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Identification of amino acid residues that determine the substrate preference of 1,3-β-galactosyl-*N*-acetylhexosamine phosphorylase

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ABSTRACT

Three amino acid residues of 1,3-β-galactosyl-*N*-acetylhexosamine phosphorylase (GalHexNAcP) were assigned as the determinants of substrate preference for galacto-*N*-biose (GNB) and lacto-*N*-biose I (LNB) based on the three-dimensional structure of the protein. Mutants of GalHexNAcP from *Bifidobacterium longum*, which acts similarly on both GNB and LNB, were constructed and characterized. V162T mutation led to an increase in the selectivity on GNB. P161S and S336A mutations independently enhanced the selectivity on LNB. The alignment of amino acid sequences suggests that the activities of most homologous sequences are predictable by comparing the corresponding three residues.

1. Introduction

Sugar phosphorylases are useful in the preparation of particular oligosaccharides because of their reversible reactions with strict regio-specificities [1,2]. 1,3-β-Galactosyl-*N*-acetylhexosamine phosphorylase [EC 2.4.1.211] (GalHexNAcP) reversibly phosphorolyzes galacto-*N*-biose (Galβ1, 3GalNAc, GNB) and/or lacto-*N*-biose I (Galβ1, 3GlcNAc, LNB) to form α-D-galactose 1-phosphate (Gal1*P*) and the corresponding *N*-acetylhexosamine (GalNAc and GlcNAc, respectively) [3]. GalHexNAcPs can be subcategorized into GNB/LNB phosphorylases (GLNBPs: acting similarly on both GNB and LNB) [4,5], GNB phosphorylases (GNBPs: preferring GNB) [6,7], and LNB phosphorylases (LNBPs: preferring LNB) based on their substrate preferences as evaluated by the ratio of k_{cat}/K_m values for GNB and LNB (GNB/LNB ratio) [8]. GNB/LNB ratios of GLNBPs are typically between 0.3 and 1, whereas those of GNBP and LNBP are typically >50 and <0.02, respectively.

GalHexNAcP was initially found as GLNBP, a cell-free extract of *Bifidobacterium bifidum* [3], and the first cloning of the gene encoding GalHexNAcP was reported on GLNBP from *Bifidobacterium longum* subsp. *longum* JCM1217 ($GLNBP_{BL}$) [4]. All the characterized GalHexNAcPs are classified in glycoside hydrolase family 112 (GH112) based on their amino acid sequences in the CAZy database [9], along with another inverting phosphorylase on β-galactosides, 1,4-β-galactosyl-L-rhamnose phosphorylase [EC 2.4.1.247] (GalRhaP) [10,11]. To date, 14 enzymes belonging to GH112 have been characterized [3–8,10–13] (Table 1). These enzymes are useful for producing specific β-galactosides because of their strict substrate specificities [13–17].

Many sequences belonging to GH112 are being registered from the genomic sequence data of various bacteria. Most of the bacteria possessing a gene encoding GalHexNAcP are commensal or pathogenic for humans and animals, probably related to the metabolism of GNB and LNB existing in the sugar chains of tissues [4]. Therefore, it is valuable to predict their enzymatic activity on the basis of the amino acid sequences in order to understand the target of such pathogenic bacteria. However, it is difficult to

Abbreviations: GalHexNAcP, 1,3-β-galactosyl-N-acetylhexosamine phosphorylase; GalRhaP, 1,4-β-galactosyl-L-rhamnose phosphorylase; GH112, glycoside hydrolase family 112; GLNBP, galacto-*N*-biose/lacto-*N*-biose I phosphorylase; $GLNBP_{BL}$, GLNBP from *Bifidobacterium longum* JCM1217; GNB, galacto-*N*-biose; GNBP, galacto-*N*-biose phosphorylase; LNB, lacto-*N*-biose I; LNBP, lacto-*N*-biose I phosphorylase.

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Table 1
List of characterized GH112 enzymes.

