Left-Sided Substrate Binding of Lysozyme: Evidence for the Involvement of Asparagine-46 in the Initial Binding of Substrate to Chicken Lysozyme†

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ABSTRACT: The "right-sided" and "left-sided" substrate binding modes at the lower saccharide binding subsites (D–F sites) of chicken lysozyme were investigated by utilizing mutant lysozymes secreted from yeast. We constructed the following mutant lysozymes; "left-sided" substitution of Asn46 to Asp, deletion of Thr47, and insertion of Gly between Thr47 and Asp48 and "right-sided" substitution of Asn37 to Gly. Analyses of their activities and substrate binding abilities showed that Asn46 and Thr47 are involved in the initial enzyme–substrate complex and Asn37 is involved in the transition state. These results support an earlier proposal that interactions between substrate and residues at the left side of lysozyme stabilize a catalytically inactive enzyme–substrate complex, while interactions between substrate and residues at the right side stabilize the catalytically active complex [Pincus, M. R., & Scheraga, H. A. (1979) *Macromolecules 12,* 633–644]. These results are also consistent with the proposed kinetic mechanism for lysozyme reaction that the rearrangement of an initial enzyme–substrate complex (β-complex) to another complex (γ-complex) is required for catalytic hydrolysis [Banerjee S. K., Holler, E., Hess, G. P., & Rupley, J. A. (1975) *J. Biol. Chem. 250,* 4355–4367].

From the crystal structures of hen egg white lysozyme (chicken lysozyme) and of various lysozyme–inhibitor complexes, it has been suggested that the active site can accommodate six saccharide units of polymeric substrate, designated subsites A, B, C, D, E, and F, and that only those saccharide molecules whose pyranose rings make contact with subsites D and E are catalytically hydrolyzed (Blake et al., 1967a,b). In these studies, only subsites A, B, and C have been directly determined from the crystal structure of the stable complex between lysozyme and a trimer of *N*-acetyl-D-glucosamine, $(NAG)_3$,[1] but subsites D, E, and F have been predicted from model building. In the proposed substrate binding mode, the D-site saccharide residue is distorted and E- and F-site saccharide residues extended below to make contact with such residues as Phe34, Asn37, and Arg114. On the other hand, Pincus and Scheraga (1979) have predicted on the basis of conformational energy calculations that there are two distinct binding modes, "left-sided" and "right-sided" modes, and that the former is more stable than the latter (Figure 1). In both modes, subsites A–C are quite similar to those determined by the crystallographic studies. As for subsite D–F, however, in the left-sided binding mode, the D-site saccharide residue is not distorted and the F-site residue contacts with such residues as Arg45, Asn46, and Thr47, while the right-sided binding mode is similar to the one proposed on the basis of X-ray crystallographic studies.

In extensive studies of the kinetics and thermodynamics of the interactions of lysozyme with the $\beta(1\text{–}4)$-linked hexamer of *N*-acetyl-D-glucosamine, $(NAG)_6$, it has been found that two types of equilibrium complexes are formed between lysozyme and $(NAG)_6$ (Banerjee et al., 1975). The first one is a "nonproductive" complex (also called α-complex) in which only three terminal saccharide residues bind to subsites A–C, and the other is a "productive" complex in which all six saccharide residues make contact with the enzyme (β-complex). The latter complex is shown to rearrange to another "productive" complex (also referred to as γ-complex) from which catalytic hydrolysis occurs (Banerjee et al., 1975). In the case of high polymer substrates such as glycol chitin (Yamada & Imoto, 1981), the formation of the α-complex (nonproductive complex) could be neglected in the kinetics because the concentration of the terminal end of substrate is very low compared with that of the polymer interior. Smith-Gill et al. (1984) have indicated that ring-necked pheasant lysozyme, in which Lys and His replace Asn113 and Arg114, respectively, has the same affinity for $(NAG)_6$ as does chicken lysozyme and that a monoclonal antibody, which binds specifically to an epitope including residues Arg45, Asn46, Thr47, Asp48, and Arg68 on the far left side of hen egg white lysozyme, is competitively displaced by $(NAG)_5$ and $(NAG)_6$. From these observations, they concluded that, in an equilibrium binding of lysozyme to polymer substrate, the right-sided binding mode is not involved but the left-sided one is. On the other hand, Muraki et al. (1989) have shown that the mutation of Arg115 in human lysozyme (corresponding Arg114 in chicken lsyozyme), a right-sided residue, affects the cleavage patterns of $(NAG)_6$. These results seem to suggest a mechanism for the lysozyme reaction in which high polymer substrate initially binds to the left-sided binding site of lysozyme, next rearranges to the right-sided one, and then is catalytically hydrolyzed.

In this paper, we prepared several mutant lysozymes in which mutations are introduced in the left- or right-sided binding site and examined the above mechanism in detail. The mutant lysozymes used were those in which Asn46 is replaced by Asp (N46D), Thr47 is deleted (47del), Gly is inserted between Thr47 and Asp48 (47'G), and Asn37 is

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[1] Abbreviations: NAG, *N*-acetyl-D-glucosamine; $(NAG)_n$, a $\beta(1\text{–}4)$-linked *n*-mer of NAG; GdnHCl, guanidine hydrochloride; GC, glycol chitin; HPLC, high-performance liquid chromatography; NAM-NAG-NAM, a $\beta(1\text{–}4)$-linked trisaccharide, *N*-acetyl-D-muramic acid-*N*-acetyl-D-glucosamine-*N*-acetyl-D-muramic acid.

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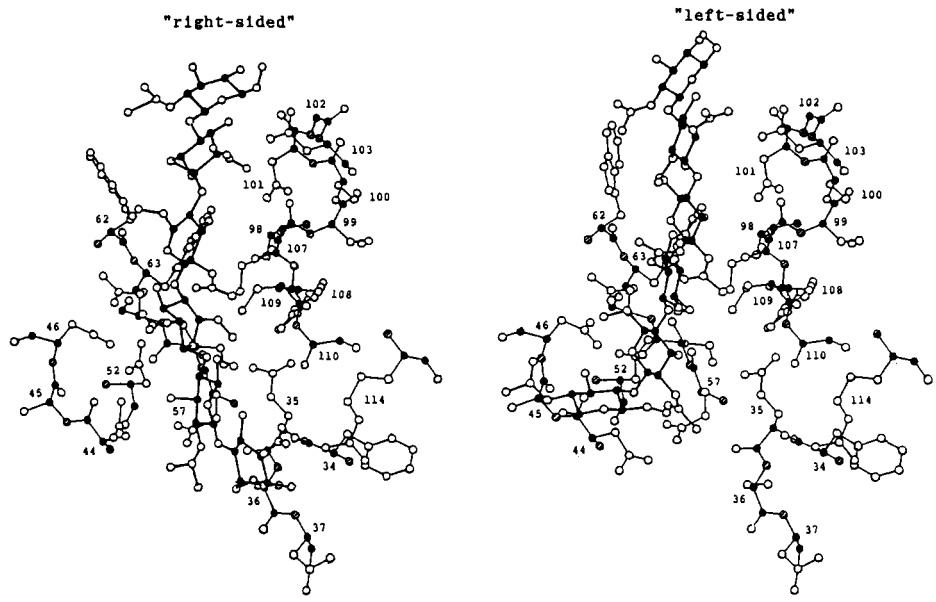


