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Reaction Mechanism and Substrate Recognition of GH-94 Phosphorolytic Enzymes

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Abstract: *Vibrio proteolyticus* chitobiose (GlcNAc-β1,4-GlcNAc) phosphorylase (ChBP) catalyzes the reversible phosphorolysis of chitobiose into α-GlcNAc-1-phosphate and GlcNAc with inversion of the anomeric configuration. ChBP and its homologues, cellobiose phosphorylase (CBP) and cellodextrin phosphorylase (CDP), were classified under the glycosyltransferase (GT) class, GT-36, on the finding that they have no hydrolytic activity. As the first known structures of a GT-36 enzyme, we determined the crystal structures of ChBP including the ternary complex with GlcNAc and SO_4. They are also the first structures of an inverting phosphorolytic enzyme in a complex with a sugar and a sulfate ion, and reveal a pseudo-ternary complex structure of enzyme-sugar-phosphate. ChBP comprises a β-sandwich domain and an $(\alpha/\alpha)_6$ barrel domain, constituting a distinctive structure among GT families. Instead, it shows significant structural similarity with glycoside hydrolase (GH) enzymes, glucoamylases (GH-15), and maltose phosphorylase (GH-65). The proposed reaction mechanism of ChBP also shows similarity with those for inverting hydrolytic enzymes with the exception of the molecules attacking the C1 atom. The similarities of overall structures and catalytic mechanisms between ChBP and GH enzymes led to the reclassification of family GT-36 into a novel GH family, namely GH-94. The substrate complex structures of ChBP also provide many structural insights into its oligosaccharide synthesis reaction such as substrate specificity.

Key words: crystallography, GH-94, phosphorolytic enzyme, reaction mechanism, structure and function relationship

Enzymes involved in the formation or cleavage of glycosyl linkages are mainly categorized into the Glycoside Hydrolase (GH) or GlycosylTransferase (GT) class (CAZy website at http://afmb.cnrs-mrs.fr/CAZY/),[1)] and each class comprises families classified on the basis of amino acid sequence similarity. GH enzymes (E.C. 3.2.1.-) are frequently employed in the hydrolysis of polysaccharides and they are very important in industries. Their reactions are substantially irreversible due to the existence of an abundance of water molecules in the reaction mixture. GT enzymes (E.C. 2.4.1.-) catalyze the transfer of sugar moieties from sugar donors (sugar-nucleotides) to specific acceptor molecules.[2)] Although 74 GT families have been described,[2,3)] their 3-D structures show only two topologies, GT-A and GT-B. Most GT enzymes are membrane-associated proteins, and present many problems for practical use for oligosaccharide synthesis. Phosphorolytic enzymes (E.C. 2.4.1.-: usually named using a combination of "the name of the substrate" and "phosphorylase") catalyze the phosphorolysis of glycosidic bonds to generate glycosyl-phosphates. Since the energy of the glycosyl-phosphate bond is not as high as that of a glycosyl-nucleotide, their reactions are reversible. Therefore, phosphorolytic enzymes can be employed for both the synthesis and degradation of sugar chains, exploiting their reversible reactions. Classification of phosphorolytic enzymes is complicated. E.C. numbers of 2.4.1.- are given for phosphorolytic enzymes because phosphorolysis is a sort of transferase reaction. Actually, some of them have GT-like 3D-structure and catalytic mechanisms which use sugar-nucleotides as sugar donors. For example, glycogen phosphorylase (GT-35) has a GT-B fold, and also exhibits a strong resemblance in its active site to a typical GT-enzyme, glycogen synthase (GT-5).[4)] However, some phosphorolytic enzymes are placed in GH families due to the similarity of their amino acid sequences with those of hydrolytic enzymes, and they have folds similar to those of GH enzymes. For example sucrose phosphorylase (GH-13) has an α-amylase-like TIM barrel fold,[5)] and maltose phosphorylase (GH-65) has a glucoamylase (GH-15)-like $(\alpha/\alpha)_6$ barrel fold (Table 1).[6)]

Chitobiose phosphorylase from *Vibrio proteolyticus* catalyzes the reversible phosphorolysis of GlcNAc-β1,4-GlcNAc (chitobiose) into α-GlcNAc-1-P and GlcNAc.[7)] ChBP was classified in family GT-36 along with cellobiose phosphorylase (CBP), cellodextrin phosphorylase (CDP) at first, because no hydrolytic activity was found in the studied members of this family. The substrate specificities of ChBP, CBP and CDP with regards to the degree of polymerization are quite different; ChBP and CBP show activity only toward disaccharides, whereas CDP phosphorolyses cellotriose or higher cellooligosaccharides.[8)] Although GT-36 was classified into a GT class,

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Table 1. Phosphorolytic enzymes in CAZy database.

