Theoretical Calculation of pH reveals an important role of Arg205 in the Activity and Stability of Streptomyces sp. N174 Chitosanase*

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Based on the crystal structure of chitosanase from Streptomyces sp. N174, we have calculated theoretical $pK_a$ values of the ionizable groups of this protein using a combination of the boundary element method and continuum electrostatics. The $pK_a$ value obtained for Arg205, which is located in the catalytic cleft, was abnormally high (>20.0), indicating that the guanidyl group may interact strongly with nearby charges. Chitosanases possessing mutations in this position (R205A, R205H, and R205Y), produced by Streptomyces lividans expression system, were found to have less than 0.3% of the activity of the wild type enzyme and to possess thermal stabilities 4–5 kcal/mol lower than that of the wild type protein. In the crystal structure, the Arg205 side chain is in close proximity to the Asp145 side chain (theoretical $pK_a$, -1.6), which is in turn close to the Arg200 side chain (theoretical $pK_a$, 17.7). These theoretical $pK_a$ values are abnormal, suggesting that both of these residues may participate in the Arg205 interaction network. Activity and stability experiments using Asp145- and Arg200-mutated chitosanases (D145A and R190A) provide experimental data supporting the hypothesis derived from the theoretical $pK_a$ data and prompt the conclusion that Arg205 forms a strong interaction network with Asp145 and Arg190 that stabilizes the catalytic cleft.

Most glycosyl hydrolases possess a carbohydrate in their catalytic center that acts as a proton donor in the catalytic process cooperating with another acidic amino acid residue. In chitinolytic enzymes, which hydrolyze β-1,4-linked polysaccharides of N-acetylglucosamine or GlcN, the catalytic carboxylic acids have been identified (1–3), and their environments within the crystal structures discussed (4, 5). The carboxylate acting as the proton donor is usually in a specific environment, favorable to efficient proton donation; for example, in hen egg white lysozyme, Glu35, which acts as the proton donor, is in a hydrophobic environment because of the van der Waals’ contacts with the indole group of Trp108 (6). This hydrophobic environment results in Glu35 possessing a $pK_a$ value (6.3) higher than normal and creates a widely extended bell shape profile in the pH activity relationship (7). Clearly, the interaction of the proton donor carboxylate with other amino acid residues is a very important structural factor in determining the maximum activity of glycosyl hydrolases.

Chitosanase from Streptomyces sp. N174 has been extensively characterized in terms of both its structure and function (8). The chitosanase substrate is a linear polysaccharide of GlcN, which exhibits a polycationic (basic) property. Consequently, it is important to investigate the electrostatic properties of the chitosanase because the acidic side chain of the enzyme might participate in the substrate recognition as well as in the catalysis. In this particular enzyme, Glu22 was found to act as a proton donor, in cooperation with Asp22, thereby activating the water molecule to attack the anomeric carbon of the glucosamine residue in substrate (2, 9). In the crystal structure of the chitosanase deduced by Marcotte et al. (10), these two carboxylates are 13.8 Å apart, indicating that the catalytic reaction takes place through an inverting mechanism (9, 11). However, as is observed with hen egg white lysozyme, the proton donor Glu22 might work independently but instead require some assistance derived from interactions with other amino acids. In fact, the carboxylate of Glu22 is in close proximity to the guanidyl group of Arg205 (see Fig. 1). To better understand the catalytic mechanism of this enzyme, we have examined in detail the environment of Glu22.

When an ionizable side chain interacts with nearby charges, this interaction should affect the $pK_a$ value of the said ionizable group. Thus, a shift in the $pK_a$ value of an ionizable side chain in protein from that of a model compound of the corresponding amino acid reflects the influence of the environment on the ionizable group. In recent years, theoretical methods for calculating the $pK_a$ values of the ionizable amino acid residues in proteins have been developed by several investigators (12–16). Most of these methods are based on the finite difference method for the calculation of electrostatic potentials in and around solvated proteins and have been improved by modifications to more accurately calculate the $pK_a$ values. Recently, Juffer et al. (17–19) have developed a novel method for calculating $pK_a$ values using the boundary element method in combination with continuum electrostatics. This method yielded theoretical $pK_a$ values very close to those obtained experimentally. For example the calculated and experimental $pK_a$ values for hen egg white lysozyme have a correlation efficient of 0.97 (19). Based on this result, the theoretical calculation of the $pK_a$ values of the ionizable groups in chitosanase should provide useful information on their environment.