	Name	Bacterial species	Strain	Accession no.	Activity	Reference
a	$GLNBP_{BL}$	*Bifidobacterium longum* subsp. *longum*	JCM1217	BAD80751.1	GLNBP	[4]
b	LnpA1	*Bifidobacterium bifidum*	JCM1254	BAD80752.1	GLNBP	[3,5]
c	LnpA2	*Bifidobacterium bifidum*	JCM1254	BAE95373.1	GLNBP	[5]
d	Blon_2174	*Bifidobacterium longum* subsp. *infanits*	ATCC15697	ACJ53235.1	GLNBP	[13]
e	Cphy_3030	*Clostridium phytofermentans*	ATCC700394	ABX43387.1	GLNBP	[10]
f	Cphy_0577	*Clostridium phytofermentans*	ATCC700394	ABX40964.1	GLNBP	[10]
g	Apre_1669	*Anaerococcus prevotii*	DSM20548	ACV29689.1	GLNBP	[12]
h		*Erysipelothrix rhusiopathiae*	ATCC19414	ZP_05748149	GNBP	[12]
i	CPF_0553	*Clostridium perfringens*	ATCC13124	ABG83511.1	GNBP	[6]
j	GnpA	*Propionibacterium acnes*	JCM6425	AB468066	GNBP	[7]
k	Smon_0146	*Streptobacillus moniliformis*	DSM12112	ACZ00636.1	GNBP	[12]
l	VV2_1091	*Vibrio vulnificus*	CMCP6	AAO07997.1	LNBP	[8]
m	Cphy_1920	*Clostridium phytofermentans*	ATCC700394	ABX42289.1	GalRhaP	[10]
n	Oter_1377	*Opitutus terae*	PB90-1	ACB74662.1	GalRhaP	[11]

predict activity by phylogenetic analysis. For example, the activities of Cphy_0577 and Cphy_3030 from *Clostridium phytofermentans* are GLNBP [10], though their amino acid sequences are located closer to GNBP from *Clostridium perfringens* [6] than to GLNBP from bifidobacteria on the genetic tree (Fig. 1).

Recently, we determined the three-dimensional structures of $GLNBP_{BL}$ by X-ray crystallography in the presence and absence of various ligands and observed the deformation of a catalytic TIM domain during substrate binding [18]. The N-terminal region in the ligand-free structure, adopts an open conformation. The binding of the acceptor molecule (GlcNAc or GalNAc) causes a small movement of an α-helix (residues 160–170: 160–170 helix) in the Ig-like domain (residues 71–180) that is inserted between β-3 and α-3 of the TIM barrel domain to form substrate-binding pocket (semi-closed conformation). Phosphate binding induces a large conformational change in the TIM barrel scaffold (closed conformation). Docking analysis strongly indicated that the structural movement is presumably required for the catalytic activity of $GLNBP_{BL}$. Thus, such structural dynamics must be considered when exploring the amino acid determinants that define the preferences of GalHexNAcP.

In this report, we selected amino acid residues related to substrate preference on the basis of the information of crystal structures around the pocket of substrate binding and analyzed the preferences of the GLNBP mutants related to the peculiar amino acid residue in GNBP and LNBP. As a result, we determined the effective amino acid residues for substrate preference based classification of GalHexNAcP.

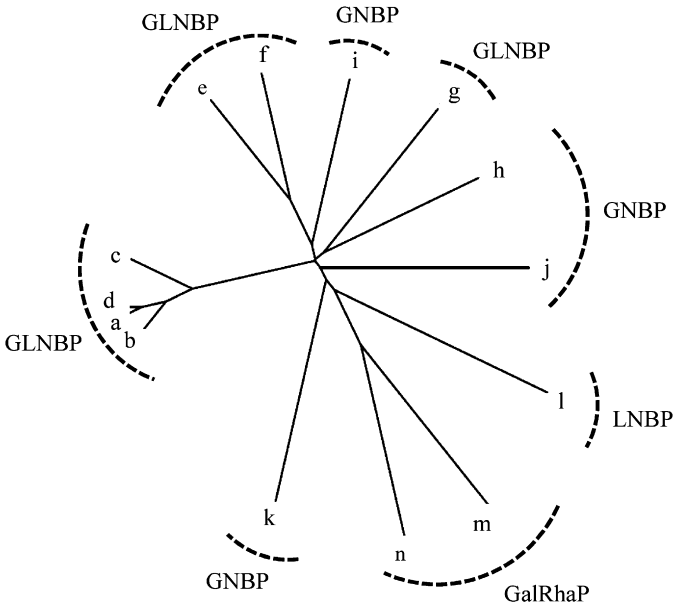

Fig. 1. Phylogenetic tree of amino acid sequences of characterized GH112 enzymes. The origins and accession numbers of the enzymes are listed in Table 1.