Class	GH-type			GT-type	
Family	GH-13	GH-65	GH-94 (former GT-36)	GT-4	GT-35
Enzyme	Sucrose phosphorylase	Maltose phosphorylase	ChBP, CBP, CDP	Trehalose phosphorylase	Glycogen phosphorylase
Hydrolytic activity	Yes	No (homologous to trehalase)	No	No	No
Fold	(α-amylase-like) $(\alpha/\beta)_8$ barrel	(Glucoamylase-like) $(\alpha/\alpha)_6$ barrel	(Glucoamylase-like) $(\alpha/\alpha)_6$ barrel	—	(glycogen synthase-like) GT-B
Cofactor requirement	—	—	—	—	Pyridoxal phosphate
Mechanism	Retaining	Inverting	Inverting	Retaining	Retaining

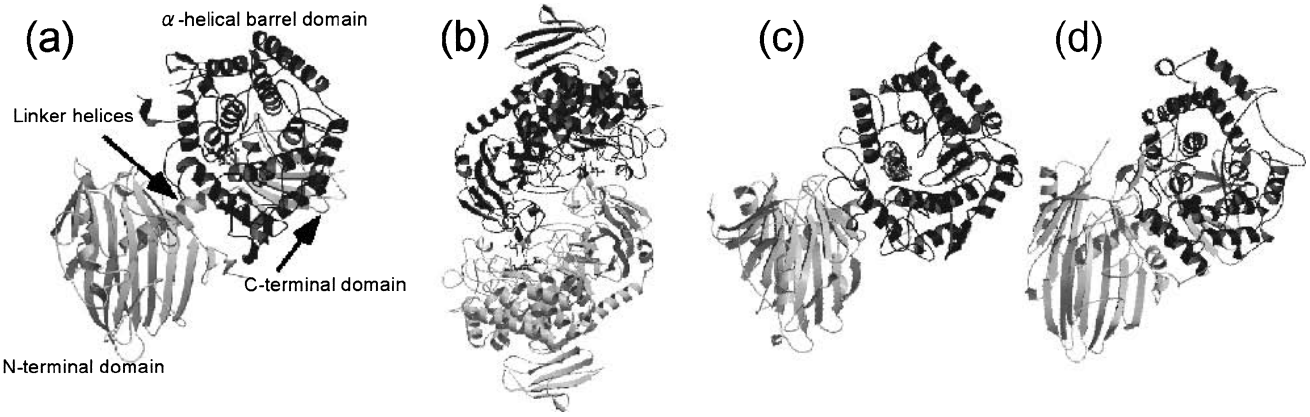

Fig. 1. Ribbon diagrams of ChBP and related structures.

(a) Overall structure of the ChBP monomer (GlcNAc complex) shown as a ribbon model. The catalytic α-helical barrel domain is colored in black, and the N-terminal domain, linker helices, and the C-terminal domain are in gray. The bound GlcNAc molecules are shown as a ball-and-stick model. (b) Ribbon diagram presentation of the ChBP dimer. The subunits are located around the crystallographic 2-fold axis. One subunit is shown in dark-grey whereas the other one is shown in light-grey. (c) A ribbon diagram of bGA (1LF9; GH-15). Bacterial and archaeal glucoamylases comprise an N-terminal β-sandwich domain, linker helices, and an α-helical barrel domain, whereas fungal glucoamylases comprise only an α-helical barrel domain. The bound acarbose molecule is shown as a ball-and-stick model. (d) A ribbon diagram of MalP (1H54; GH-65). The domain constitution is identical with that of ChBP.

it does not require a nucleotide moiety such as pyridoxal phosphate or a sugar nucleotide for the reaction like typical GT enzymes. Besides, on amino acid sequential analysis, no evidence has been obtained that GT-36 has either a GT-A or GT-B fold.[2,9] Recently, we have revealed that ChBP has a fold and catalytic mechanism similar to those of the GH enzyme, leading to reclassification of GT-36 under GH-94.[10]

Crystallography.