Intramolecular interactions in proteins can also be studied...

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by unfolding experiments comparing wild type and mutated proteins. The thermal unfolding of Streptomyces sp. N174 chitosanase has been investigated using CD and fluorescence spectroscopy (20) and led to the observation that mutagenesis of the tryptophan residues that participate in the hydrophobic interaction in the core structure not only destabilizes the protein but also enhances the formation of molten globules in its unfolding process. Such an interesting phenomenon in the unfolding process might afford significant information on the intramolecular interactions of the chitosanase.

In this study, we attempt to identify the amino acid residue that makes the largest contribution to the catalytic activity of Glu22 in Streptomyces sp. N174 chitosanase. We initially calculate the pKa values of the ionizable amino acid residues using the method reported by Juffer et al. (19) and then use this data in conjunction with the crystal structure to select a site to mutate. Examination of the enzymatic activities and stabilities of the mutated chitosanases leads us to conclude that Arg205 is essential for sustaining the catalytic potential of Glu22 in this chitosanase.

### EXPERIMENTAL PROCEDURES

#### Materials—
Wild type chitosanase from Streptomyces sp. N174 was purified as described previously (21, 22). Glucosamine oligosaccharides ([GlcN]n) (n = 3–6) were purchased from Calbiochem-Novabiochem Co.

#### Theoretical Calculation of pKa Values of Ionizable Groups in the Chitosanase—
pKa values were calculated by the method reported by Juffer et al. (19). The structure of the chitosanase was taken from the Brookhaven Protein Data Bank (chain A from entry 1CHK) (10). A surface enclosing the protein is described as a triangulated surface and acts as a dielectric boundary such that mutual polarization effects between the solvent and the protein are included. The surface roughly follows the solvent-accessible surface of the protein. The triangulation algorithm employed in this work is described elsewhere (17). Partial charges were taken from Gromacs (23). Calculations were performed with an ionic strength of 0.15 M, the dielectric constant of the protein and 78.5 for the solvent.

#### Site-directed Mutagenesis—
The mutations were introduced into the csn gene using the Altered Site System (Promega, Madison, WI) in S. lividans Hopwood (John Innes Institute, Norwich, UK). The vector pALTER-1 gyrA96, Dson, WI) was subcloned in pFD666 vector, and transformed into Streptomyces sp. N174 TK24 was kindly provided by D. A. Hopwood (John Innes Institute, Norwich, UK). The vector pALTER-1 (Promega) was used for site-directed mutagenesis, whereas the shuttle vector pFD666 was used for expression of wild type and mutated genes in S. lividans as described previously (24).

#### Site-directed Mutagenesis—
The mutations were introduced into the csn gene using the Altered Site System (Promega, Madison, WI) in which we subcloned the wild type chitosanase gene in pALTER-1, creating pALTER-son (2). The mutagenic oligonucleotides, CTGGTGCTCCACGGCGCTGGTGTCG for R205A, GGTGTCCACGTGGCT-creating pALTER- gene using the Altered Site System (Promega, Madison, WI) in

#### Thermal Unfolding Experiments—
Thermal unfolding curves of the chitosanases were experimentally obtained in 50 mM sodium phosphate buffer, pH 7.0, using a Jasco J-720 spectrophotometer (cell length, 0.1 cm). The CD value at 222 nm was monitored while raising the temperature of the solution at a rate of 1 °C/min. The temperature was directly calculated by HPLC using a gel filtration column of TSK-GEL G3000 (25). The enzymatic activity on glucosamine hexasaccharide ([GlcN]6) was determined by HPLC using a gel filtration column of TSK-GEL G3000 (25).

