J. Appl. Glycosci., **52**, 183−189 (2005)

Proceedings of the Symposium on Amylases and Related Enzymes, 2004

Substrate-Binding Mode of Bacterial Chitosanases

(Received December 2, 2004)

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Abstract: Mode of substrate-binding of chitosanases from *Streptomyces* sp. N174 (N174 chitosanase) and *Bacillus circulans* MH-K1 (MH-K1 chitosanase) was examined by site-directed mutagenesis and physicochemical techniques, including thermal unfolding, fluorescence spectroscopy, and X-ray crystallography. Asp57 located at the central portion of the binding cleft of N174 chitosanase was mutated to asparagine and alanine (D57N and D57A), and the relative activities of the mutated enzymes were 72 and 0.5% of that of the wild type, respectively. Thermal unfolding experiments in the presence of $(GlcN)_n$ clearly indicated the importance of Asp 57 for substrate binding. Kinetic analysis of $(GlcN)_6$ degradation catalyzed by N174 chitosanase suggested that Asp57 is most likely to participate in the substrate binding at subsite −2 through hydrogen bonding as well as electrostatic interaction. On the other hand, for MH-K1 chitosanase, we focused our attention on Tyr148 and Lys218, which are located at the bottom of the binding cleft and at the flexible loop forming the edge of the binding cleft, respectively. These residues were mutated to serine (Y148S) and proline (K218P), respectively, and the enzymatic activities of Y148S and K218P were found to decrease to 12.5 and 0.16% of the wild type. When $(GlcN)_3$ binding ability to the chitosanase was evaluated from the change in tryptophan fluorescence intensity, the binding abilities of Y148S and K218P were found to be reduced from that of the wild type by 1.0 and 3.7 kcal/mol of binding free energy, respectively. The crystal structure of K218P revealed that the main chain and side chain structures of the loop comprising Lys218 are affected by the mutation. Thus, we concluded that the flexible loop comprising Lys218 plays an important role in substrate binding, and that the role of Tyr148 is less important but significant, probably due to stacking interaction.

Key words: chitosanase, substrate binding, fluorescence, thermal unfolding, X-ray crystallography

Chitosanase is an enzyme that belongs to the glycoside hydrolase family and is characterized by its ability to catalyze the hydrolytic cleavage of chitosan, a polycationic carbohydrate derived from chitin by partial or complete deacetylation. Chitosan is a mixed polysaccharide containing β-1,4-linked residues of β-D-glucosamine (GlcN) and *N*-acetyl-β-D-glucosamine (GlcNAc). The differences in the mechanism of chitosan hydrolysis among the various groups of enzymes, such as lysozymes, chitinases, and chitosanases, were examined by experiments that analyzed the structure (*i.e.*, the sequence) of the oligosaccharide products from enzymatic hydrolysis. From these sequences, the cleavage specificity of several enzymes could be deduced. Fukamizo *et al*.[1] proposed classifying chitosanases as enzymes that hydrolyze chitosan without splitting the linkage GlcNAc-GlcNAc. Conversely, chitinases could cleave the GlcNAc-GlcNAc linkage, but not the GlcN-GlcN linkage. Chitosanases were further subdivided into three classes according to their cleavage specificity; class I enzymes could split both GlcN-GlcN and GlcNAc-GlcN linkages; class II enzymes could split only GlcN-GlcN linkages; and class III enzymes could split both GlcN-GlcN and GlcN-GlcNAc linkages. The recognition mechanisms of the chitosanases are thus complicated, making it difficult to unequivocally distinguish from the other chitinolytic enzymes. This situation lead us to examine the mode of substrate binding of chitosanases.

A number of chitosanases have been isolated from various bacteria and fungi, and their genes have been cloned and sequenced. These chitosanases belong to various GH (glycosyl hydrolase) families, GH-5, GH-8, GH-46, GH-75 and GH-80 according to the amino acid sequences.[2] Among these chitosanases sequenced thus far, the enzymes from *Streptomyces* sp. N174 (N174 chitosanase) and *Bacillus circulans* MH-K1 (MH-K1 chitosanase), belonging to family GH-46, have been most intensively studied based on their X-ray crystal structures.[3,4] In this article, we review the recent findings from the substrate binding experiments of family GH-46 chitosanases conducted in our laboratory.

N174 Chitosanase.