The non-labeled ChBP protein was expressed in *Escherichia coli* strain BL21 gold and purified using Ni-NTA Superflow (QIAGEN). The selenomethionine-labeled enzyme was expressed in methionine auxotroph *E. coli* strain B834 (DE3) (Novagen) in medium containing selenomethionine. The purification procedures for the selenomethionine-labeled enzyme were the same as those for the non-labeled enzyme. ChBP crystals were obtained at 4°C using the hanging-drop vapor-diffusion method by mixing 5 μL of a protein solution with 5 μL of a reservoir solution comprising 0.1 M HEPES Na-OH (pH 8.0), 0.17 M $CaCl_2$ and 30−40% (v/v) PEG400. Crystals of the selenomethionine-labeled ChBP were crystallized in the presence of 1 mM DTT. Crystals of the GlcNAc complex were obtained by co-crystallization using a reservoir solution containing 25 mM GlcNAc. Crystals of the GlcNAc-SO_4 complex were obtained by co-crystallization using a reservoir solution containing 10 mM GlcNAc and 10 mM ammonium sulfate.

The crystal structure of ChBP was determined by multiple wavelength anomalous dispersion (MAD) of selenomethionine labeled ChBP and refined in the native (substrate-free) form, a GlcNAc complex, and a ternary complex with GlcNAc and SO_4 at 1.8, 1.6 and 2.0 Å resolution to R factor (R_{free}) of 16.2 (18.6) 15.7 (17.9) and 16.3% (19.5%), respectively. The coordinates for the native, GlcNAc complex, and GlcNAc-SO_4 structures have been deposited in the Protein Data Bank under accession codes 1V7V, 1V7W and 1V7X, respectively. Figure 1(a) shows a ribbon diagram of the overall monomer structure of the GlcNAc complex of ChBP. Each crystal belongs to the same space group $C2$, and contains one monomer molecule per asymmetric unit. A dimer is formed from one monomer each from adjacent asymmetric units located around the crystallographic 2-fold axis (Fig. 1(b)).

Overall structure.

The structure of ChBP comprises a complex architecture consisting of four distinct regions: an N-terminal β-

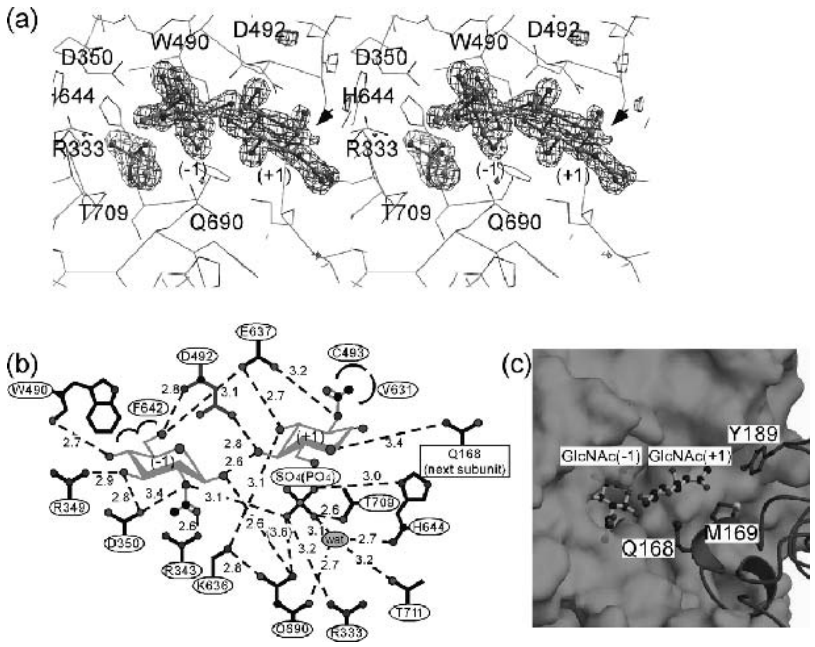

Fig. 2. Active site of ChBP.