#### Steady State Kinetics—
Kinetic constants (kcat and km) were obtained from Eadie-Hofstee plots of the initial velocities as the increase in reducing sugar concentration and the concentration (µM/ml) of substrate chitosan.

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#### Analysis of the Unfolding Curves—

#### Calculated pKa values of the ionizable side chains of Streptomyces sp. N174 chitosanase

| pKa | Residue | pKa | Residue |
|-----|---------|-----|---------|
| 3.7 | Glu171  | 3.7 | Glu172 |
| 4.5 | Glu16  | 4.5 | Glu162 |
| 4.8 | Glu162 | 4.8 | Glu163 |
| 4.8 | Glu163 | 4.8 | Glu164 |
| 5.1 | Glu17  | 5.1 | Glu171 |
| 6.2 | Glu172 | 6.2 | Glu173 |
| 6.3 | Glu173 | 6.3 | Glu174 |
| 7.1 | His8  | 7.1 | His9  |
| 8.6 | His9  | 8.6 | His10 |
| 10.1 | Lys85 | 10.1 | Lys86 |
| 12.7 | Lys81 | 12.7 | Lys82 |
| 10.6 | Lys82 | 10.6 | Lys83 |
| 11.0 | Lys83 | 11.0 | Lys84 |
| 13.2 | Lys89 | 13.2 | Lys90 |
| 13.5 | Lys90 | 13.5 | Lys91 |
| 13.2 | Lys91 | 13.2 | Lys92 |
| 13.0 | Lys92 | 13.0 | Lys93 |
| 11.0 | Lys93 | 11.0 | Lys94 |
| 16.2 | Tyr35 | 16.2 | Tyr36 |
| 16.4 | Tyr36 | 16.4 | Tyr37 |
| 12.1 | Tyr37 | 12.1 | Tyr38 |

The abbreviations used are: (GlcN)n, β-1,4-linked oligosaccharide of GlcN with a polymerization degree of n; HPLC, high pressure liquid chromatography.
ence in $T_m$ between the mutant and wild type proteins and $\Delta S_m$ (wild type) is the entropy change of the wild type protein at $T_m$. The unfolding curves obtained by CD were analyzed by a three-state transition model, and the thermodynamic parameters were calculated as described above for the individual transition phases (20).

Molecular Modeling of Mutated Chitosanases—Molecular modeling of mutant chitosanase was performed on a Silicon Graphics (Personal Iris) station using SYBYL molecular modeling software version 5.4. Total energy (bond stretching energy, angle bending energy, out of plane bending, van der Waals’, and electrostatic energy) was calculated for the whole molecule. The conformation of the mutated chitosanase molecule was modeled by local energy minimization.

RESULTS AND DISCUSSION

Theoretical Calculation of $pK_a$ Values—Theoretical $pK_a$ values of the ionizable side chains of Streptomyces sp. N174 chitosanase were deduced using the method of Juffer et al. (19). To date, most theoretical calculations of protein $pK_a$ values have been done for relatively small proteins such as lysozyme, ribonuclease, and trypsin inhibitor. Here we report the successful calculation of $pK_a$ values for chitosanase, a protein of 26 kDa. To our knowledge, this is the largest protein for which $pK_a$ values have been calculated using the boundary element method (28). The calculated $pK_a$ values are listed in Table I.