N174 chitosanase is classified under class I chitosanases according to its splitting specificity,[5] and its properties have been reviewed by Fukamizo and Brzezinski.[6]

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Abbreviations: GlcN, 2-amino-2-deoxy-D-glucopyranose; $(GlcN)_n$, β-1,4-linked oligosaccharide of GlcN with a polymerization degree of *n*; GlcNAc, 2-acetamido-2-deoxy-D-glucopyranose; d.a., degree of acetylation; MH-K1 chitosanase, chitosanase from *Bacillus circulans* MH-K1; N174 chitosanase, chitosanase from *Streptomyces* sp. N174.

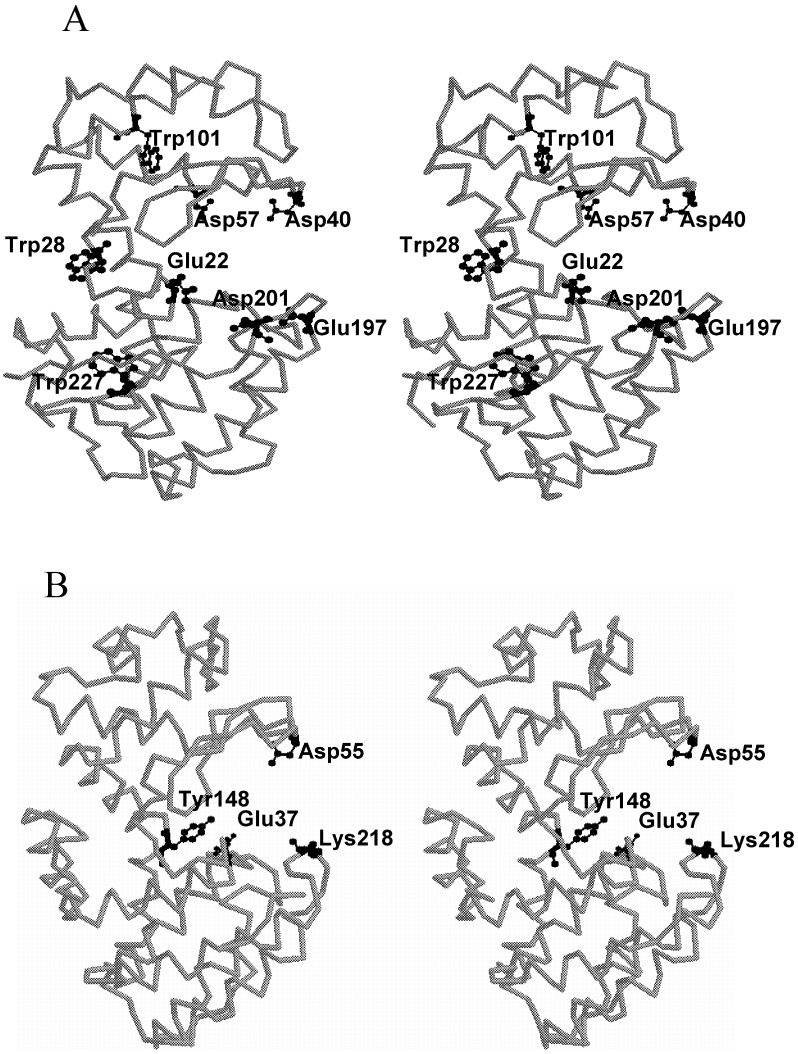

Fig. 1. Stereo views of x-ray crystal structures of N174 chitosanase (A) and MH-K1 chitosanase (B).

Glu22 and Asp40 are the catalytic residues of N174 chitosanase, and Glu37 and Asp55 are those of MH-K1 chitosanase. Other residues highlighted in the structures are the mutated residues, which are described in the text.

Its three-dimensional structure was solved by X-ray crystallography (PDB code, 1CHK),[3] which revealed that the enzyme has two globular upper and lower domains, which generate the active site cleft for the substrate binding as shown in Fig. 1A. The catalytic residues were found to be Glu22 and Asp40 by site-directed mutagenesis.[7] From the distribution of electrostatic potential on the enzyme surface, the entire region of substrate-binding cleft was found to be densed with electronegative charges. Thus, we supposed that carboxylic amino acid residues could play an important role in determining the mode of substrate-binding of this chitosanase. At least 12 carboxylic residues are found in the active site cleft of N174 chitosanase[3] and could participate in the binding of the positively charged chitosan substrate in both specific and non-specific ways. A model of the binding of glucosamine hexasaccharide, $(GlcN)_6$, to chitosanase has been proposed[3] adopting a (-4, -3, -2, -1, $+1$ and $+2$)-type enzyme-substrate interaction typical of hen egg white lysozyme.[8] According to this model, three carboxylic residues could be directly involved in links with the substrate, promoting the binding to low acetylation degree chitosan by chitosanase. The three residues are Asp57, Glu197 and Asp201, and localize to subsites -2, -1 and $+2$, respectively. Among the subsites, subsite -2 is thought to make a major contribution to substrate binding of this type of glycosyl hydrolases, and Asp57 is observed to be conserved in all chitosanases belonging to family GH-46. Thus, we decided to prepare two mutated enzymes, in which Asp57 is replaced with asparagine (D57N) and alanine (D57A).