FIGURE 1: Two distinct binding modes between chicken lysozyme and $(NAG)_6$ (Pincus & Scheraga, 1979).

replaced by Gly (N37G). We found that the replacement of Asn46 in the left-sided region by Asp stabilizes the initial enzyme–substrate complex but does not affect the transition state, while the replacement of Asn37 in the right-sided region by Gly does not affect the formation of the equilibrium enzyme–substrate complex but increases the activity. These observations are completely consistent with the above mechanism for lysozyme reaction.

MATERIALS AND METHODS

Materials. Wild-type lysozyme was prepared by expression and secretion from yeast as described previously (Inoue et al., 1992a). CM-Toyopearl 650M, a cation-exchange resin for purification of secreted lysozymes, was obtained from Tosoh (Tokyo). A cation-exchange column of Asahipak ES-502C (7.8 × 100 mm) was obtained from Asahi Kasei Co. (Tokyo). *Micrococcus luteus*, a substrate of lysozyme, was from Sigma. Glycol chitin, a synthetic substrate of lysozyme (Yamada & Imoto, 1981), and chitin-coated Celite, an affinity adsorbent for lysozyme (Yamada et al., 1985a), were prepared as described elsewhere. A β(1–4)-linked trimer of *N*-acetyl-D-glucosamine, $(NAG)_3$, was prepared according to the method of Rupley (1964). All other chemicals were of analytical grade for biochemical use.

Mutant Lysozymes. Mutations were introduced into the chicken lysozyme gene by site-directed mutagenesis as described previously (Miki et al., 1987). The structures of the mutagenic primers used for the replacement of Asn46 by Asp (N46D), the deletion of Thr47 (47del), the insertion of Gly between Thr47 and Asp48 (47'G), and the replacement of Asn37 by Gly (N37G) were 5'-CAAACCGTGACACCATGGATCCACCGACTAG-3', 5'-CAAACCGTAACGATGGTTCCACCG-3', 5'-CAAACCGTAACACCGGTGATGGTTCCACCG-3', and 5'-CAAAATTCGAATCCGGATTCAACACC-3', respectively. The mutations were verified by dideoxy sequencing as described previously (Miki et al., 1987).

The expression and secretion of mutant lysozymes from yeast as mature forms were carried out according to a method described previously (Inoue et al., 1992a), using a yeast *Saccharomyces cerevisiae* strain AH22 (Hinnen et al., 1978) as host, a plasmid pAM82 (Miyanohara et al., 1983) as an expression vector, and the yeast invertase signal peptide as a secretion-promoting signal (Taussig et al., 1983; Chang et al., 1986). Briefly, yeast *S. cerevisiae* AH22 cells harboring pAM82 derivative, which carries DNA coding for a fused protein between an yeast invertase signal peptide and a mature form of mutant lysozyme inserted in the direction under control of the repressible acid phosphatase promoter (PHO5) of yeast, were cultivated in modified Burkholder minimal media (Toh-e et al., 1973) in the presence of a limited amount of phosphate (0.6 mM KH_2PO_4) at 30 °C for 125 h.

The lysozyme secreted in the culture supernatant was isolated by cation-exchange chromatography on a column (1.3 × 40 cm) of CM-Toyopearl 650M which was eluted with a gradient of 500 mL of 0.05 M phosphate buffer and 500 mL of the same buffer containing 0.5 M NaCl at pH 7 and 4 °C. The lysozyme thus eluted was collected, dialyzed against distilled water, and then lyophilized.

Analytical Methods. Amino acid analysis were performed on a Hitachi 835 amino acid analyzer after hydrolysis of samples in 6 N HCl under vacuum at 110 °C for 20 h. The NH_2-terminal sequences of protein and peptide samples were determined with an Applied Biosystems Model 473A protein sequencer.

Circular dichroism (CD) spectra were measured with a Jasco J-720 spectropolarimeter at 20 °C using 1.3×10^{-5} M protein in 0.01 M potassium phosphate buffer (pH 7).

Digestions of reduced and S-carboxymethylated lysozymes with TPCK-trypsin (Worthington) and separation of the resulting peptides on reversed-phase HPLC were accomplished as described by Yamada et al. (1985b).

Affinity HPLC of lysozymes for the determination of their substrate binding abilities was performed on a chitin-coated Celite column (4 × 100 mm) which was eluted with a gradient of 20 mL of 0.1 M acetate buffer (pH 5.5) and 20 mL of 1 M acetic acid, both containing 0.25 M NaCl, at a flow rate of 0.5 mL/min and 0 °C (Yamada et al., 1985a).

The dissociation constants of lysozymes for the binding to $(NAG)_3$ in 0.1 M acetate buffer at pH 5.5 and 40 °C were determined by UV difference spectroscopy (Dahlquist et al., 1966) with a Hitachi 150-20 double-beam spectrophotometer.

Activity Measurements. Activities of lysozymes against *M. luteus* (lytic activities) were determined turbidimetrically at 450 nm in 0.05 M potassium phosphate buffer at pH 7.0 and 30°C.