(a) Stereoview of a wireframe model of ChBP, and the $|F_{obs}|-|F_{calc}|$ electron density of the bound GlcNAc molecules, chloride and sulfate. GlcNAc(−1) and GlcNAc(+1) are shown as a ball-and-stick model and labeled (−1) and (+1), respectively. The chloride ion is shown as a grey sphere. The sulfate ion is shown as a ball-and-stick model. A $|F_{obs}|-|F_{calc}|$ omit map of GlcNAc molecules and chloride in the GlcNAc complex, and that of sulfate in the GlcNAc-SO_4 complex structure are shown with contouring at 3.0 σ. The residues involved in GlcNAc recognition (D350 and W490), sulfate recognition (R333, H644 and T709) and catalysis (D492) are labeled. GlcNAc(−1) takes on the β-anomer configuration whereas GlcNAc(+1) takes on a mixture of α- and β-anomer configurations (indicated by an arrow). (b) Schematic drawing of the atoms and interactions involved in the recognition of GlcNAc molecules and sulfate in the GlcNAc-SO_4 complex structure. Broken lines and semicircles indicate hydrogen bonds and hydrophobic interactions, respectively. Q168 of the next subunit also contributes to the active site pocket formation and substrate recognition. (c) Active site pocket formation through the dimeric interaction. The molecular surface of one subunit of ChBP and a ribbon diagram of the next subunit are shown. Bound GlcNAc molecules and the residues in the active site pocket formation are shown as a ball-and-stick model.

sandwich domain (residues 1–270; N-terminal domain), a helical linker region (271–302), an $(\alpha/\alpha)_6$ barrel fold domain (310–724; α-helical barrel domain), and a C-terminal β-sheet domain (303–309, 725–801; C-terminal domain). Folds similar to the N-terminal domain are found in β-galactosidase (GH-2)[11,12] and 4-α-glucanotransferase (GH-57),[13] but their functions are unknown. The N-terminal domain is connected to the α-helical barrel domain by a linker region including two helices (comprising residues 272–279 and 283–301), which forms a 90° elbow through a short turn. The linker helices are followed by a β-strand comprising residues 303–309, which forms a β-sheet with 5 β-strands in the C-terminal domain. The α-helical barrel domain predominantly consists of α helices, 12 of which are arranged in two concentric layers that form an $(\alpha/\alpha)_6$ barrel. Bound GlcNAc molecules in the complex structure indicated that the α-helical barrel domain is the catalytic domain of ChBP (Fig. 2(a)). The C-terminal domain forms a two-layered jelly roll fold consisting of seven strands comprising residues 725–801 and a strand comprising residues 306–309. Surprisingly, the domain constitution of ChBP is almost identical to that of the bacterial glucoamylase from *Thermoanaerobacterium thermosaccharolyticum* (bGA; GH-15; Fig. 1(c))[14] and maltose phosphorylase from *Lactobacillus brevis* (MalP; GH-65; Fig. 1(d)).[6] These enzymes can be structurally aligned, although their amino acid sequences are too divergent to produce an alignment. The high level of Dali[15] scores (against bGA, Z-score=24.2, rmsd=4.0 Å for 545 residues; against MalP, Z-score=16.2, rmsd=4.3 Å for 566 residues) indicated that the reaction mechanism of ChBP could be evolutionarily related to those of bGA and MalP.

Reaction mechanism of ChBP.

The reaction mechanism of ChBP can be elucidated by comparing its active site structure with the GH-15 bGA and GH-65 MalP structures. The α-helical barrel domain of the GlcNAc complex (residues 310–724) was superimposed on those of bGA (295–684) and MalP (316–683) (Fig. 3(a)). D492 of ChBP, which is involved in the highly conserved region of all GH-94 (former GT-36) enzymes,[8] superposed well as to both the catalytic residues of bGA (E438, general acid) and MalP (E487, putative general acid), and these residues are also located at the topologically identical loops. D492 of ChBP is also assumed to be a general acid, as judged from the fact that the D492A/N mutants of ChBP exhibited no detectable activity. On the other side of the active site, at the position of the general base of an inverting hydrolase (E636 of bGA), no candidate for general base residue is present in inverting phosphorolytic enzymes (MalP and ChBP). In the case of the MalP enzyme, the phosphate ion at this position is thought to act as the nucleophile instead of a hydroxyl ion activated by the general base residue.[6] A similar reaction mechanism can be assumed for another inverting phosphorolytic enzyme, ChBP, as described below. In the ChBP structure, the Q690 residue, which is