Enzymatic activities of Asp57-mutated chitosanases toward chitosan.

After purification of the wild type and mutated chitosanases, CD spectra of the enzymes were obtained in 50 mM sodium phosphate buffer, pH 7.0, using a Jasco J-720 spectropolarimeter (cell length, 0.1 cm) at 20°C. D57N and D57A were found to retain most of their wild type global conformation as judged from the spectra. Enzymatic activities of the mutated chitosanases toward $(GlcN)_6$ substrate were measured, and the relative activities of D57N and D57A were found to be 72 and 0.5% of that of the wild type, respectively. Clearly, the mutation of Asp57 affects the enzymatic activity. Steady state kinetic parameters could not be obtained because of their strong substrate inhibition.

Chitosan binding ability of chitosanases as determined by thermal unfolding experiments.

To obtain thermal unfolding curves of chitosanases in either the presence or absence of $(GlcN)_n$, the CD value at 222 nm was monitored while raising the temperature at a rate of 1°C per min. One hundred molar excess of the saccharide was added to the enzyme solution for examining the binding effect on the thermal stability. At first, E22Q chitosanase, in which the catalytic residue Glu22 is mutated to glutamine, was tested for its binding ability. The hydrolytic activity of E22Q was known to be lower than 0.1% of that of the wild type enzyme.[7] We found that the transition temperature (T_m) increased in the presence of $(GlcN)_n$,[9] and that the stabilization effect became larger with the increasing chain length of the added oligosaccharide.[10] Similar experiments with the D57A chitosanase revealed that protein stability is not enhanced upon addition of $(GlcN)_3$. Furthermore, the enhancement of stability in D57A upon the addition of either $(GlcN)_6$ or chitosan (d.a. $<$ 0.01; DP $\fallingdotseq$20) was much less intense than that measured with E22Q.[9] Control HPLC revealed only negligible hydrolysis of the saccharides added during the unfolding experiments. This suggested that the observed differences in unfolding behaviour between the E22Q and D57A chitosanases are due to a significant impairment of substrate binding ability. For D57N, the binding experiment has not been done because of its hydrolytic effect on the saccharide added.

Hexasaccharide digestion experiments.

When the wild type enzyme was incubated with $(GlcN)_6$, the substrate was almost completely degraded within 30 min, producing predominantly $(GlcN)_3$ and $(GlcN)_2$ + $(GlcN)_4$ in smaller amounts. D57N completely hydrolyzed $(GlcN)_6$ within 3 h, producing $(GlcN)_3$, $(GlcN)_2$ and $(GlcN)_4$ in a similar distribution to that of the wild type. On the other hand, as long as 60 h was required for complete digestion of $(GlcN)_6$ using an equivalent amount of D57A chitosanase. The product distribution produced by the D57A mutant was different from that of the wild type enzyme, yielding smaller amounts of $(GlcN)_2$ and $(GlcN)_4$ as compared to the amount of $(GlcN)_3$ than did the wild type enzyme.

Theoretical analysis of the experimental time-course.

Theoretical analysis of the reaction time-course was performed using the reaction model reported previously,[11] in which the bond cleavage (k_1) and the hydration (k_2) processes are assumed to be time-dependent, but all other steps are time-independent binding processes (Fig. 2). Binding constants are defined for all possible binding modes, and were calculated from the unitary binding free energy changes of individual subsites by assuming additivity of the free energy values. Having observed that trimeric products are preferentially generated from the hexameric substrate, we tested a model assuming that the chitosanase has the subsite arrangement, −3, −2, −1, +1, +2 and +3. The value of the binding free energy change of each subsite was estimated using the optimization technique based on the modified Powell method[12] employing the cost function,

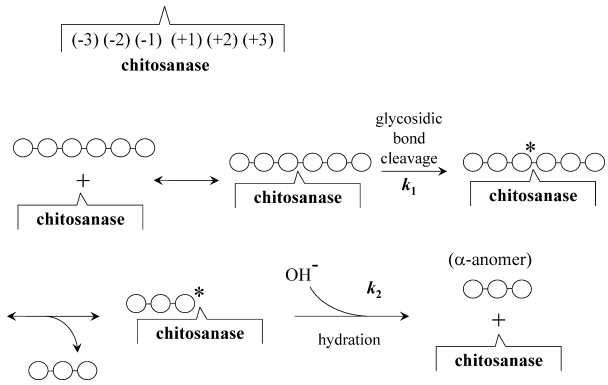

Fig. 2. Reaction model scheme of $(GlcN)_6$ hydrolysis catalyzed by N174 chitosanase.