Activities of lysozymes against glycol chitin (GC activities) were measured in 0.1 M acetate buffer at pH 5.5 and 40 °C according to the literature (Yamada & Imoto, 1981) with slight modifications to improve the accuracy. That is, 1 mL of glycol chitin in 0.1 M acetate buffer (0.5 mg/mL) was preincubated at 40 °C for 10 min in a stoppered test tube with gentle shaking. To the solution was added 0.1 mL of lysozyme in the same buffer (4–38 μg/mL, depending on the activity); the sample was mixed briefly with a vortex mixer and incubated at 40 °C for 30 min. After the reaction, 2 mL of potassium ferricyanide in 0.5 M sodium carbonate (0.5 g/mL) was added and mixed briefly, and the mixture was incubated at 80 °C for 1 h to complete the oxidation of reducing sugar with ferricyanide. A control solution was obtained by the same procedure except that the lysozyme solution was added after the addition of the ferricyanide solution. After cooling, the decrease in absorbance at 420 nm of the mixture compared with the control solution (ΔA_{420}) was determined. Since the value of ΔA_{420} increased linearly with increase of the amount of lysozyme added up to about 0.3 absorbance unit, the concentrations of mutant lysozymes so as to give $\Delta A_{420} < 0.3$ absorbance unit were employed.

pH dependence of GC activities of wild-type and N46D lysozymes in a pH range from 3.0 to 7.5 at 40 °C and ionic strength 0.1 M were also determined in the same manner. Buffers were prepared by mixing two solutions in various ratios to adjust the desired pH's. The first solution contained 0.05 M acetic acid, 0.0125 M phosphoric acid, and 0.1 M NaCl, and the other contained 0.05 M sodium acetate, 0.0125 M K_2HPO_4, and 0.0125 M NaCl. Activities were expressed relative to wild-type lysozyme at pH 5.5.

By use of various substrate concentrations, apparent k_{cat} and K_m values for GC activity of wild-type or N46D lysozyme at pH 4.0, ionic strength 0.1 M, and 40 °C were also determined by this oxidation method of reducing sugar, assuming the values of ΔA_{420} obtained from 30-min enzymatic reactions to be proportional to the initial rates at the respective concentrations of glycol chitin.

Estimation of the pK_a of Asp46 in N46D Lysozyme by Cation-Exchange HPLC. To detect the difference in net charge between wild-type and N46D lysozymes at pH 4, 5, and 6, their retention times in cation-exchange HPLC on a column of Asahipak ES-502C (7.8 × 100 mm) were compared by a coinjection method. The column was eluted with a gradient formed from 20 mL of 0.1 M acetate buffer and 20 mL of the same buffer containing 1 M NaCl (pH 4 and 5) or a gradient formed from 20 mL of 0.05 M phosphate buffer and 20 mL of the same buffer containing 0.5 M NaCl (pH 6) at a flow rate of 0.5 mL/min.

Spectroscopic Titrations of Glu35 and Asp52. The pK_as of Glu35 and Asp52 of free lysozyme and of its $(NAG)_3$ complex in 0.1 M KCl at 30 °C were determined by a titration curve obtained from the pH dependence of the tryptophyl absorbance [protein concentration 8.4×10^{-5} M in the absence of $(NAG)_3$] at 301 nm and of the tryptophyl fluorescence [protein concentration 1.5×10^{-6} M in the presence of 1.2×10^{-4} M $(NAG)_3$] at 330 nm (excited at 280 nm) as described previously (Inoue et al., 1992a,b).

Unfolding Equilibrium. Unfolding equilibria of lysozymes by guanidine hydrochloride (GdnHCl) were measured at pH 5.5 and 35 ± 0.2 °C by fluorescence at 360 nm (excited at 280 nm) as described previously (Inoue et al., 1992a,b). As for N46D lysozyme, its pH dependence in a pH range of 3.5–5.5 at 35 °C was also determined. The protein concentration was 9.3×10^{-7} M. All buffers used were 0.1 M sodium acetate adjusted to respective pHs (pH 3.5–5.5) with HCl.

Table I: Comparison of Primary Structures of Chicken, Human, and Rat Lysozymes and of Human α-Lactalbumin around the Mutated Residues

	residue number[a]							
	36	37	38	45	46	47	47′	48
chicken lysozyme	Ser	Asn	Phe	Arg	Asn	Thr		Asp
human lysozyme	Ser	Gly	Tyr	Tyr	Asn	Ala	Gly	Asp
rat lysozyme	Ser	Asn	Tyr	Tyr	Asp	Pro	Gly	Asp
human α-lactalbumin	Ser	Gly	Tyr	Glu	Asn			Asn

[a] Numbering is that of chicken lysozyme.

RESULTS

Preparations of Mutant Lysozymes. Mutant lysozymes were prepared by utilizing a yeast expression and secretion system described previously (Inoue et al., 1992a). In order to avoid global conformational changes due to the introduction of mutations, the mutations that would influence the substrate binding ability of lower subsites E and F of lysozyme at either the left or right side were chosen on the basis of comparison of primary sequences of c-type lysozymes (c-lysozymes) and an α-lactalbumin, both of which compose the c-lysozyme superfamily (Jolles & Jolles, 1984; Nitta & Sugai, 1989; also see Table I). One mutant lysozyme, N46D lysozyme, was selected because Asn46, a left-sided residue, is a highly conserved residue but in rat lysozyme it is replaced by Asp. Similarly, 47del and 47′G lysozymes were chosen as left-sided mutants, because human α-lactalbumin has a deletion at position 47 and human and rat lysozymes have an insertion of Gly between positions 47 and 48. As a right-sided mutant, N37G lysozyme was chosen because Asn37 is replaced by Gly in human lysozyme.

Each mutant lysozyme was secreted from yeast in the medium by 125-h cultivation of yeast *S. cerevisiae* AH22 cells harboring a pAM82 derivative that carries DNA coding for a fused protein between an yeast invertase signal peptide and a mature form of mutant lysozyme at 30 °C (Inoue et al., 1992a). The amounts of mutant lysozymes secreted in the media were found to be 1.6, 0.7, 2.0, and 4.2 mg/L for N46D, 47del, 47′G, and N37G lysozymes, respectively, while 5.0 mg/L wild-type lysozyme was secreted under the same conditions. Each mutant lysozyme was purified from the culture supernatant by cation-exchange chromatography.

The NH_2-terminal amino acid sequences of the purified enzymes were all identical to that of native lysozyme obtained from hen egg white as before (Inoue et al., 1992a,b). Their amino acid compositions were all consistent with those expected from the mutations introduced (data not shown). In order to confirm further the mutations in the respective mutant lysozymes, they were reduced, S-carboxymethylated, digested with TPCK-trypsin, and subjected to peptide mapping on reversed-phase HPLC. In Figure 2, the elution patterns of the peptides obtained from mutant lysozymes as well as that from wild-type lysozyme are shown. Clearly, only the peptides containing designed mutations showed the altered elution positions. The amino acid compositions and amino acid sequences of these peptides were completely consistent with those expected (data not shown). Thus, we concluded that the lysozymes secreted from yeast were all those having the correct NH_2 termini and the respective mutations as designed.