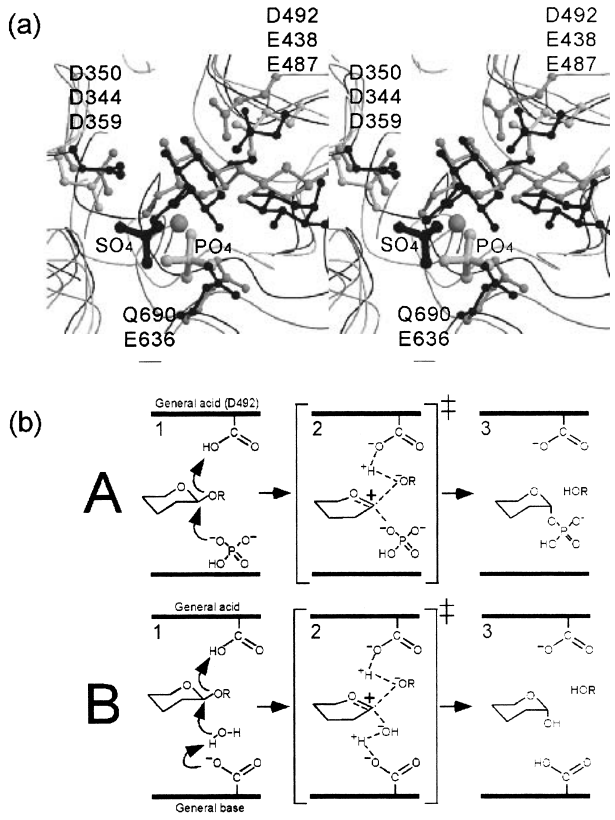

Fig. 3. Reaction mechanism of ChBP.

(a) Stereoview of ChBP, bGA and MalP superimposed at the active site. Backbone traces of ChBP, bGA and MalP are colored black, gray and white, respectively. Bound GlcNAc molecules in ChBP (black) and acarbose in bGA (gray) are shown as a wire-frame model. The structurally conserved residues are shown as a ball-and-stick model, and labeled in the order corresponding to ChBP, bGA and MalP. Sulfate in ChBP and phosphate in MalP are also shown as a ball-and-stick model, and the water molecule in bGA, which attacks the glycosidic bond, is shown as a sphere. The structures were superimposed using the rotation and translation vectors generated with the Dali server. (b) Schematic reaction mechanisms of ChBP and hydrolytic enzymes. (A) ChBP; (B) inverting GHs.

fully conserved in GH-94 enzymes, is located at almost the same position of E636 in bGA (rmsd=1.0 Å), and sulfate is bound near this residue in the GlcNAc-SO4 complex structure, whereas a chloride ion is bound in the GlcNAc complex structure (Fig. 2(a)). The sulfate ion is located on the α-face, in a similar position to the phosphate ion of MalP (rmsd = 2.7 Å). Therefore, we presume that the area around the sulfate ion is also the binding site for the phosphate ion. In other words, the GlcNAc-SO_4 complex structure can be regarded as a pseudo ternary complex of ChBP, a sugar, and phosphate. The distance between the carboxyl group of D492 and the carbamoyl group of Q690 is 8.8 Å, and the topology of D492-GlcNAc-SO_4-Q690 in ChBP is very similar to that of E438-acarbose-water-E636 in bGA. Therefore, we conclude that the enzymatic phosphorolysis of ChBP begins with the direct nucleophilic attack by phosphate on the glycosidic bond with the aid of D492, which donates a proton to the glycosidic oxygen atom, and then proceeds through an oxocarbenium cation-like transition state as shown in Fig. 3(b)A. The proposed reaction mechanism for ChBP, an inverting phosphorolytic enzyme, is similar to that for inverting GHs illustrated in Fig. 3(b)B.[16] The only difference between the mechanisms is as follows: The water molecule that attacks C1 of the glycoside is activated with the aid of the general base residue in the inverting GH reaction (Fig. 3(b)B2), whereas such activation of the phosphate ion is not necessary for the reaction of ChBP.

Interestingly, the ChBP structure is the first non-GT-A/B structure within the families currently classified as GT. However, ChBP shows notable structural similarity to the hydrolytic enzymes, both in the overall fold and catalytic center. In addition, PSI-BLAST analyses indicate a low but significant sequence similarity between GH-94 (former GT-36) and GH-15 enzymes (B. Henrissat, personal communication). Accordingly, family GT-36 was reclassified as a novel GH family (GH-94) in the CAZy database.