The calculated $pK_a$ value for Arg$_{205}$ is greater than 20.0, a result that usually indicates that the side chain is involved in a strong interaction with nearby charges (19, 29). Examination of the crystal structure indicates that Glu$_{22}$ is located at about 3 Å from Arg$_{205}$ (Fig. 1), and could possibly interact with Arg$_{205}$ (10). However, the calculated $pK_a$ value for Glu$_{22}$ is normal (3.4), and mutation of Glu$_{22}$ has been shown to have little effect on the stability of the chitosanase (29). Therefore, it is unlikely that the Arg$_{205}$ side chain interacts with that of Glu$_{22}$. A closer examination of the crystal structure indicates that Glu$_{22}$ is not only close to Arg$_{205}$ but also to a hydrophobic residue, Val$_{14}$. It is a generally accepted rule that a hydrophobic environment can raise the $pK_a$ of an acidic residue. This might compensate for the effect of the proximity of Arg$_{205}$, which would tend to lower the $pK_a$. Thus, the normal $pK_a$ value calculated for Glu$_{22}$ might be the result of this polar-apolar environment.

In hen egg white lysozyme, an abnormal $pK_a$ value of 6.3 was experimentally obtained for Glu$_{25}$, which acts as the proton donor (6, 7), and this is regarded as an important factor in this enzyme ability to retain its activity over a wide pH range. As described above, the corresponding proton donor carboxylate, Glu$_{22}$, has a normal theoretical $pK_a$ value of 3.4 in chitosanase. The pH activity profile of chitosanase (21) indicates that the enzyme is quite active even at higher pH values (~6.0), suggesting that the actual $pK_a$ value of the proton donor carboxylate should be higher than the theoretical $pK_a$ value of 3.4. The theoretical $pK_a$ values were obtained on the basis of the crystal structure of the free enzyme. However, the $pK_a$ value for the catalytic carboxylate estimated from the experimental pH activity profile is that for the chitosanase-substrate complex in which the conformation of the catalytic cleft is altered from that of the free enzyme because of substrate binding. In fact, the distance between Glu$_{22}$ and Asp$_{40}$ is 13.8 Å in the free state but is estimated to be only 12.0 Å in the complexed structure obtained by energy minimization (10). Such a conformational change induced by substrate binding might increase the hydrophobicity of the environment around the Glu$_{22}$ side chain, thereby raising its $pK_a$, and resulting in the considerable activity observed at higher pH values.

Arg$_{118}$ and Asp$_{119}$ were found to have theoretical $pK_a$ values of >20 and 0.8, respectively, suggesting a strong interaction between these adjacent residues. Similar interactions might exist between His$_9$ (theoretical $pK_a$ of 10.3) and the C terminus (theoretical $pK_a$ of 0.8) and between Tyr$_{143}$ (theoretical $pK_a$ of >20.0) and Asp$_{133}$ (theoretical $pK_a$ of 1.2); however, these interactions are not directly involved in catalytic activity. For this reason, we decided to focus our attention on Arg$_{205}$, which is considered to be directly involved in the catalytic activity.

Activity of the Arg$_{205}$-mutated Chitosanases—To investigate the role of Arg$_{205}$ in chitosanase activity, we examined the enzymatic activities of four chitosanases mutated in this residue (R205H, R205Y, R205A, and R205K). Initially, we examined the in vivo activity of S. lividans clones transformed with mutated chitosanase genes by the formation of clearing halos on chitosan medium (Fig. 2). All S. lividans clones carrying the Arg$_{205}$-mutated genes showed no halo (colonies 2, 3, 7, and 8), whereas that carrying the wild type gene formed a wide and clear halo (colony 1). Next, we tried to produce these mutant chitosanases using 1% glucosamine liquid medium. R205A, R205H, and R205Y could be produced and purified by the method reported previously (2) but not R205K. The R205K-mutated enzyme was also produced at levels comparable with the other mutants but could not be purified because of its high tendency to precipitate at the moderately acidic pH levels used in the purification procedure. Most likely, the protein was misfolded, thereby exposing hydrophobic amino acids at the molecular surface.