Properties of Mutant Lysozymes. As shown in Table II, none of the mutant lysozymes prepared here was completely devoid of activity, suggesting that no large global conformation changes were induced by the introduction of the present

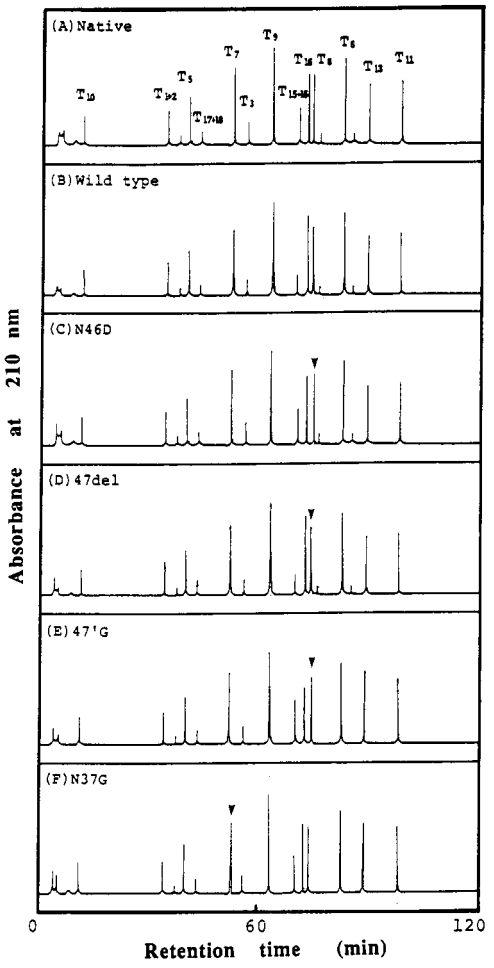


FIGURE 2: Reversed-phase HPLC of tryptic peptides obtained from reduced and S-carboxymethylated lysozymes on a column (4 × 250 mm) of Wakopak 5C18. The column was eluted with a gradient formed from 50 mL of 1% acetonitrile and 50 mL of 50% acetonitrile, both containing 0.1% concentrated HCl at a flow rate of 0.6 mL/min. (A) From native lysozyme; (B) from wild-type lysozyme; (C) from N46D lysozyme; (D) from 47del lysozyme; (E) from 47'G lysozyme; (F) from N37G lysozyme. T refers to the tryptic peptides and peptide numbering is from the N-terminal peptide. The arrows represent the peptides containing mutations.

Table II: Comparisons of Some Properties of Wild-Type and Mutant Chicken Lysozymes

	activity (%)		substrate binding		stability
lysozyme	lysis[a]	GC[b]	K_d (10^{-5} M) for $(NAG)_3$[c]	rel retention time[d]	$C_{1/2}$[e] (M)
wild type	100	100	1.4	1	3.61
N46D	27.6	8.8	1.3	1.4	3.30
47del	57.8	12.6	2.0	0.88	2.82
47'G	110.1	30.6	1.4	1	3.25
N37G	122.2	125.3	1.5	1	3.52

[a] Against *M. luteus* in 0.05 M phosphate buffer at pH 7.0 and 30 °C. [b] Against glycol chitin in 0.1 M acetate buffer at pH 5.5 and 40 °C. [c] Dissociation constant of the complex between lysozyme and $(NAG)_3$ in 0.1 M acetate buffer at pH 5.5 and 40 °C. [d] Relative retention time in the affinity HPLC on a chitin-coated Celite column at 0 °C. [e] Concentration of GdnHCl needed to induce 50% denaturation in 0.1 M acetate buffer at pH 5.5 and 35 °C. Details are given in the text.

mutations. No large global conformational changes in mutant lysozymes were suggested by their CD spectra (Figure 3). However, the activity of lysozyme against glycol chitin at pH 5.5 and 40 °C (GC activity) was considerably decreased by the left-sided mutations (N46D, 47del, and 47'G lysozymes). The lytic activity of lysozyme against *M. luteus* at pH 7 and 30 °C was also decreased by these mutations with the exception

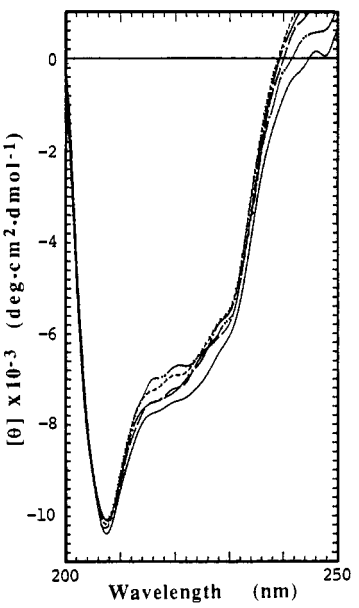


FIGURE 3: Circular dichroism spectra of wild-type lysozyme (—), N46D lysozyme (—•—), 47del lysozyme (– – –), 47'G lysozyme (—··—), and N37G lysozyme (– – –) in 0.01 M phosphate buffer (pH 7) at 20 °C.

of 47'G lysozyme, an insertion mutant. On the other hand, N37G lysozyme, a right-sided mutant, was more active than wild-type lysozyme in both assays.

The dissociation constants (K_ds) of N46D, 47'G, and N37G lysozymes for binding to $(NAG)_3$ at pH 5.5 and 40 °C were almost the same as that of wild-type lysozyme (Table II). Since $(NAG)_3$ is known to form a complex with lysozyme occupying the upper subsites (A, B, and C subsites) of lysozyme out of six saccharide binding subsites named A–F (Blake et al., 1967b), these results indicate that the structure around the upper saccharide binding subsites (A–C subsites) of lysozyme was not altered by these mutations. On the other hand, the binding ability of 47del lysozyme for $(NAG)_3$ was slightly (by about 0.2 kcal/mol) lower than that of wild-type lysozyme, suggesting that the deletion of Thr47 causes a small conformational change at the upper saccharide binding subsites of lysozyme.

The relative retention times of lysozymes in affinity chromatography on a column of chitin-coated Celite at 0 °C are also shown in Table II. Since chitin, a substrate, occupies the whole saccharide binding sites (A–F subsites), the relative retention time on a chitin-coated Celite column should reflect the stability of the enzyme–substrate complex or the substrate binding ability of the enzyme in the equilibrium binding. As shown in Table II, 47'G and N37G lysozymes have the same affinities for chitin as does wild-type lysozyme. Since the saccharide binding abilities of the upper subsites (A–C subsites) in these lysozymes are the same as in wild-type lysozyme, these observations suggest that neither the inserted Gly residue between Thr47 and Asp48 in the left side nor the deleted side chain of Asn37 in the right side is involved in the equilibrium binding to the substrate at the lower subsites (D–F subsites).