$$F = \sum_i \sum_n \left[(GlcN)^e{}_{n,i} - (GlcN)^e{}_{n,i}\right]^2$$

where e and c are the experimental and calculated values, n is the size of the oligosaccharide and i the reaction time. In calculation of the reaction time-course, the value of the rate constant k_1 (for cleavage of the glycosidic linkage) was assumed to be dependent on the size of the substrate, and the k_{cat} values obtained from the steady state kinetic analysis with oligosaccharide substrates, 50 s^{-1} for $(GlcN)_4$, 50 s^{-1} for $(GlcN)_5$ and 200 s^{-1} for $(GlcN)_6$ (Fukamizo *et al*., unpublished data), were allocated to the individual k_1 values. As previously reported,[5] the N174 chitosanase catalysis takes place through an inverting mechanism, in which the hydration occurs almost concurrently with the bond cleavage. Thus, the higher value, 1000 s^{-1}, was tentatively allocated to k_2, the rate constant for hydration.

Starting with roughly estimated values, optimization was conducted for the binding free energy values of the individual subsites using the experimental time-course of hexasaccharide degradation catalyzed by the wild type enzyme. Finally, the value of the cost function attained the minimum when calculated with the values, −0.7, −4.7, +3.4, −0.5, −2.3 and −1.0 kcal/mol, for individual subsites −3, −2, −1, +1, +2 and +3, respectively. Next, the time-course for D57N was analyzed in a similar manner. Fixing the rate constants at the values estimated for wild type enzyme, optimization was conducted starting from the free energy values estimated for the wild type enzyme. In this case, however, only the values for subsites −3, −2 and −1 were changed, because the other subsites (+1, +2 and +3) are located too far from the Asp57 residue and the Asp57 mutation is unlikely to affect the free energy values of these subsites. Values of −0.7, −3.8 and +3.4 kcal/mol for subsites −3, −2 and −1 of D57N were found to yield a minimum cost function. Optimization of the reaction time-course obtained for the D57A mutant was conducted in a manner analogous to that with the D57N mutant. However, the value of the cost function did not decrease as much as in the case of the D57N mutant. The overall reaction rate of the calculated time-course was much higher than the experimental one. Therefore the optimization procedure was repeated using decreased k_1 values. With 5 s^{-1} for $(GlcN)_4$, 5 s^{-1} for $(GlcN)_5$ and 20 s^{-1} for $(GlcN)_6$, the fit between the experimental data and the calculated reaction

Table 1. Kinetic parameter values obtained from theoretical analysis of the time-course of hexasaccharide degradation.

Enzyme	Rate constants (s^{-1}) k_1			k_2	Binding free energy changes (kcal/mol)					
	$(GlcN)_4$	$(GlcN)_5$	$(GlcN)_6$		(−3)	(−2)	(−1)	(+1)	(+2)	(+3)
Wild type	50	50	200	1000	−0.7	−4.7	+3.4	−0.5	−2.3	−1.0
D57N	50	50	200	1000	−0.7	−3.8	+3.4	−0.5	−2.3	−1.0
D57A	5	5	20	1000	−1.7	0.0	+4.5	−0.5	−2.3	−1.0

time-course was satisfactory, and −1.7, 0.0 and +4.5 kcal/mol were deduced as the optimized values for subsites −3, −2 and −1, respectively. All of these values for kinetic parameters obtained in the theoretical analysis are listed in Table 1.

The substitution of the $-CH_2-COO^-$ in Asp57 with either $-CH_2-CO-NH_2$ (in D57N) or $-CH_3$ (in D57A) affects the substrate-binding ability, suggesting that the interaction of Asp57 with the substrate is electrostatic in nature and is eventually accompanied by hydrogen bonding. Marcotte *et al*.[3] suggested that there are two possible contacts between Asp57 and the sugar residue at subsite −2; one is an electrostatic interaction with the sugar amine, and the other is a hydrogen bond with the hydroxyl oxygen at C3 of the pyranose ring. Both interactions would be lost in the D57A mutant, while hydrogen bonding would be conserved in the D57N mutant. This explanation is consistent with the observed decrease in the free energy value at subsite −2 (Table 1).

Fluorescence analysis of substrate binding.