When the enzymatic activities were determined using the purified enzymes and chitosan as substrate, only R205A was found to have a measurable level of activity. Its $K_m$ and $k_{cat}$ values were determined to be 0.014 (wild type, 0.029) mg/ml and 0.83 (wild type, 727.5) min$^{-1}$, respectively. The overall activity ($k_{cat}/K_m$) of R205A was 0.24% that of the wild type enzyme. Similar results were obtained using (GlcN)$_n$ as substrate. The reaction time course profiles are shown in Fig. 3. The relative activity of R205A toward (GlcN)$_n$ was 0.16% of that of the wild type enzyme. As observed with chitosan, both R205H and R205Y showed no activity with (GlcN)$_n$. On the basis of these results, we conclude that Arg$_{205}$ is essential for the activity of the chitosanase.

Thermal Stabilities of the Arg$_{205}$-mutated Chitosanases—Thermal unfolding curves of the mutated chitosanases, obtained by monitoring CD at 222 nm are shown in Fig. 4A. The individual profiles were biphasic showing a first cooperative phase followed by a second phase that is less cooperative. Each
arginine mutation was found to greatly lower the transition temperature of the first transition phase and to enhance the formation of the intermediate state. Unfolding curves were also obtained by monitoring tryptophan fluorescence, and the results are shown in Fig. 4. These unfolding profiles differ from those obtained by CD and agree with theoretical curves obtained with the three-state transition model. At 38 °C, the mutant chitosanases were completely unfolded as judged from their tryptophan environments (Fig. 4B), whereas 25–35% of the total α-helices were still folded as observed in Fig. 4A, indicating that the intermediate is in a molten globule-like structure. These phenomena are very similar to those obtained with tryptophan-mutated chitosanases (20), for which the unfolding data were successfully explained using a three-state transition mechanism. For this reason, we tried to analyze our data using a three-state model identical to that reported by Honda et al. (20). The theoretical unfolding curves that best agreed with the experimental ones are represented by the solid lines in Fig. 4A, and the corresponding thermodynamic parameters are listed in Table II. The loss of stability is about 5 kcal/mol for each mutant chitosanase in the first transition phase, a value that cannot be explained by disruption of a single intramolecular interaction. It is likely that the Arg205 mutation results in the disruption of a strong interaction network formed by the Arg205 side chain, in agreement with the explanation for the theoretical pKₐ value for Arg²⁰⁵.

Restoration of the Stability of the Mutant Chitosanase by Oligosaccharide Binding—Honda et al. (29) reported that the Tₘ value of an inactive mutant chitosanase increases when individual GlcN oligosaccharides are added to the enzyme solution. The extent of this increase is essentially proportional to the length of the oligosaccharide added. When we performed

ARG. 205 in Streptomyces sp. N174 Chitosanase
Table II
Thermodynamic parameters for unfolding transition of wild type and Arg205-mutated chitosanases

| Enzyme | Tm (°C) | ΔTm (°C) | ΔHm (kcal/mol) | ΔSm (kcal/mol/°C) | ΔGm (kcal/mol) | Tm (°C) | ΔTm (°C) | ΔHm (kcal/mol) | ΔSm (kcal/mol/°C) | ΔGm (kcal/mol) |
|--------|---------|----------|----------------|------------------|----------------|---------|----------|----------------|------------------|----------------|
| R205Y  | 32.8    | -11.2    | 160            | 0.523            | -5.2          | 41.0    | -10.5    | 87             | 0.277            | -3.6           |
| R205H  | 32.0    | -11.5    | 145            | 0.475            | -5.4          | 42.2    | -9.3     | 85             | 0.270            | -3.2           |
| R205A  | 34.0    | -9.2     | 145            | 0.471            | -4.3          | 44.2    | -7.3     | 65             | 0.200            | -2.5           |
| Wild type | 44.0    | 0        | 148            | 0.467            | 0             | 51.5    | 0        | 110            | 0.339            | 0              |

Interaction Network Formed by Arg205—A closer examination of the chitosanase x-ray crystal structure was made to further understand the interaction network of Arg205. As described above, the catalytic residue Glu22 is located very close to the Arg205 side chain (Fig. 1) but is not involved in the unusually strong interactions of Arg205. Conversely, the Arg205 side chain is about 4 Å from that of Asp145 (Fig. 6A), and the theoretical pK_a value for Asp145 was found to be -1.6 (Table I), an abnormal negative value. This indicates a strong interaction, such as a buried salt bridge, between Arg205 and Asp145.