Along with the decreased saccharide binding ability at the upper subsites [K_d for the binding to $(NAG)_3$], the relative retention time of 47del lysozyme also decreased. When compared with our previous results, which indicated the correlation between the relative retention times on a chitin-coated Celite column and the value of K_d for the binding to $(NAG)_3$ in Trp108 mutant lysozymes (Inoue et al., 1992a), the decreased relative retention time in 47del lysozyme (0.88)

can be mostly explained by the decreased affinity of A–C subsites, suggesting that the side chain of Thr47 is not involved in the equilibrium binding to substrate at the lower subsites.

N46D lysozyme has increased binding to chitin, despite the fact that it has almost the same binding strength to $(NAG)_3$ as does wild-type lysozyme (Table II). Therefore, it is concluded that the saccharide binding ability of the lower subsites (D–F subsites) in N46D lysozyme is stronger than in wild-type lysozyme.

These results strongly suggest that Asn37, a right-side residue, is not in contact with the substrate in the equilibrium binding but Asn46, a left-side residue, is. That is, the substrate initially binds to the left side of the lower part of the active-site cleft as predicted by Pincus and Scheraga (1979).

Table II also indicates the concentration of GdnHCl needed to induce 50% denaturation of each lysozyme (the midpoint of the GdnHCl denaturation, $C_{1/2}$) at pH 5.5 and 35 °C. The results indicate that the stabilities of these lysozymes against GdnHCl at pH 5.5 and 35 °C decrease in the order wild type > N37G > N46D > 47′G > 47del. It is interesting that this order seemed similar to that of the amounts of lysozymes secreted from yeast (see above). Anyway, all mutant lysozymes were found to be moderately stable, consistent with the above suggestion that the mutations introduced here do not alter the overall structure of lysozyme.

Titration of Catalytic Carboxyl Groups (Glu35 and Asp52) by Tryptophyl Absorbance and Fluorescence. X-ray crystallographic studies of lysozyme indicate that Asn46, substituted by Asp in N46D lysozyme, is in close contact with Asp52, one catalytic residue, in the crystal (Blake et al., 1967a; Diamond, 1974; Ramanadham et al., 1990). In N46D lysozyme, the ionization of the aspartic acid introduced would influence the pK_as of Asp52 and/or Glu35 as a result of electrostatic repulsion.

As mentioned previously (Inoue et al., 1992a), the pK_as of Glu35 and Asp52 in lysozyme free from a substrate and those in the lysozyme–$(NAG)_3$ complex can be determined by the pH dependence of the tryptophyl absorbance at 301 nm and by the pH dependence of the tryptophyl fluorescence of lysozyme in the presence of $(NAG)_3$, respectively, because Trp108 is in van der Waals contact with Glu35 (Imoto et al., 1972), which is further connected with Asp52 by a hydrogen-bond network through two bound water molecules (Diamond, 1974; Ramanadham et al., 1990). Thus, we determined the pK_as of Glu35 and Asp52 in N46D lysozyme in the presence and absence of $(NAG)_3$ by spectroscopic titrations of tryptophyl absorbance and fluorescence.

Figure 4 shows the pH dependence of the absorbance at 301 nm of N46D lysozyme as well as that of wild-type lysozyme in 0.1 M KCl at 30 °C. Transitions observed at around pH 4 and 6 are of the ionizations of Asp52 and Glu35, respectively (Itani et al., 1975). The pK_a of Glu35 was lowered from 6.1 to 5.7 and that of Asp52 was lowered from 3.8 to 3.2 by the substitution of Asn46 by Asp.

Figure 5 shows the pH dependence of the intensities of tryptophyl fluorescence at 330 nm (excited at 280 nm) of wild-type and N46D lysozymes in 0.1 M KCl containing 1.2×10^{-4} M $(NAG)_3$ at 30 °C. Just as in the titration curves obtained from the tryptophyl absorbance of lysozymes in the absence of $(NAG)_3$, two transitions were observed. From the large transitions observed around pH 6, the pk_as of Glu35 in $(NAG)_3$ complexes of wild-type and N46D lysozymes were determined to be 6.4 and 6.0, respectively. The pK_a of Glu35 in N46D lysozyme was increased by the presence of $(NAG)_3$, as in the case of wild-type lysozyme. The small transition at

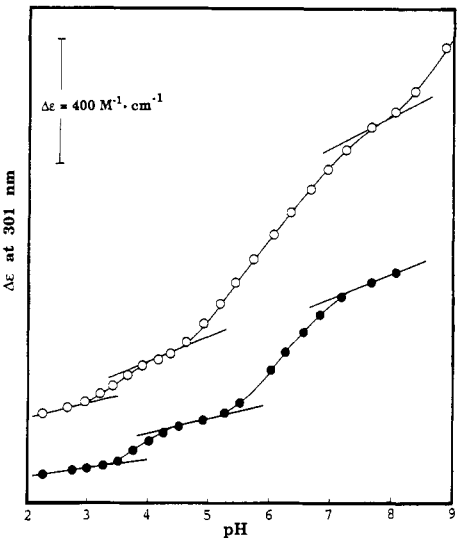


FIGURE 4: pH dependences of the absorbance at 301 nm of wild-type (●) and N46D (○) lysozymes in 0.1 M KCl at 30 °C.