2. Experimental

2.1. Amino acid sequences

Multiple alignments of the amino acid sequences of GH112 proteins including GalHexNAcP and GalRhaP were performed using the ClustalW program (http://www.genome.jp/tools/clustalw/). Phylogenetic tree was drawn from the results using the neighbor-joining method.

2.2. Preparation of mutant enzymes

A $GLNBP_{BL}$ expression plasmid prepared in a previous study [4] was used as the template DNA. Mutations were introduced into the expression plasmid according to the construction manual of the QuikChange® site-directed mutagenesis kit (Stratagene, La Jolla, CA) using KOD plus DNA polymerase (Toyobo, Osaka, Japan), restriction endonuclease (*Dpn* I; Stratagene), and synthesized primers shown in Table 2. After confirming the mutation in DNA sequence, each mutated plasmid was used to transform *Escherichia coli* BL21 (DE3). Each transformant was cultivated at 30 °C with shaking in 20 mL LB medium containing 50 μg/ml kanamycin until the absorbance reached 0.5 at 600 nm. Protein production was induced by the addition of isopropyl-1-thio-β-D-galactoside at a final concentration of 0.5 mM, followed by incubation for a further 20 h at 30 °C with shaking. The cells were harvested by centrifugation at 15,000 × *g* for 10 min, suspended in 20 mM MOPS buffer (pH 7.5), and sonicated (Branson Ultrasonics Corporation, Danbury, CT). Then, the cell debris was removed by centrifugation at 17,000 × *g* for 30 min. Each enzyme was purified using a Ni-NTA spin column (Qiagen, Hilden, Germany), as instructed by the manufacturer. The

Table 2
Primers used for site-directed mutagenesis.

	Nucleotide sequence (5′ → 3′)
P161S-forward	gcctacatcatatgggactcggtcgagatg
P161S-reverse	catctcgaccgagtcccatatgatgtaggc
V162T-forward	catctgggatccgaccgagatgtacaacc
V162T-reverse	ggttgtacatctcggtcggatcccagatg
S336A-forward	gtggtcggtgccatcggcgacggcaccaccacccgcatgatc
S336A-reverse	gtcgccgatggcaccgaccacggcgtccaggccgagttcgtc

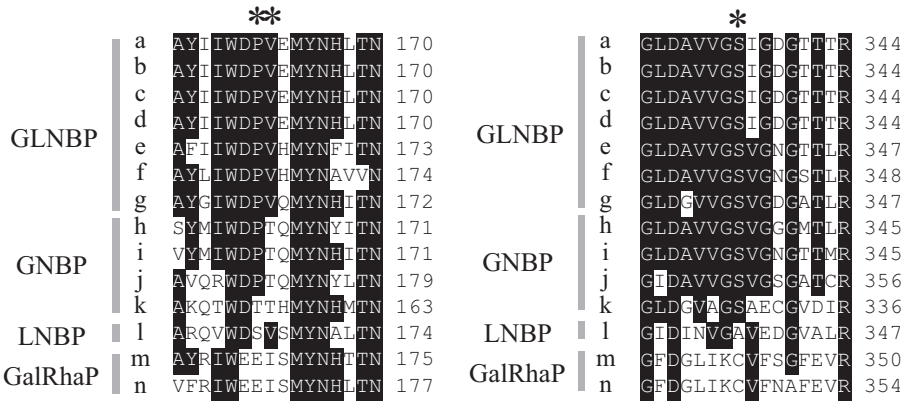

Fig. 2. Multiple alignment of amino acid sequences of GH112 proteins characterized substrate preference around P161/V162 and S336 of $GLNP_{BL}$. Conserved amino acid residues are highlighted. Asterisks show the residues corresponding to P161, V162, and S336 of $GLNP_{BL}$. The origins and accession numbers of the enzymes are listed in Table 1.

homogeneity of the purified enzymes was confirmed by SDS-PAGE. Protein concentrations were determined using theoretical extinction coefficients calculated from the amino acid sequence of the proteins [19].

