*CBP, the two tryptophans come close to each other (8.0 Å), and then (GlcNAc)₂ has been sandwiched between the two tryptophan residues, forming tight stacking interactions. This explains why *Vh*CBP has much higher affinities toward chitin oligosaccharides as compared with GH18 and GH19 chitinases. As seen from the sequence alignment shown in Fig. 1, most amino acids interacting with (GlcNAc)₂ are conserved between *Vh*CBP and *Vc*CBP, except Glu-10/Asp-9, which is only a conservative mutation. The binding affinity of *Vc*CBP toward (GlcNAc)₂ is most likely strong as in the case of *Vh*CBP.

Although Ni²⁺ ion was found at the central part of the lower domain, it was far from the (GlcNAc)₂-binding site. It is unlikely that the Ni²⁺ ion is directly involved in the (GlcNAc)₂ binding. In the crystal structure of *Vc*CBP, Mg²⁺ or Mn²⁺ is located in the lower domain; however, the metal-binding site is

Figure 6. A, stereo representation of the close-up view of (GlcNAc)₂ bound to VhCBP. The main chain structure of VhCBP is colored in magenta for the upper domain and in cyan for the lower domain. The bound (GlcNAc)₂ is represented by ball-and-stick model colored in magenta. The $2F_{\text{obs}} - F_{\text{cal}}$ omit map for (GlcNAc)₂ is colored in gray and is contoured at 1σ . The Trp-363 side chain from the lower domain interacts with the non-reducing end GlcNAc, whereas the Trp-513 side chain from the upper domain interacts with the reducing end GlcNAc through CH- π stacking interactions. B, stereo view of the interactions between VhCBP and (GlcNAc)₂. The bound (GlcNAc)₂ is shown as stick model colored in magenta, and the amino acid residues interacting with (GlcNAc)₂ are shown as stick models colored in cyan. Water molecules are represented by white spheres. Hydrogen bonds are represented as dotted lines. NRE and RE represent the non-reducing end GlcNAc and the reducing end GlcNAc, respectively.

markedly different from that of VhCBP. The role of metal ion in the function of CBP is still unclear.

In the binding experiments of VhCBP, we tested only chitin and its oligosaccharides. In the complexed structure, both acetamido groups in bound (GlcNAc)₂ are recognized by VhCBP in addition to the hydroxyl groups of the pyranose rings (Fig. 6B). In the complexed structure, both acetamido groups in bound (GlcNAc)₂ made substantial interactions with polar residues in the sugar-binding pocket. These functional groups are predicted to play a crucial role in sugar specificity of VhCBP. The deacetylated oligosaccharides may interact, but weak interactions with VhCBP are expected because of the lack of the crucial acetamido groups.

Bacterial chitooligosaccharide-binding proteins are part of the chitin degradation pathway. The proteins usually recognize homochitooligomers not branched sugars. Structural inspection of VhCBP suggested a small, narrow sugar-binding pocket, with both ends of the sugar-binding sites not open to accom-

modate long-chain sugars or branched glycans. The feature of the sugar-binding pocket appears to determine the substrate specificity of this protein.

Conformational changes of periplasmic SBPs, such as ribose-binding protein, allose-binding protein, leucine/isoleucine/valine-binding protein, and leucine-binding protein, were intensively studied by X-ray crystallography (22–25). These SBPs adopt a similar fold made of two lobes, which are connected by one or more polypeptide chains, forming a hinge. Most SBP ligands were found to bind to this hinge region between the lobes. The binding of a ligand brings about a dramatic conformational change from opened form to closed form, and the ligand is then clamped between the two lobes. The structural findings for VhCBP including the binding mechanism and the conformational change are consistent with those of periplasmic SBPs reported to date. Thus, these structural aspects appeared to be a general feature among the periplasmic SBPs. However, multiple conformations were found in opened structures of

ribose- and allose-binding proteins (23, 24), suggesting that the conformational changes of these SBPs do not take place through a one-step transition from open to close conformation. In *VhCBP*, however, we failed to obtain the crystal structure of the open form. Further structural studies should be conducted to identify the mechanism of conformational change in CBPs from bacteria. Hollenstein *et al.* (26) reported that the SBP–ligand complex in closed form possesses a protein-binding surface not present in the open form, suggesting that ligand binding followed by the conformational change may be essential for SBP to be recognized by membrane-bound transporter protein. To further characterize our *VhCBP* protein, we are now trying to observe the interaction between *VhCBP* and the transporter protein from *V. harveyi*, in addition to the structural studies.