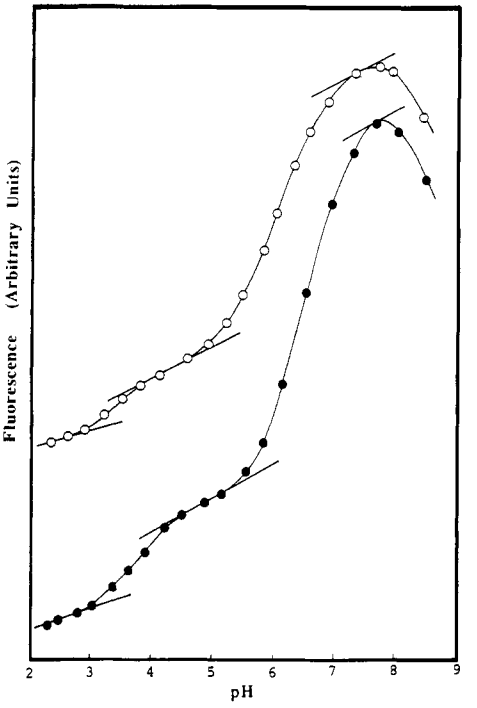


FIGURE 5: pH dependence of the fluorescence intensities at 330 nm of wild-type (●) and N46D (○) lysozymes in 0.1 M KCl containing 1.2×10^{-4} M $(NAG)_3$ at 30 °C. For excitation, 280-nm light was used.

around pH 3.5 was considered to be that of the ionization of Asp52 because this transition was not observed in D52N lysozyme (Inoue et al., 1992a). The pK_a values of Asp52 in the presence of $(NAG)_3$ determined from these titrations were 3.8 for wild-type and 3.2 for N46D lysozyme, which were the same as those in the absence of $(NAG)_3$. Thus, the pK_a of Asp52 in N46D lysozyme is shown to be abnormally low (3.2 compared with the normal intrinsic pK_a of 3.9). Although we did not expect the abnormally low pK_a of Asp52 in N46D lysozyme, the results may suggest that the aspartic acid residue introduced in N46D lysozyme (Asp46) is also in close contact with Asp52 so as to decrease the pK_a of Asp52.

Estimation of the pK_a Value of Asp46 in N46D Lysozyme by Means of Cation-Exchange HPLC and pH Dependence of the Stability. Since Asp46 in N46D lysozyme is shown to be in close contact with Asp52 and to lower the pK_a of Asp52,

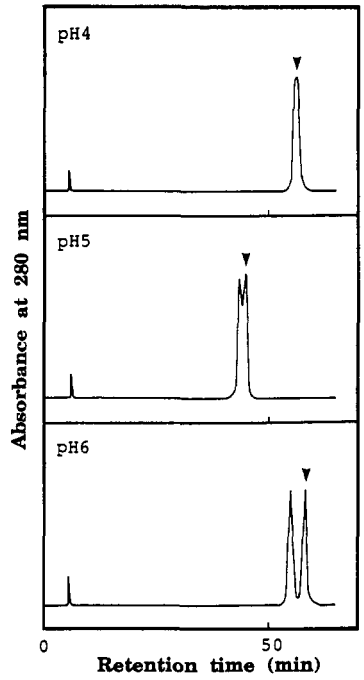


FIGURE 6: Difference in molecular surface charge between wild-type and N46D lysozymes on cation-exchange HPLC (Asahipak ES-502C, 7.8 × 100 mm) at pH 4, 5, and 6. Arrows indicate the peaks of wild-type lysozyme. Details are given in the text.

Table III: Parameters Characterizing the GdnHCl Denaturation of Wild-Type and N46D Lysozymes at 35 °C

	wild type		N46D	
pH	$C_{1/2}$ (M)	ΔG_D (H_2O)[a] (kcal/mol)	$C_{1/2}$ (M)	ΔG_D (H_2O)[a] (kcal/mol)
3.5	2.71	7.34	2.88	7.80
4.0	3.26	8.83	3.27	8.86
4.5	3.69	9.99	3.43	9.30
5.0	3.63	9.84	3.35	9.08
5.5	3.61	9.78	3.30	8.94

[a] Calculated using the average m value (m = 2.71 kcal mol^{-1} M^{-1}).

the pK_a of Asp46 is also expected to be influenced by the ionization of Asp52. Thus, we roughly estimated the pK_a value of Asp46 by comparing the retention times between wild-type and N46D lysozymes by their coinjection on cation-exchange HPLC at pH 4, 5, and 6 (Figure 6). Clearly, N46D lysozyme was coeluted with wild-type lysozyme at pH 4 and separated slightly at pH 5 and completely at pH 6 from wild-type lysozyme. These results suggest that the pK_a of Asp46 is approximately 5.

Recently we have shown that the curve-fitting analysis of the pH dependence of the conformational stability of a mutant lysozyme determined by the extrapolation from the GdnHCl denaturation experiments (Pace, 1975) is a good method to determine the unknown pK_A value of a specific residue (Inoue et al., 1992a,b). Thus, we carried out the same analysis for N46D lysozyme at pH 3.5–5.5 and 35 °C to determine the pK_a of Asp46 in N46D lysozyme. In Table III, the values of $C_{1/2}$ and extrapolated free energy of unfolding in the absence of GdnHCl [$\Delta G_D(H_2O)$; $\Delta G_D(H_2O) = mC_{1/2}$; m = 2.71 kcal mol^{-1} M^{-1}] for N46D lysozyme at pH 3.5–5.5 and 35 °C are shown. For comparison, those values for wild-type lysozyme are also shown in Table III. To analyze the data of N46D lysozyme, we assumed the pK_a of Asp52 in the folded state of N46D lysozyme to be 3.2 (see above) and other requisite pK_a values to be the same as those in wild-type lysozyme (Inoue et al., 1992a,b). Thus, the pK_a of Asp46 in N46D lysozyme was determined to be 4.7. This value is abnormally high compared with the value of 3.9 for a normal aspartic acid residue (Roxby & Tanford, 1971) and is consistent with the results estimated from the retention times on cation-exchange HPLC (Figure 6). These results again suggest that Asp46 in N46D lysozyme is in close contact with Asp52 and that the ionized Asp52 destabilizes the ionized form of Asp46 by electrostatic repulsion.

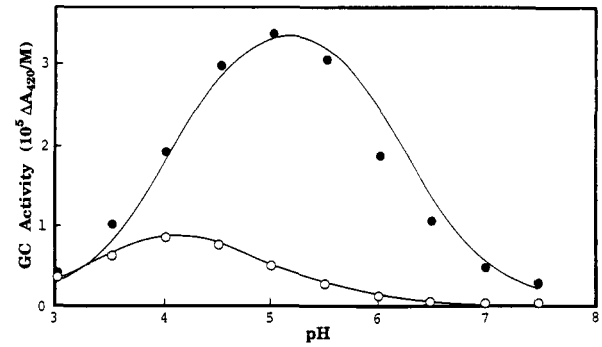
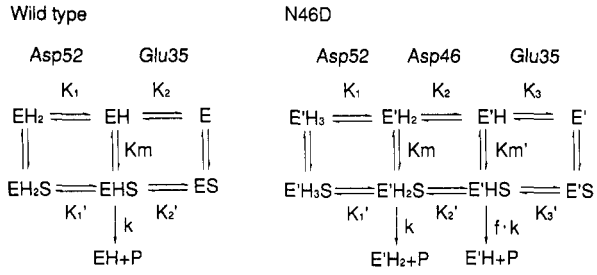


FIGURE 7: pH dependence of GC activities of wild-type (●) and N46D (○) lysozymes at ionic strength 0.1 and 40 °C. Solid lines are theoretical ones.