2.3. Enzyme assays

GNB and LNB were enzymatically synthesized as reported in previous studies [16,17]. Enzyme activity for GNB and LNB was measured by the quantification of Gal1P produced in their phosphorolytic reaction using the assay method reported previously [20]. Reactions were done at 30 °C and in a reaction mixture containing 100 mM MOPS buffer (pH 7.5), 10 mM phosphate buffer (pH 7.0), and various concentrations of GNB/LNB. The kinetic parameters were calculated by curve fitting the experimental data with the theoretical equation, using the GraFit computer program [21].

3. Results and discussion

3.1. Selection of mutated amino acid residues

We reported in a previous study that the following amino acid residues in $GLNBP_{BL}$ were possibly involved in the recognition of GlcNAc and GalNAc: P161, V162, Y165, N166, S336, R358, Y362, and F364 [18]. From the results of multiple alignments for GH112 enzymes (Fig. S1, Fig. 2), five amino acid residues, Y165, N166, R358, Y362, and F364 were completely conserved, indicating that these residues are not determinants of substrate preference. Amino acid residues corresponding to V162 of $GLNBP_{BL}$ are conserved as Val among GLNBPs and LNBP, but Val is substituted by Thr in all the characterized GNBPs. However, the residues corresponding to P161 and S336 are conserved among most GLNBPs and GNBPs, but are substituted by Ser and Ala, respectively, in LNBP. Interestingly, alternations of the side-chain rotamers of P161 and S336 induced by substrate binding are observed in each conformation state, as is described below. These three amino acid residues in GalRhaP differed from those of GalHexNAcP, but were conserved among GalRhaPs (Glu/Ile/Cys) (Fig. 2). Therefore, we speculated that these three amino acid residues, P161, V162, and S336, are determinants of substrate preference of GalHexNAcP.

3.2. Mutation of $GLNBP_{BL}$ into GNBP-type

The P161S, V162T, S336A and P161S/S336A mutants of $GLNBP_{BL}$ were constructed and characterized. Kinetic parameters and the GNB/LNB ratio of each mutant are summarized in Table 3. The k_{cat}/K_m value of $GLNBP_{BL}$-V162T on GNB was approximately half of that of the wild type, which was caused by a decrease in the k_{cat} value. However, its k_{cat}/K_m value on LNB was approximately one-sixth of that of the wild type because of the drastic increase in the K_m value on LNB. As a result, the mutation at V162 increased the GNB/LNB ratio 3-fold, which indicates the promotion of GNB preference by limiting the affinity against LNB. Thus, the residue is considered to be an essential residue to determine whether GalHexNAcP can phosphorolize LNB efficiently.

The side chain of V162 is located close to the axial O4 atom of the GalNAc, bound at the subsite +1 [18]. When a Thr residue with a hydroxyl group in the side chain is introduced at the corresponding position to V162, a polar HexNAc-binding pocket that can accept the axial hydroxyl group of GalNAc at the C4 position becomes available. The V162T mutation drastically decreased the ratio of the K_m value on GNB against that of LNB (wild type, 2.8; V162T, 0.31), which supports the above assumption.

Chao et al. [12] determined that the same mutation did not affect the preference for GlcNAc and GalNAc as the acceptor in the reverse reaction by measuring the activity at a fixed concentration. The difference is probably attributable to the fact that the reaction rates were measured corresponding to the k_{cat} and not the k_{cat}/K_m values. Chao et al. also reported that the V162I mutation only causes a drastic decrease in the reaction rate with GalNAc. The introduction of a bulky side chain probably causes a steric hindrance for axial O4 of GalNAc; this observation strongly supports speculation that V162 is a determinant residue of whether the enzyme can accept GalNAc and/or GlcNAc. Some other residues were also assumed to participate in the preference for GNB, because the GNB/LNB ratio of the V162T mutant (2.7) is still much lower than that of other GNBPs (>50).