Fluorescence spectra of N174 chitosanase were recorded with an excitation wavelength 295 nm using a Hitachi F-3010 spectrofluorometer. The enzyme preparation was dialyzed against 0.06 M Tris-HCl buffer pH 7.0 containing 0.1 M NaCl, before the measurement. When $(GlcN)_2$ or $(GlcN)_3$ was added to the chitosanase solution, the fluorescence intensity clearly decreased in a concentration-dependent manner (Fukamizo *et al*., manuscript in preparation). The relative decreases in the fluorescence intensity were plotted against the substrate concentration to obtain the saturation curves, which was then employed for Scatchard analysis to obtain the binding constants and the binding free energy changes. These values are listed in Table 2. The affinity of $(GlcN)_3$ was found to be similar to that of $(GlcN)_2$. Such low molecular weight oligosaccharides might bind to subsites −3∼−2 or to subsites +2∼+3, and avoid being in contact with subsites −1 and +1, which have very low affinities of sugar residue binding. If this is the case, $(GlcN)_3$ would bind to the enzyme occupying only two subsites and the terminal residue would extend beyond the substrate-binding cleft. This situation might result in the similarity between the binding constants of $(GlcN)_2$ and $(GlcN)_3$. When D57A was used instead of the wild type, the change in the fluorescence intensity upon addition of $(GlcN)_n$ was too small to analyze quantitatively the binding ability.

Table 2. Binding constants and binding free energy changes of $(GlcN)_n$ binding to individual chitosanases.

Chitosanase	Ligand	K_{assoc} (M^{-1})	ΔG (kcal/mol)
N174 Chitosanase			
Wild type	$(GlcN)_2$	1410	−6.6
Wild type	$(GlcN)_3$	1110	−6.4
MH-K1 Chitosanase			
Wild type	$(GlcN)_3$	2009	−6.8
Y148S	$(GlcN)_3$	341	−5.8
K218P	$(GlcN)_3$	3.4	−3.1

Conformational change induced by the oligosaccharide binding.

The change in fluorescence intensity induced by saccharide binding reflects the conformational change around the tryptophan indole groups of the enzyme protein. N174 chitosanase has three tryptophan residues, Trp28, Trp101 and Trp227, which are localized to the hinge in between the two domains, the upper domain, and the lower domain, respectively, as shown in Fig. 1A. When each of the tryptophan residues was mutated to phenylalanine, the fluorescence change induced by the saccharide binding was significantly suppressed. In particular, the suppression was found to be the most intensive in Trp28-mutated chitosanase (Fukamizo *et al*., manuscript in preparation). This suggests that the conformational change induced by saccharide binding is most intensive around the Trp28 indole side chain, which is localized to the hinge region. The saccharide binding might induce a conformational change, which seems to narrow the binding cleft. In this case, local conformation around the hinge region might be most intensively affected by the movement of the upper and lower domains. This situation appears to result in most intensive conformational change around Trp28.

MH-K1 Chitosanase.

MH-K1 chitosanase is a 29-kDa extracellular protein composed of 259 amino acids,[13] and its X-ray crystal structure has already been determined by the multiwavelength anomalous diffraction method (PDB code, 1QGI).[4] The enzyme belongs to the glycosyl hydrolase family identical to that of N174 chitosanase; hence, these two enzymes have a similar fold in their crystal structures (Figs. 1A and B).[4] According to the splitting specificity, however, the MH-K1 enzyme is classified under class III,[14] whereas N174 chitosanase is put into class I. Catalytic residues, Glu37 and Asp55, are located in a similar configuration as in the case of N174 chitosanase. Thus, the mechanism of catalytic reaction and substrate recognition of MH-K1 chitosanase might be similar to those of N174 chitosanase. On the other hand, a closer examination of the crystal structure of MH-K1 chitosanase revealed that the side chain of Tyr148 is located in between two α-helices forming the backbone of the substrate bind-

ing cleft (Fig. 1B) and is the sole aromatic amino acid residue located on the surface of the substrate binding cleft. This residue might be an additional candidate, which participates in substrate binding, because aromatic side chains have been recognized to interact with the pyranose ring of the substrate by a stacking effect.[15] Another candidate as the binding residue is Lys218, which is located in the flexible loop of the lower edge of the opened binding cleft (Fig. 1B). Such a flexible loop at or near the binding cleft has been recognized to play an important role in substrate binding in glycosyl hydrolases.[16,17] Thus, we replaced Tyr148 with Ser (Y148S) to examine the role of the aromatic side chain, and then replaced Lys218 with Pro (K218P) to examine the flexible loop.

Enzymatic activities of the mutant chitosanases.