Scheme I



Comparison of Kinetics for Catalytic Hydrolysis of Glycol Chitin between Wild-Type and N46D Lysozymes. Asn46, a left-side residue, was found to be involved in the equilibrium binding to substrate at the lower subsites. Furthermore, N46D lysozyme is much less active against glycol chitin at pH 5.5 and 40 °C than wild-type lysozyme, despite the fact that the former shows a higher binding strength to the substrate than the latter. In order to explain these observations, the kinetics of the hydrolysis of glycol chitin catalyzed by wild-type and N46D lysozymes were compared in detail.

First of all, the pH dependence of GC activities of these lysozymes was determined at 40 °C and a concentration of glycol chitin of 0.455 mg/mL. As shown in Figure 7, the pH–activity profiles were very different. One of the large differences caused by the mutation from Asn46 to Asp is the shift of the optimum pH from about 5 to 4, and the other is the decreased maximal activity. The pH dependence of the lysozyme reaction is known to be mostly explained by the pK_as of two catalytic carboxyl groups, Glu35 and Asp52 (Banerjee et al., 1973). A well-accepted mechanism is that Glu35, having a higer pK_a, serves as a general acid catalyst in its unionized form and Asp52, having a lower pK_a, stabilizes the oxocarbonium ion intermediate in its ionized form by electrostatic interactions (see Scheme I, left side) (Blake et al., 1967b; Imoto et al., 1972). According to this mechanism, the shift of the optimum pH from 5 to 4 in N46D lysozyme is caused by the decreased pK_as of Glu35 and Asp52. However, as shown above, the pK_as of Glu35 and Asp52 in wild-type and N46D lysozymes are 6.1 and 3.8, and 5.7 and 3.2, respectively, and therefore this large shift in the optimum pH

(about 1 pH unit) cannot be explained only by the decreased pK_as of Glu35 and Asp52 in N46D lysozyme.

Another possibility is that the ionization of Asp46 would be reflected in the pH–activity profile of N46D lysozyme in such a manner that the negative charge of Asp46 changes the position of the ionized carboxyl group of Asp52 by electrostatic repulsion from the optimal position for catalysis. In fact, the pK_a of Asp46 in N46D lysozyme was shown to be 4.7, and therefore it would be rather reasonable if the pH dependence of the GC activity of N46D lysozyme is mainly determined by the ionizations of Asp46 and Asp52 (see Scheme I, right side); that is, the form with ionized Asp52 and Asp46 and protonated Glu35 is much less active than the form with ionized Asp52 and protonated Asp46 and Glu35.

Next, we determined the apparent catalytic constants, $k_{cat,app}$, and the apparent Michaelis constants, $K_{m,app}$, for lysozyme-catalyzed hydrolysis of glycol chitin at pH 4.0 and 40 °C with respect to wild-type and N46D lysozymes by using various concentrations of glycol chitin. The values of $k_{cat,app}$ and $K_{m,app}$ thus determined were 4.05 ± 10^5 ΔA_{420} M^{-1} (30 min)$^{-1}$ and 0.55 mg/mL for wild-type lysozyme and 1.07×10^5 ΔA_{420} M^{-1} (30 min)$^{-1}$ and 0.12 mg/mL for N46D lysozyme. As expected, N46D lysozyme showed a smaller $k_{cat,app}$ and a similar $K_{m,app}$ than wild-type lysozyme. However, it is interesting that the values of $k_{cat,app}/K_{m,app}$ were almost identical between these lysozymes, suggesting that the enzyme–substrate complex is more stable in N46D lysozyme than in wild-type lysozyme but the transition state is not so different between them at pH 4.0. This situation has been expected for a mutation that increases the stability of a dominant nonproductive enzyme–substrate complex (Jencks, 1975).

Analysis of Kinetics. Banerjee et al. (1973) have shown that the pH dependence of the lysozyme-catalyzed hydrolysis of $(NAG)_6$ can be completely explained by considering only the ionizations of Asp101 and the two catalytic groups Glu35 and Asp52, where the ionization of Asp101 participates only in the substrate binding. This may also be true for the GC activity of wild-type lysozyme. As shown above, in the case of N46D lysozyme, the ionization of Asp46 besides those of Asp101, Glu35, and Asp52 should be considered. On the basis of these assumptions, we then attempted a complete description of the pH–rate profiles of wild-type and N46D lysozymes (Figure 7) on the basis of the mechanisms shown in Scheme I. Abbreviations used in Scheme I are as follows. In the case of wild-type lysozyme, EH_2, EH, and E are forms with both protonated Asp52 and Glu35, ionized Asp52 and protonated Glu35, and both ionized Asp52 and Glu35 in the free enzyme, respectively; EH_2S, EHS, and ES are corresponding forms of the enzyme–substrate complex; K_1, K_2, and K_1', K_2' are acidic constants for Asp52 and Glu35 in the free enzyme and the enzyme–substrate complex, respectively; K_m is the dissociation constant of EHS for the substrate (S) binding when Asp101 is completely ionized; and k is the first-order rate constant for EHS to give EH and P (product). In this mechanism, only EHS is assumed to be reactive. Similarly, in the case of N46D lysozyme, $E'H_3$, $E'H_2$, E'H, and E' are forms with all protonated Asp52, Asp46, and Glu35, ionized Asp52 and protonated Asp46 and Glu35, ionized Asp52 and Asp46 and protonated Glu35, and all ionized Asp52, Asp46 and Glu35 in the free energy, respectively; $E'H_3S$, $E'H_2S$, E'HS, and E'S are the corresponding forms in the enzyme–substrate complex; K_1, K_2, K_3 and K_1', K_2', K_3' are the acidic constants for Asp52, Asp46, and Glu35 in the free enzyme and enzyme–substrate complex, respectively; K_m and k_m' are the dissociation constants of $E'H_2S$ and E'HS for the substrate binding when Asp101 is ionized, respectively; and k and fk are the first-order rate constants for $E'H_2S$ and E'HS to give P. In this case, E'HS is assumed to be factor f (less than 1) as reactive as $E'H_2S$.