Structural insights into substrate specificity for ChBP.

1. Preference to the glycosidic bond.

Three inverting enzymes, ChBP, MalP and bGA, can be superimposed well as to their catalytic centers and flanking subsites (−1) and (+1). However, their substrate specificities are clearly different; ChBP and all GH-94 enzymes act on β-1,4 glycosidic bonds, whereas the latter two enzymes, as well as all GH-65 and GH-15 enzymes, act on α-glycosidic bonds. When the GlcNAc complex of ChBP and the acarbose complex of bGA were compared, GlcNAc(+1) of ChBP and the 6-deoxyglucose moiety at subsite (+1) of bGA were found to be bound in similar directions (Fig. 3). On the other hand, the sugar rings of GlcNAc(−1) of ChBP and the valienamine moiety at subsite (−1) of bGA are almost completely flipped. C1, C2, C3, C4, C5 and O5 atoms of GlcNac(−1) overlap with C6, C5, C4, C3, C2 and C1 of the valienamine moiety at subsite (−1) of bGA, respectively. The N2 and O3 atoms of GlcNAc(−1) are hydrogen-bonded with D350 of ChBP, whereas the O4 and O6 atoms of the valienamine moiety are recognized by the corresponding residue of bGA, D344. Therefore, ChBP and bGA (and probably also MalP) recognize the flipped sugar moiety at subsite (−1), so they can act on the opposite anomer despite the fact that their substrates are attacked by a phosphate/hydroxyl ion from the same side.

2. Specificity for sugar components.

Figure 2b is a schematic drawing of the surrounding residues interacting with the sulfate ion and GlcNAc molecules. The substrate specificity as to the OH direction of GlcNAc(−1) is strict, because O3, O4 and O6 form tight hydrogen bonds with one or more residues. The OH groups of GlcNAc(+1) are also recognized by a number of residues. On the other hand, the recognition of the N-acetyl group of GlcNAc(−1) lacks the hydrophobic interaction with the methyl group, although a hydrogen bond is formed between R343 and the carbonyl group of GlcNAc(−1). This loose interaction compared with that for the N-acetyl group of GlcNAc(+1) seems to lead to the compatibility between Glc-1-P and GlcNAc-1-P as the sugar donor.[8] On the other hand, the methyl group of the N-acetyl group of GlcNAc(+1) forms hydrophobic inter-

actions with V631 and C493, corresponding to the strict specificity as to the N-acetyl group in GlcNAc(+1). In particular, V631 seems to be a crucial residue to express the specificity of ChBP, because other GT-36 enzymes such as CBP and CDP have a tyrosine instead of the valine.

The electron density map around GlcNAc(+1) reflects a mixture of the α- and β-GlcNAc molecules. Occupancy refinement of the O1 atom revealed that GlcNAc(+1) consists of 65% α- and 35% β-GlcNAc. Considering the abundance ratio of GlcNAc in solution (α: β=6:4),[17] subsite (+1) shows less selectivity for the anomeric configuration. In fact, this ambiguity in anomeric selectivity at the acceptor site has been confirmed by determining the anomeric ratio of GlcNAc formed during the phosphorolysis of equilibrated chitobiose.[17]

3. Specificity for the degree of polymerization.

The dimer structure of ChBP with crystallographic 2-fold symmetry (Fig. 1(b)) seems to correspond to the dimeric form of this enzyme in solution.[8] The two subunits are tightly connected through hydrophobic interactions and a number of hydrogen bonds. On dimer formation, 3200 $\AA^2$ of the solvent-accessible surface area, which comprises 14% of the total monomer surface, is buried. The major contact area at the dimer interface comprises the α-helical barrel and N-terminal domains. The active site is also located at this dimer interface. The active site cleft of the α-helical barrel domain, which alone appears to be appropriate for longer oligosaccharides, is covered by the two helices (residues 160–170) in the N-terminal domain of the next subunit, forming pocket type subsites, which is appropriate for chitobiose (Fig. 2(c)). The substrate specificity of GH-94 enzymes with regard to the degree of polymerization seems to be determined by the subunit interaction formed by the N-terminal domain of the next subunit. Actually, there is relatively low sequence identity in the N-terminal domain between ChBP and CDP (17%), with many insertions and deletions, compared with that between ChBP and CBP (35%). On the other hand, the N-terminal domains of the MalP and bGA enzymes do not contribute to their subunit interactions.[6,14]

Other GH-94 enzymes.