An amino acid sequence alignment (Fig. 7) of known chitosanases belonging to the family 46 of glycosyl hydrolases (31) reveals that the arginine residue at position 205 and the aspartic acid at position 145 are conserved in all chitosanases, strongly suggesting that these two residues interact through an electrostatic interaction. In fact, an S. lividans clone carrying an Asp145-mutated chitosanase gene (D145A) showed no enzymic activity (colony 5, Fig. 2). This lack of activity may be due to incorrect folding of the mutant protein that results in strong suppression of its secretion. Because the amount of D145A produced in liquid medium was extremely low, the thermal stability of the mutant could not be tested. Extensive efforts to produce and purify D145A yielded only sufficient protein for the activity determination, which was found to be negligible.

Upon further examination of the x-ray crystal structure, we noticed a close proximity between Asp145 and Arg190 (Fig. 7), which is considerably higher than normal for model arginine compound (12.0). This arginine residue is conserved for all family 46 chitosanases sequenced to date (Fig. 7); therefore, it was mutated to alanine (R190A), and the activity and stability of the resulting mutant were determined. The S. lividans transformant containing the R190A gene exhibited a small but clear halo on chitosan medium (colony 6, Fig. 2). The k_cat and K_m values using chitosan as substrate were determined to be 0.57 (wild type, 727.5), 0.85 (wild type, 0.029), respectively, whereas the overall activity (k_cat/K_m) was found to be very low (0.003% of that of the wild type). The T_m value as determined from the unfolding experiment by CD was 37.7 °C, i.e. 6.3 °C lower than that of the wild type enzyme. The effect of this mutation on the stability is not as strong as in the case of the Arg205 mutation, suggesting that Arg190 likely participates in the interaction network, but might be located at the terminal end of the network. These results support the idea that Arg205, Asp145, and Arg190 participate in the interaction network at the catalytic cleft of the chitosanase. These three residues belong to three different α-helices (yellow regions in Fig. 7), suggesting an important role of the interaction network in maintaining the stability of chitosanase. Molecular modeling of the Arg205 mutation to alanine showed an additional interesting phenomenon; that is, after energy minimization, Asp145 completely changed its position and could no longer interact with Arg190.

The mutation to alanine at position 205 is at the origin of a cascade of events; specifically, once the link between Arg205 and Asp145 is broken, the Asp145-Arg190 linkage is coincidently disrupted an interaction network composed of a couple of intramolecular interactions involving the Arg205 side chain. This interaction network likely stabilizes the proper conformation of the catalytic cleft.
broken. This explains why a single mutation in Arg^{205} causes an extraordinarily large change in thermal stability. The disruption of the interactions between the three \( \alpha \)-helices might reduce the cooperativity of the unfolding transition, accumulating the molten globule-like intermediates observed in the unfolding pathway (Fig. 4).