CD spectra of the purified mutant enzymes exhibited profiles similar to that of the wild type, indicating that the global conformation is not affected by the mutations. The mutated chitosanases were subjected to activity determination using the substrate, 20% acetylated chitosan. The enzymatic activities of Y148S and K218P were found to decrease to 12.5 and 0.16% of that of the wild type enzyme, respectively. Acitivity measurements were also conducted by TLC analysis using the substrate, $(GlcN)_6$. The wild type enzyme produced $(GlcN)_2$, $(GlcN)_3$ and $(GlcN)_4$, of which the amounts were almost equal to each other. This suggests that the enzyme hydrolyzes $(GlcN)_6$ with an endo-splitting manner. When the Y148S mutant was used instead of the wild type, the rate of the degradation of $(GlcN)_6$ was much lower than that of the wild type enzyme reaction, but no appreciable change was found in the product distribution. In the K218P mutant, products were hardly detected, indicating that the mutant enzyme does not have any enzyme activity.

Thermal unfolding experiments.

Thermal unfolding experiments were conducted to examine the saccharide binding ability using MH-K1 chitosanase and its mutated enzymes. The transition temperature of the unfolding transition (T_m) of the wild type protein was determined to be 53.9°C. When the $(GlcN)_3$ solution was added to the wild type enzyme, the T_m value increased by 2.2°C. The increase in T_m is due to the binding interaction between the enzyme and the trisaccharide.[10] When Y148S and K218P were used instead of the wild type, the increase in T_m upon the addition of $(GlcN)_3$ was not observed. $(GlcN)_3$ does not correctly interact with Y148S and K218P.

Fluorescence analysis of substrate binding.

Fluorescence spectra of MH-K1 chitosanase were obtained in a condition similar to that for N174 chitosanase. When $(GlcN)_3$ was added to the chitosanase solution, the fluorescence intensity clearly decreased in a concentration-dependent manner (Fukamizo *et al*., manuscript in preparation). The relative decreases in the fluorescence intensity were plotted against the substrate concentration to obtain the saturation curves. From the Scatchard analysis of the saturation curves, the binding constants of the wild type, Y148S, and K218P were calculated to be 2009 ± 15,341 ± 01, and 3.4 ± 02 M^{-1}, respectively, corresponding to −6.8, −5.8 and −3.1 kcal/mol of binding free energy changes. The binding abilities of both mutant enzymes were significantly reduced when compared with the wild type enzyme.

Substitution of Tyr148 with Ser reduced the binding affinity of $(GlcN)_3$ by about 1.0 kcal/mol of binding free energy. The free energy difference, 1.0 kcal/mol, can be regarded as the free energy contribution of the stacking interaction between the aromatic ring of Tyr148 and the pyranose ring of $(GlcN)_3$. The lower binding ability of Y148S can be confirmed as well from the thermal unfolding experiments. Thus, the activity decrease in Y148S (→ 12.5%) should be derived at least partly from the suppression of binding ability. It is reasonable to presume that the mutation of Tyr148 might not only affect the binding ability at the subsite but impair the catalytic efficiency by affecting the conformational state of the catalytic cleft. In fact, in *Streptomyces* sp. N174 chitosanase, the mutation of Asp57, which participates in the sugar residue binding at −2 site, affects not only the binding affinity at the corresponding subsite but the rate constant for cleavage of β-1,4-glycosidic linkage (Table 1). A similar effect might possibly occur in the Tyr148 mutation of MH-K1 chitosanase. For rationalizing the 3.7 kcal/mol decrease in K218P, we had to examine the crystal structure of the mutated enzyme.

X-ray crystal structure of the mutant chitosanase.

To identify the structural factor resulting in the lower binding ability, we tried to solve the crystal structure of K218P. Crystallization of the mutated enzyme was performed at 20°C by the sitting drop vapor diffusion method using ammonium sulfate as the precipitant. Data collection, phase determination, and structure refinement were done by the method reported previously.[4] After the refinement procedure, we obtained the final structure as shown in Fig. 3A, which shows superposition of Cα traces of the wild type and K218P. It appears that a global conformation is not significantly affected by the mutation. Closer examination of the structures, however, reveals that the flexible loop comprising Lys218 is shifted by the mutation, and the side chain structure of the flexible loop is also considerably affected by the mutation as shown in Fig. 3B. In particular, the orientation of the side chain of Asn217 is greatly rearranged; hence, the hydrogen bonding network is disrupted by the mutation. The hydrogen bonding network of Tyr219 was also found to be disrupted by the Lys218 mutation. This situation would result in the lowest binding ability of K218P.