In the GH-94 enzymes, CBP from *Cellvibrio gilvus* is the most studied enzyme in terms of substrate specificity, reaction mechanism, and application for oligosaccharide synthesis.[9] We have also crystallized the CBP from *Cellvibrio gilvus*, and crystallographic refinement is currently in progress.[18]

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GH-94 加リン酸分解酵素の反応機構と基質認識

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Vibrio proteolyticus 由来キトビオースホスホリラーゼ（ChBP）はキトビオース（GlcNAc）$_2$ のβ-1,4 グリコシド結合を加リン酸分解し，α-GlcNAc-1-リン酸と GlcNAc を生成する反転型加リン酸分解酵素である．ChBP はセロビオースホスホリラーゼ（CBP），セロデキストリンホスホリラーゼとアミノ酸配列の相同性を有し，ともに加水分解活性を全く持たないため，グリコシルトランスフェラーゼ GT-36 に分類されていた．加リン酸分解酵素は逆反応である糖リン酸エステルを供与体とした糖転移反応も触媒し，この反応を用いて加リン酸分解酵素を実用的なオリゴ糖合成酵素として利用することも可能である．本研究は加リン酸分解酵素の応用研究のための触媒反応，基質認識機構の構造基盤の獲得を目的とした．GT-36 として初めて ChBP の立体構造を明らかにしたところ，その構造はβ-サンドイッチドメイン，リンカーヘリックス，$(\alpha/\alpha)_6$ バレルドメイン（触媒ドメイン），β-シートドメインから成り，既知のグリコシルトランスフェラーゼとは全く異なるドメイン構造を有する一方，加水分解酵素 GH-15･グルコアミラーゼ，GH-65･マルトースホスホリラーゼと高い構造の相同性を有していた．また，GlcNAc（基質），硫酸イオン（リン酸アナログ）の三者複合体構造から，反転型加リン酸分解酵素の反応機構は，グリコシド結合を求核攻撃する分子がリン酸である点を除けば，反転型加水分解酵素の反応機構と同じであることを明らかにした．ChBP と GH-15，GH-65 との立体構造，活性中心部位の構造，反応機構の類似性から，GT-36 は GH-94 に再分類されることになった．これは，GT に分類されていた酵素が立体構造の解明により GH に再分類された最初の例である．

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〔質問〕 横浜市大　梶原

酵素触媒部位の輸送経路はどうなっているのでしょうか？

〔答〕

立体構造をみますと，キトビオースホスホリラーゼの活性中心部位は分子表面に露出しておりますので，特に構造変化を必要とすることなく基質が結合できると思います．本酵素は酵素反応の解析結果から sequential bi bi 機構を取ることが明らかになっておりますが，基質結合の順番については明らかになっておりません．立体構造をみますと，サブサイト−1 は活性中心ポケットの奥，サブサイト＋1 はポケットの入り口にあり，＋1 に糖が結合すると−1 への基質の出入りはできなくなると思われます．そのため，合成方向の反応では，まずサブサイト−1 に GlcNAc-1-リン酸が結合して，次に糖受容体・GlcNAc がサブサイト＋1 に結合する順番になっていると思われます．一方，リン酸結合部位はサブサイトに糖が結合してもあまり影響がありません．そのため立体構造からは，分解方向の反応におけるキトビオースとリン酸の結合順序についての知見は得られませんでした．

〔質問〕 江崎グリコ・生化研　藤井

キトビオースホスホリラーゼはセロビオースホスホリラーゼと同様にフィードバック制御が起きるのでしょうか？　また，セロデキストリンホスホリラーゼもダイマー構造を取るのでしょうか？

〔答〕

キトビオースホスホリラーゼによるキトビオースの加リン酸分解反応では，生成物である GlcNAc による生成物阻害がみられます．また，合成方向の反応でも GlcNAc による阻害がみられます．このような糖受容体による阻害は，他の GH-94 の加リン酸分解酵素にもみられる現象であり，糖受容体が糖供与体の結合サイト（サブサイト−1）に競合するために起こると考えられております．また，現在諸性質が解析されている *Clostridium stercorarium*，*Clostridium thermocellum* 由来のセロデキストリンホスホリラーゼは二量体構造をとると報告されております．