3.3. Mutation of $GLNBP_{BL}$ into LNBP-type

The P161S mutation caused an increase in the K_m values on both GNB and LNB by approximately 3-fold of those of the wild type (Table 2). However, the mutation only slightly increased the k_{cat} value on LNB, while that on GNB decreased by approximately half, as compared to those of the wild type. As a result, the mutation decreased the GNB/LNB ratio to 0.26, which indicates an increase in its LNB selectivity by 3.5-fold. The S336A mutation caused a

Table 3
Kinetic parameters of each enzyme on GNB and LNB.

	K_m (mM)	k_{cat} (s^{-1})	k_{cat}/K_m (mM^{-1} s^{-1})	GNB/LNB ratio
GLNBP from *B. longum* JCM1217 ($GLNBP_{BL}$) and its mutants				
Wild type				
GNB	7.7 (±0.5)	65 (±3)	8.5 (±0.3)	0.88
LNB	2.8 (±0.2)	27 (±1)	9.3 (±0.4)	
V162T				
GNB	6.7 (±0.4)	27 (±1)	4.0 (±0.1)	2.7
LNB	22 (±2)	33 (±2)	1.5 (±0.1)	
P161S				
GNB	27 (±2)	33 (±2)	1.2 (±0.1)	0.26
LNB	8.4 (±0.3)	39 (±1)	4.6 (±0.1)	
S336A				
GNB	20 (±3)	3.5 (±0.3)	0.17 (±0.01)	0.15
LNB	13 (±2)	15 (±1)	1.1 (±0.1)	
P161S/S336A				
GNB	120 (±13)	3.7 (±0.3)	0.031 (±0.001)	0.095
LNB	46 (±3)	15 (±1)	0.34 (±0.01)	
GNBP[a]				
C. perfringens				
GNB	1.9 (±0.3)	6.0 (±0.3)	3.2 (±0.3)	59
LNB	26 (±3)	1.4 (±0.1)	0.054 (±0.002)	
P. acnes				
GNB	140 (±20)	103 (±12)	0.73 (±0.02)	68
LNB	1300 (±500)	14 (±5)	0.01 (±0.01)	
LNBP[b]				
V. vulnificus				
GNB	10 (±1)	2.2 (±0.2)	0.22 (±0.01)	0.017
LNB	3.6 (±0.4)	45 (±2)	13 (±1)	

[a] Adopted from Ref. [7].
[b] Adopted from Ref. [8].

drastic decrease in the k_{cat} value on GNB, whereas the K_m values on both GNB and LNB were increased, resulting in a 6-fold increase in the selectivity of LNB (GNB/LNB ratio of 0.15). The kinetic parameters of the double mutation of P161S and S336A appear to be synergetic effects of each mutation, i.e., the K_m values of the double mutant against both GNB and LNB increased 16-fold (the increments of P161S and S336 are 3.5- and 3-fold, respectively). The double mutation caused a 10-fold increase in the selectivity on LNB, suggesting that both the residues are independently related with the selectivity of LNB.

Notably, P161 and S336 are involved in the structural movements of GLNBP (open, semi-closed, and closed conformations) induced by substrate binding [18]. The obvious difference between the open and semi-closed conformations is the rotamer of P161. P161 is located at the root of the 160–170 helix. The movement of the helix was observed in acceptor bound structures and it seemed to fix the HexNAcs in subsite +1. Thus, the induced fit of the helix correlates to the alternative rotamer switching of P161 (Fig. 3). P161S mutation presumably increases the flexibility of the 160–170 helix, which enables various conformations between open and semi-closed conformations. This increase in the flexibility may cause a steric hindrance with GNB more severely than with LNB, because the C4 axial hydroxyl group of the GalNAc moiety in GNB (the corresponding position in LNB is occupied by proton) is located close to the side chain of Val162.