A lysine residue positively charged in the moderate pH region cannot generally be regarded as a candidate for the amino acid residue responsible for substrate recognition or binding in chitosanases, because of the polycationic property of chitosan substrate. In this study, however, we found that the mutation of Lys218 strongly impaired the enzymatic activity (0.16%), and that the binding constant of $(GlcN)_3$ severely decreased in K218P by about 3.7 kcal/mol of the binding free energy. Lys218 is likely very important for substrate binding. The crystal structure of K218P revealed that the main chain and side chain struc-

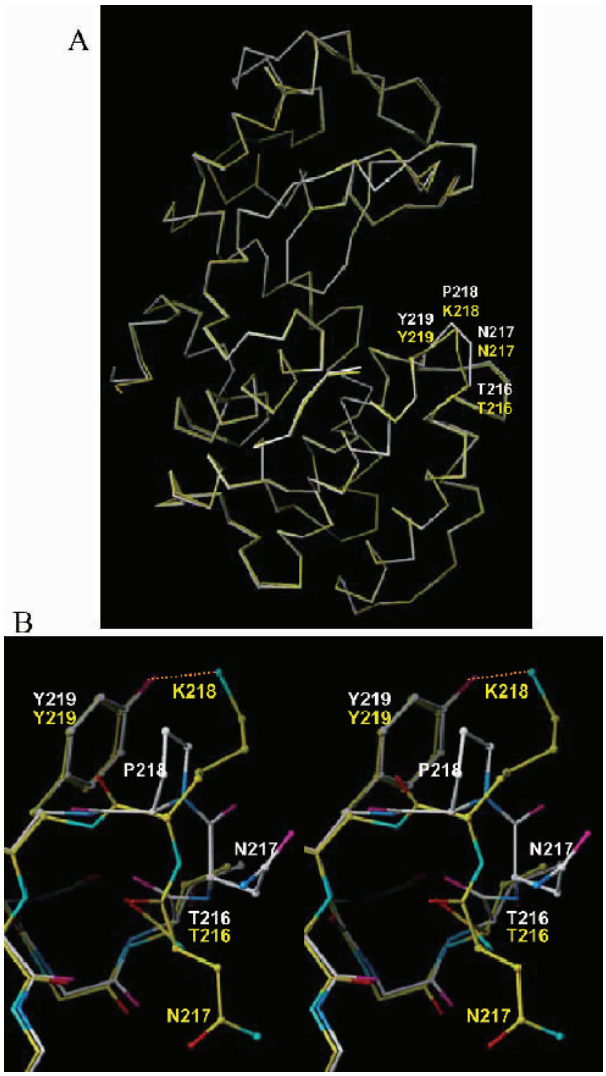

Fig. 3. Superposition of the crystal structures of the wild type and K218P of MH-K1 chitosanase.

(A) Cα traces of the wild type (yellow) and K218P (white), (B) stereo view of the close-up of the mutation position (Lys218). Labels are amino acid residues for the wild type (yellow) and K218P (white).

tures of the loop comprising Lys218 are considerably affected by the mutation; hence, a hydrogen bonding network with the neighboring amino acid residues (Asn217 and Tyr219) is disrupted by the mutation. In the crystal structure of the wild type chitosanase, the loop comprising Lys218 is located at the lower edge of the binding cleft (Fig. 1B), and the temperature factor of this loop region was found to be relatively high.[4] It has been recognized that such a flexible loop plays an important role in substrate binding in most carbohydrate active enzymes. The flexible loop of human salivary amylase is considered to assist the catalysis during the transition state and to play a role in releasing the product from the active site.[16] When an oligosaccharide inhibitor binds to *Bacillus cereus* amylase, the flexible loop (residues 93–97) was found to largely move and cover the bound inhibitor.[17] Similarly, the flexible loop comprising Lys218 in MH-K1 chitosanase might perform such an important action toward the substrate for correctly positioning the substrate molecule. Mutation of Lys218 to proline severely altered the conformation of the flexible loop resulting in the lowest binding ability.

Conclusion.