Experimental procedures

Materials

Solid α -chitin and β -chitin originated from crab and squid pen, respectively, were purchased from Marine BioResources Co. Ltd. (Samut Sakhon, Thailand), and their derivatives, colloidal chitins, were prepared by the method of Roberts and Selitrennikoff (27). Chitin oligosaccharides, (GlcNAc)_{*n*} (*n* = 2–6), were purchased from Seikagaku Biobusiness Co. (Tokyo, Japan). *E. coli* strain Origami (DE3) cells and the expression vector pET23a(+) were from GeneScript Co. (Piscataway, NJ). Ni²⁺-affinity resin, nickel-nitrilotriacetic acid, was also purchased from GeneScript Co. HiTrap Q and 16/60 Superdex 200 were from GE Healthcare (Chicago, IL). All other reagents were of analytic grade.

Expression plasmid

The pET23a(+) plasmid containing a synthetic gene encoding *VhCBP* fused with the His₆ tag at the C terminus was obtained from GeneScript Co. and designated as pET23a(+)-*VhCBP*. The sequence of the *VhCBP*-encoding region was amplified by PCR and confirmed to contain the exactly correct sequence of *VhCBP* from the restriction fragment profile.

Protein expression and purification

The expression plasmid, pET23a(+)-*VhCBP*, was transformed into *E. coli* strain Origami (DE3) cells, which were inoculated into 10 ml of LB broth containing ampicillin/kanamycin and grown overnight at 30 °C, while shaking at 200 rpm. After centrifugation at 4,500 rpm and 4 °C for 20 min, the cells were harvested, resuspended in 10 ml of LB broth containing ampicillin/kanamycin, and then transferred to 1 liter of LB broth containing ampicillin. The culture was incubated at 37 °C, shaking at 200 rpm, until optical density at 600 nm reached 0.7. After cooling down the culture by sitting in ice-cold water for 20 min, isopropyl β -D-1-thiogalactopyranoside was added to a final concentration of 0.5 mM to induce expression. The culture was allowed to incubate at 25 °C for 16 h and then centrifuged at 4,500 rpm for 15 min to harvest the cells. The cells obtained were resuspended in 20 mM sodium phosphate, pH 7.4, containing 50 mM NaCl, 1 mM phenylmethylsulfonyl fluoride, 10% glycerol, 0.1% Triton X-100, and 3 units of DNase and then sonicated for 10 min. The cell debris was removed by centrifuge

at 12,000 rpm and 4 °C for 20 min, and the supernatant was applied onto a nickel-nitrilotriacetic acid column (2 ml of recharged resin) equilibrated with 20 mM sodium phosphate buffer, pH 7.4, containing 50 mM NaCl and 10 mM imidazole. After washing the column with 100 ml of the equilibration buffer, the adsorbed protein was eluted with 30 ml of the same buffer containing 150 mM imidazole. The eluted protein fractions were pooled and dialyzed against 20 mM sodium phosphate, pH 7.4, containing 50 mM NaCl. The resultant protein solution was filtered with a 0.45- μ m membrane filter and applied onto a HiTrap Q column (5 ml) equilibrated with the dialysis buffer. After washing the column with the same buffer, the adsorbed proteins were eluted with a linear gradient elution from 50 mM to 0.5 M NaCl in the same buffer. The protein fractions were pooled and finally purified by a gel-filtration column of 16/60 Superdex 200. After confirming a single protein band on SDS-PAGE (28) (Fig. 2A), the purified *VhCBP* fractions were stored at 4 °C and used for subsequent experiments. Protein concentration was determined by reading absorbance at 280 nm, using the extinction coefficients, 103,375 M^{−1} cm^{−1}, calculated from the equation proposed by Pace *et al.* (29).

Chitin-binding assay

Binding experiments using insoluble chitins (colloidal α -chitin and colloidal β -chitin) were conducted at 4 °C. A reaction mixture (500 μ l) comprising 5 μ g of *VhCBP*, 1.0 mg of colloidal chitin, and 20 mM Tris-HCl buffer, pH 8.0, containing 150 mM NaCl was incubated for 30 min and then centrifuged at 12,000 rpm and 4 °C for 10 min. The supernatant (100 μ l) was mixed with the Coomassie Brilliant Blue G-250 dye solution, and the protein concentration was determined by the method of Bradford (30). The amount of bound protein was calculated by subtracting the free protein content at the equilibrium from the initial protein content and then converted to the bound protein fraction (%). *VhChiA*, a chitinase A from *V. harveyi*, was used as a positive control of these experiments, because the enzyme protein was reported to have a high affinity toward the insoluble chitin (18).