The side-chain hydroxyl group of S336 forms a hydrogen bond with the side-chain nitrogen atom of R358, which plays a significant role in catalysis, as confirmed by mutagenesis [18]. Docking analysis strongly suggests that R358 recognizes phosphate, and the binding site accommodation for phosphate is formed only when $GLNBP_{BL}$ adopts the closed conformation (Fig. 4) [18]. The hydrogen bond between S336 and R358 presumably plays a key role in the stabilization of the closed conformation. Thus, the S336A mutation causes similar increases in the K_m values of both GNB and LNB, which can be explained by an increase in flexibility at the phosphate binding site structure. The S336A mutation also causes drastic decreases in the k_{cat} values of GNB, which cannot be readily

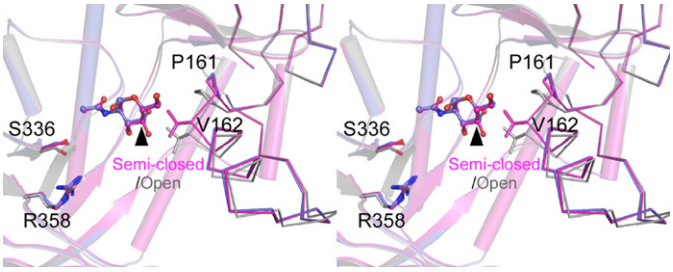

Fig. 3. Stereoview of open- (gray; PDB 2ZUS) and semi-closed conformations [PDB 2ZUT (GlcNAc complex; magenta) and 2ZUU (GalNAc complex; blue)]. TIM barrel domain and Ig-likedomain (including 160–170 helix) are shown as cartoon and backbone representation, respectively. Side chains of P161, V162, S336, and R358 are shown as stick models. Bound GalNAc/GlcNAc is shown as a ball-and-stick model, and its C4 atoms are indicated by arrows.

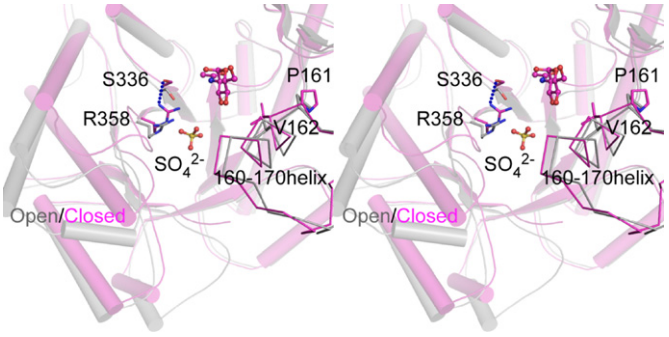

Fig. 4. Stereoview of structures of open- (gray; PDB 2ZUS) and closed-conformation (magenta; PDB 2ZUW). TIM barrel domain and Ig-like domain (including 160–170 helix) are shown as cartoon and backbone representation, respectively. Side chains of P161, V162, S336, and R358 are shown as stick models. Bound GlcNAc and sulfate (phosphate mimic) is shown as a ball-and-stick model. Hydrogen bond interactions formed in closed conformation between side chains of S336 and R358 are shown by dotted line.