Protein-carbohydrate interactions have been investigated using glycoside hydrolases resulting in the partial elucidation of their mechanisms.[15] Chemical modification and site-directed mutagenesis studies[18] have suggested a key role for tryptophan residues because of both their ability to stack and their non-polar interaction with the pyranose ring of substrates. In the case of the protein-chitosan interaction, however, the mechanism is thought to be significantly different, because of the polycationic properties of the chitosan. Electrostatic interaction might be one of the critical factors of the molecular recognition. In fact, the side chain of Asp57 of N174 chitosanase participates in binding the sugar residue at subsite -2, most probably through both electrostatic and hydrogen bonding interactions. Although hexasaccharide substrate mainly binds to the entire binding cleft, occupying subsites -3 to $+3$ of the chitosanase (Fig. 2), the Asp57 interaction with the sugar residue at subsite -2 is the most important for the binding of chitosan oligosaccharide to chitosanase. Similarly, in MH-K1 chitosanase, carboxylic amino acids seem to be important for substrate binding. In this chitosanase, however, other factors controlling the substrate-binding were found by site-directed mutagenesis studies. The lower enzymatic activities of the mutant enzymes from MH-K1 chitosanase, Y148S and K218P, were found to be derived from the decrease in substrate binding ability. The crystal structure of K218P revealed that the mutation alters the main chain and side chain structures of the flexible loop comprising Lys218 and disrupts a hydrogen bonding network with the neighboring amino acid residues, Asn217 and Tyr219. The loop comprising Lys218 and the neighboring residues plays an important role in substrate binding, and the role of Tyr148 is less important but significant, probably due to stacking interaction.

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細菌キトサナーゼの基質結合機構

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Streptomyces sp. N174 および *Bacillus circulans* MH-K1 由来のキトサナーゼの基質結合機構を明らかにするために，部位特異的変異導入，熱変性実験および蛍光実験などの方法を用いて，基質結合に関与するアミノ酸残基の同定を試みた．*Streptomyces* sp. N174 キトサナーゼの場合，X 線結晶構造およびその構造中における静電ポテンシャル分布より Asp57 が基質結合に関与するアミノ酸残基として最も可能性が高いと考えられた．そこで，この酸性アミノ酸残基を特異的に変異させたキトサナーゼを生産し，その酵素学的性質を調べた．Asp57 変異酵素（D57A）の酵素活性を調べてみると，野生型と比べ著しく減少した（0.5%）．野生型では，キトサンの結合に伴い熱変性の転移温度がかなり高くなるのに対し，D57A では大きな転移温度の上昇はみられなかった．明らかに Asp57 は基質結合に関与する重要なアミノ酸であることがわかった．本酵素によるオリゴ糖加水分解反応のモデリングを行い，各サブサイトの親和力を推定してみたところ，Asp57 の変異は（−2）サブサイトの親和力に大きく影響することがわかった．*Bacillus circulans* MH-K1 キトサナーゼの場合，クレフト内に存在する唯一の芳香族アミノ酸である Tyr 148 の部位特異的変異を行い（Y148S），また，基質結合クレフトの開口部分に存在する Lys218 の変異も行い（K218P），これら変異酵素の解析を行った．それらの変異酵素の蛍光スペクトル変化に基づいて，基質結合性を調べてみたところ，Y148S は1.0 kcal/mol ほど，K218P は3.6 kcal/mol ほどの基質結合力の減少が起こっていた．以上より，Tyr 148 は基質のピラノース環と有意に相互作用を行っていること，および，クレフト開口部に存在する Lys218 を含むループ構造は基質結合に重要であることがわかった．

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〔質問〕　江崎グリコ・生化研　高田

1）基質と酵素を混合し，温度を上昇させながら CD スペクトルをとり，「間接的に基質結合を評価」という実験がありましたが，酵素分子のゆらぎ，基質分子のゆらぎ等，多くのファクターが関与するのではないでしょうか？

2）上の実験の場合，基質が切断されてしまった場合は，どのように評価するのですか？

〔答〕

1）厳密な意味ではそのようなファクターを考慮すべきですが，実験条件を満たしさえすれば，Schellman が定式化した基質結合と T_m との関係は成立しますので（*Biopolymers*, **14**, 999−1018（1975）），T_m の上昇が基質との結合力を反映するものと考えることができます．これまで，ニワトリ卵白リゾチームや放線菌キトサナーゼでその典型的な実験結果が得られています（Pace and McGrath: *J. Biol. Chem*., **255**, 3862−3865（1980）; Honda *et al*., *FEBS Lett*., **411**, 346−350（1997））．本稿の中で言及している熱変性による結合実験はすべて，これら典型的な例と同じ条件で行っていますので，T_m の上昇に基づく基質結合性の評価は妥当であると考えています．

2）この実験は，基本的には，グリコシド結合の切断が起こらないような条件で行うべきです．例えば，触媒基を特異的に変異させた酵素を用いたり，あるいは基質側に何らかの化学修飾を施して結合はするが分解反応は起こらないというような基質を用いるべきです．本稿の実験では，不活性の変異酵素あるいは，分解されないオリゴ糖（三糖）を用いていますので，グリコシド結合の切断の可能性は全くないものと考えます．