Similar Arginine Residues in the Catalytic Cleft in Other Related Enzymes—Based on their three-dimensional structures, it has been proposed by Monzingo et al. (33) that \textit{Streptomyces} sp. N174 chitosanase, phage T4 lysozyme, barley chitinase, and goose egg white lysozyme belong to the same structural superfamily. Recently, Saito et al. (34) reported the crystal structure of chitosanase from \textit{Bacillus circulans} MH-K1, which exhibits a very close similarity to that of \textit{Streptomyces} sp. N174 chitosanase and should therefore be included in the superfamily proposed by Monzingo et al. (33). Comparison between the crystal structures of these two chitosanases indicates that \textit{B. circulans} chitosanase has a similar orientation of the Arg-Asp-Arg network in the catalytic cleft (Fig. 6B). Arg^{205}, Asp^{145}, and Arg^{190} of the \textit{Streptomyces} enzyme are replaced by Arg^{228}, Asp^{172}, and Arg^{210} in the \textit{B. circulans} enzyme; hence, the network is completely conserved in the two chitosanases. When the structure of phage T4 lysozyme (35) is superimposed on that of \textit{Streptomyces} sp. N174 chitosanase (33), the Arg^{145} residue of T4 lysozyme occupies a position analogous to that of Arg^{205} of the chitosanase, whereas Asn^{101} of the lysozyme can correspond to Asp^{145} of the chitosanase (Fig. 6C). At least a part of the network is therefore conserved between the chitosanase and T4 lysozyme. In barley chitinase (36), Arg^{215} is very close to the catalytic residue Glu^{67} and also interacts with Glu^{203} (Fig. 6D). Both Arg^{215} and Glu^{203} are completely conserved in all known chitinases of plant or bacterial origin belonging to family 19 of glycosyl hydrolases (not shown). The interaction between Arg^{215} and Glu^{203} is very similar to the

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**Table III**

| Enzyme | First transition phase | Second transition phase |
|--------|------------------------|------------------------|
|        | \( T_m \) | \( \Delta T_m \) | \( \Delta H_m \) | \( \Delta S_m \) | \( \Delta G_m \) | \( T_m \) | \( \Delta T_m \) | \( \Delta H_m \) | \( \Delta S_m \) | \( \Delta G_m \) |
| R205Y  | 32.8 | -11.2 | 160 | 0.523 | -5.2 | 41.0 | -10.5 | 87 | 0.277 | -3.6 |
| R205Y + (GlcN)\textsubscript{3} | 37.5 | -6.5 | 130 | 0.419 | -3.0 | 47.2 | -4.3 | 133 | 0.415 | -1.5 |
| R205Y + (GlcN)\textsubscript{4} | 39.0 | -5.0 | 120 | 0.385 | -2.3 | 47.6 | -3.9 | 140 | 0.437 | -1.3 |
| R205Y + (GlcN)\textsubscript{5} | 44.7 | -0.7 | 130 | 0.409 | 0.3 | 51.3 | -0.2 | 135 | 0.416 | -0.1 |
| R205Y + (GlcN)\textsubscript{6} | 43.8 | 0.2 | 145 | 0.458 | -0.1 | 50.0 | -1.5 | 122 | 0.378 | -0.5 |
| Wild type | 44.0 | 0 | 148 | 0.467 | 0 | 51.5 | 0 | 110 | 0.339 | 0 |

The values were obtained from experimental unfolding curves shown in Fig. 5.
Arg205-Asp145 interaction in Streptomyces sp. N174 chitosanase. However, the Glu67-Arg215-Glu203 network of barley chitinase could not be superimposed on the Glu22-Arg205-Asp145 network in Streptomyces sp. Chitosanase, indicating that the former is not structurally equivalent to the latter, although it is probably functionally equivalent. Chen et al. (37) reported that a tetrad of ionizable amino acids is important for catalysis in barley β-glucanase. Glu288 is the catalytic residue of the enzyme, and the tetrad consists of Glu231, Glu279, Lys282, and Glu288. Lys282 and Glu279 form an ion pair or salt bridge, and the dipole is located close to the catalytic residue, Glu288. Therefore, the Lys282-Glu279 dipole of the β-glucanase is analogous to the Arg205-Asp145 dipole in Streptomyces sp. N174 chitosanase. Clearly, the presence of an arginine residue near the catalytic carboxylate seems to play an important role in the catalytic reaction of various glycosyl hydrolases.

In conclusion, theoretical pKa calculations based on the method of Juffer et al. (19) yield useful information on the states of the ionizable groups in proteins. When applied to Streptomyces sp. N174 chitosanase, the calculated pKa values agree well with the results obtained by activity determinations and unfolding experiments. Both the theoretical and experimental data strongly suggest that Arg205 plays a very important role in stabilizing the catalytic cleft of Streptomyces sp. N174 chitosanase.

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