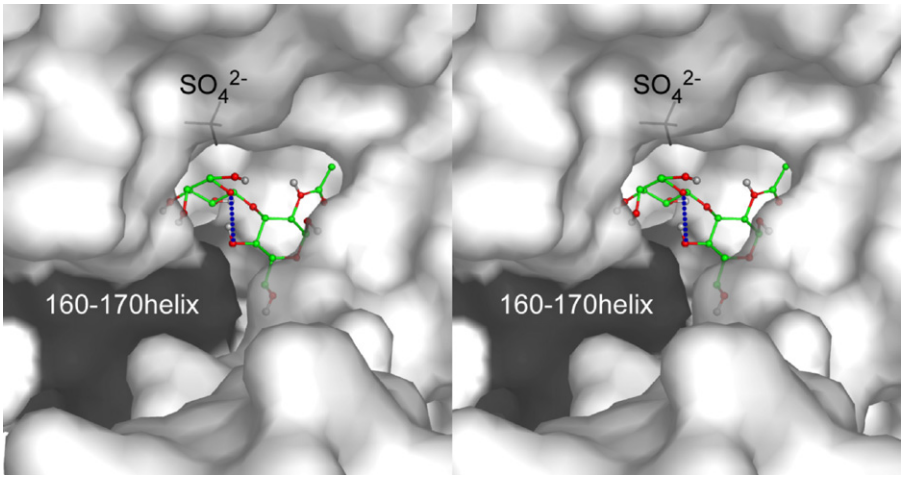

Fig. 5. Stereoview of docking result of LNB into $GLNBP_{BL}$ closed conformation. The transparent surface of $GLNBP_{BL}$ is in white, and the molecular surface of 160–170 helix is in black. Bound sulfate (phosphate mimic) is shown as a line model. The LNB structure of the best docked result by AutoDock is shown as a ball-and-stick model, and the intramolecular hydrogen bond (the distance of 2.8 Å) between O5 (ring oxygen) of Gal and O4 of GlcNac is shown by dashed line.

explained by the structural differences of the enzymes. One possible explanation is that an intramolecular hydrogen bond exists only in LNB that helps the catalytic reaction. A hydrogen bond between O4 of the GlcNAc residue and O5 of the Gal residue (the ring oxygen) is predicted by the docking analysis of LNB and GLNBP (Fig. 5) [18]. The presence of the same hydrogen bond was also observed in the crystal structure of the GNB/LNB binding protein [22] and predicted by the molecular dynamics simulations of LNB in water [23]. For the reaction mechanism of β-glycosidases, it is believed that distortion of the sugar ring at subsite −1 is required for efficient catalysis, mainly because steric hindrance from the hydrogen atom at the anomeric carbon should be removed for nucleophilic attack [24]. The intramolecular hydrogen bond of LNB possibly fixes the Gal moiety to adopt a distorted conformation suitable for efficient catalysis in the destabilized substrate-binding site. The hydrogen bond is not available in GNB because of the differences in the C4 hydroxyl group of GalNAc residue. In the S336A mutant, therefore, the absence of the hydrogen bond to Arg358 destabilized the closed conformation and affected the k_{cat} value of/for GNB more strongly than of/for LNB.

3.4. Estimation of substrate preference

From the results described above, the amino acid residues to classify GalHexNAcP into GLNBP, GNBP, and LNBP can be defined as follows: GLNBP (the positions of 161/162/336 in $GLNBP_{BL}$ numbering are Pro/Val/Ser), GNBP (Pro/Thr/Ser), and LNBP (Ser/Val/Ala). In addition, these residues in GalRhaP are Glu/Ile/Cys.

A number of sequences belonging to GH112 have been registered from the genomic sequence data of various bacteria. The alignment of the GH112 sequences (Fig. S2) shows that most GH112 proteins obey one of the four patterns, which suggests that their activities are predictable from their amino acid sequences. Only a few exceptional sequences do not agree with the four patterns, and their patterns are within Glu/Ile/Gly or Thr/Thr/Ser. The former resembles the pattern of GalRhaP, being different only in the third position. Recently, Chao et al. characterized a GH112 enzyme from *Streptobacillus moniliformis* of Thr/Thr/Ser pattern and it was determined to be GNBP [12]. The first residue, Thr, resembles the residue for LNBP, Ser. The latter two, Thr and Ser, match the Thr/Ser pattern of GNBP. The GNB/LNB ratio of the enzyme was 13, which was considerably smaller than those of the other typical GNBPs (>50) [6,7]; this difference can be attributed to the mixed pattern in the three residues.

4. Conclusion

We identified that one residue in the amino acid sequence of GalHexNAcP corresponded to the preference for GNB and that two residues independently corresponded to the preference for LNB. These findings help to directly predict the substrate preference of GalHexNAcP homologues by comparing the corresponding three amino acid residues.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.molcatb.2011.09.004.

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