ITC experiments

The *VhCBP* solution (80–90 μ M) in 20 mM potassium phosphate buffer (pH 8.0) was degassed, and its concentration was determined. Individual (GlcNAc)_{*n*} (where *n* = 1, 2, 3, 4, 5, and 6) (0.1 mM) were dissolved in the same buffer, and the solution pH was adjusted to 8.0. Then the (GlcNAc)_{*n*} solution was degassed and loaded into a syringe, whereas the protein solution (0.2028 ml) was loaded into the sample cell after confirming the solution pH 8.0. Calorimetric titration was performed with an iTC200 system (Microcal, Northampton, MA) at 4 °C. Aliquots (1.0–2.0 μ l) of the ligand solution were added to the sample cell with a stirring speed of 1000 rpm. Titrations were completed after 40 injections. For analysis of the ITC data, the Origin software installed in an ITC instrument was used. The one-set-of-sites model was employed to fit the experimental data. The other details of the data analysis were previously described (21).

Crystallization and data collection

Crystal trials were set up by the sitting-drop, vapor-diffusion method with Morpheus and structure screen kits (Molecular Dimensions Limited, Suffolk, UK) using a Mosquito robot (TTB Labtech). Crystals plated were incubated at 20 °C, and small crystals grown were observed under several conditions within 3 days of incubations. Further optimization was carried out manually using the hanging-drop, vapor-diffusion method. 1 μ l aliquot of the protein solution (10–15 mg/ml in 10 mM HEPES, pH 7.5, 150 mM NaCl) was mixed with 1.5 μ l of a reservoir solution. Bipyramidal crystals were observed with Morpheus conditions B4 (0.09 M Halogens (NaF, NaBr, NaI), 0.1 M buffer 2 (HEPES/MOPS), pH 7.5, and 37.5% MPD_P1K_P3350 mix). The crystals were collected, briefly transferred to a solution of mother liquor containing 20% PEG400, and then flash-frozen in liquid nitrogen. One of the crystals diffracted at the highest resolution of 1.36 Å, and the structure was solved in PHENIX (31) using 1ZU0 as a molecular replacement model. For data collection under cryogenic conditions, the crystals were briefly transferred to the mother liquor and were then flash-cooled by a nitrogen stream at 95 K. The data set of the crystals was collected at 95 K at the Beamline I04-1 (Diamond Light Source, Didcot, UK). The resulting data set was processed with Dials and scaled with Aimless (32) in PHENIX. The crystals belong to the triclinic space group $P3_12_1$, with unit cell dimensions of $a = 54.68$ Å, $b = 54.68$ Å, $c = 306.45$ Å, $\alpha = 90.0^\circ$, $\beta = 90.0^\circ$, and $\gamma = 120.0^\circ$. The processing statistics are summarized in Table 2.

Structure determination and refinement

Initial phasing and modeling was done using AUTOSOL within PHENIX (31). Further model building was performed using the program COOT (33). The phase for VhCBP was obtained by molecular replacement using MOLREP (34) with the structure of VcCBP in complex with (GlcNAc)₂ (PDB code 1ZU0) as the search model. The analyses of the electron density map $F_{\text{obs}} - F_{\text{cal}}$ and $2F_{\text{obs}} - F_{\text{cal}}$ and model building were carried out in COOT and restrained refinement in REFMAC5 (35). The geometry of the final model was validated by MolProbity (36). The final $2F_{\text{obs}} - F_{\text{cal}}$ omit map, contoured at 3.0 σ , clearly showed the electron density map for (GlcNAc)₂ with full occupancy. The structures and electron density maps of all the refined structures were created and displayed by PyMOL (37). The refinement statistics are summarized in Table 2.

Author contributions—W. S. and T. F. conceptualization; W. S. resources; W. S., A. R., D. B., and B. v. d. B. data curation; W. S. and B. v. d. B. formal analysis; W. S., B. v. d. B., and T. F. supervision; W. S. funding acquisition; W. S., A. R., D. B., B. v. d. B., and T. F. validation; W. S., N. S., A. R., D. B., and Y. K. investigation; W. S., N. S., A. R., and T. F. visualization; W. S., N. S., A. R., D. B., Y. K., and B. v. d. B. methodology; W. S. and T. F. project administration; W. S. and B. v. d. B. writing-review and editing; T. F. writing-original draft.

Acknowledgment—We gratefully acknowledge the Centre for Scientific and Technological Equipment, Suranaree University of Technology for providing the facilities for carrying out this research.

Note added in proof—Fig. 3, A and F, was inadvertently duplicated in the version of this article that was published as a Paper in Press on February 14, 2018. This error has now been corrected and does not affect the results or conclusions of this work.

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