Table IV: pK_a Values of Glu35, Asp52, and Asp101 (and Asp46 for N46D) in the Free (pK_a) and Substrate-Bound (pK_a') Enzymes Used for Analyses of the pH–GC Activity Profiles of Wild-Type and N46D Lysozymes

	wild type		N46D	
	pK_a	pK_a'	pK_a	pK_a'
Asp52	3.8[a]	3.8[b]	3.2[a]	3.2[b]
Glu35	6.0[a]	6.2[b]	5.7[a]	6.0[b]
Asp101[c]	4.3	3.4	4.3	3.4
Asp46			4.7[d]	4.6[e]

[a] From the pH dependence of absorbance (Figure 4). [b] From the pH dependence of fluorescence in the presence of $(NAG)_3$ (Figure 5). [c] From Kuramitsu et al. (1975). [d] From the pH dependence of stability. [e] Estimated from the pH dependence of GC activity.

In order to analyze the pH–activity profiles of wild-type and N46D lysozymes, we must know the pK_as of Glu35, Asp52, and Asp101 (and that of Asp46 for N46D lysozyme as well) in the enzyme–substrate complexes in addition to those in the free enzymes. The pK_a values of Glu35 and Asp52 (and Asp46) in the free enzymes have been already given above. Since it has been shown that the pK_as of Glu35 and Asp52 in the enzyme–substrate complex are well approximated by those in the enzyme–$(NAG)_3$ complex (Banerjee et al., 1973; Banerjee & Rupley, 1973), we employed the values determined above (Figure 5). Asp101 is located at the upper part of the active-site cleft (Blake et al., 1967b; also see Figure 1) and participates only in the substrate binding (Banerjee & Rupley, 1973; Kuramitsu et al., 1975). As shown above, the upper part of the substrate binding site is not perturbed by the mutation of Asp46 to Asp. We employed pK_as of 4.3 and 3.4 for Asp101 in both wild-type and N46D lysozymes as those in the free enzymes and the enzyme–substrate complexes, respectively (Banerjee & Rupley, 1973). As for the pK_a of Asp46 in the N46D lysozyme–substrate complex, we estimated it to be 4.6 (see below). These pK_a values are summarized in Table IV.

For wild-type lysozyme, using the values of $k_{cat,app}$ [4.05×10^5 ΔA_{420} M^{-1} (30 min)$^{-1}$] and $K_{m,app}$ (0.55 mg/mL) at pH 4.0 and using the respective pK_a values shown in Table IV, k and K_m were calculated to be 6.10×10^5 ΔA_{420} M^{-1} (30 min)$^{-1}$ and 0.22 mg/mL, respectively. The solid curve shown in Figure 7 for wild-type lysozyme is the theoretical curve thus obtained. The good correlation between the theoretical curve and the experimental data indicates that the respective pK_a values in the enzyme–substrate complex employed here are reasonable.

For N46D lysozyme, since the factor f and pK_a of Asp46 in the enzyme–substrate complex ($-\log k_2'$ or pK_2' in Scheme I) are not known, k and K_m (or K_m') cannot be calculated in the same manner. Therefore, these values were determined by curve-fitting analysis. Thus, f, pK_a of Asp46 in the enzyme–substrate complex, k, K_m, and K_m' were determined to be 0.2, 4.6, 1.44×10^5 ΔA_{420} M^{-1} (30 min)$^{-1}$, 0.05 mg/mL, and 0.04 mg/mL, respectively. The solid curve for N46D lysozyme in Figure 7 is simulated with these parameters. These results indicate that the substrate binding abilities of the E'H and $E'H_2$ forms are similar, but the E'HS form is only 20% (f = 0.2) as reactive as the $E'H_2S$ form. In other words, the ionization of Asp46 does not affect the substrate binding ability of N46D lysozyme but decreases its activity by about 80%.

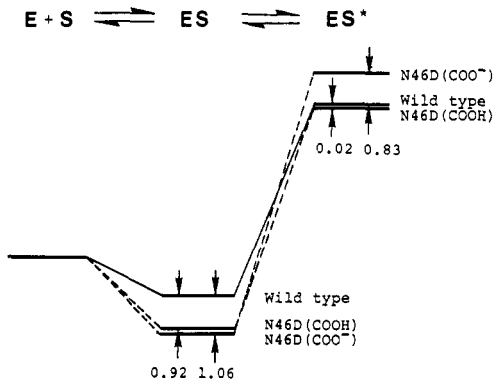


FIGURE 8: Free energy diagrams for the hydrolysis of glycol chitin catalyzed by wild-type and N46D lysozymes at 40 °C. (E + S), ES, and ES* represent the ground state, the Michaelis complex between enzyme and substrate, and the transition state of the complex, respectively. N46D-COOH and N46D-COO⁻ represent the protonated and ionized forms of Asp46 in N46D lysozyme, respectively. Only the difference in free energy (kilocalories per mole) for each state is shown.

DISCUSSION

From the kinetic parameters obtained in the hydrolysis of glycol chitin catalyzed by wild-type and N46D lysozymes, the relationship of ground state (E + S), enzyme–substrate complexes (ES), and transition states (ES*) for wild-type (K_m and k) and N46D lysozymes (K_m and k, K_m' and fk) are depicted in Figure 8 as free energy diagrams. Figure 8 shows that the replacement of Asp46 with protonated Asp stabilizes the enzyme–substrate complex by about 1 kcal/mol relative to the ground state but does not affect the energy level of the transition state. These results clearly indicate that Asn46, a left-side residue, is involved in the initial substrate binding but not in the transition state at all.

Figure 8 also indicates that the replacement of Asn46 with ionized Asp stabilizes the enzyme–substrate complex by 1.06 kcal/mol and destabilizes the transition state by 0.83 kcal/mol. These observations seem to contradict the conclusion that Asn46 is not involved in the transition state. However, if one assumes that a hydrogen-bond network composed of the side chains among Asp52, Asn44, Asn46, and Asn59 (Ramanadham et al., 1990) plays a role in maintaining the optimal catalytic position of Asn52, this contradiction may be explained by the possibility that the negative charge of Asp46 causes Asp52 to move away from its optimal position due to electrostatic repulsion. In this meaning, it is interesting to seek the effect of the mutation of Asn44 or Asn59 to Asp on lysozyme activity.

As mentioned in Results, the substrate binding ability of the lower saccharide binding subsites (D–F subsites) of lysozyme is decreased by the deletion of Thr47 (47del lysozyme) but not by the insertion of Gly between Thr47 and Asp48 (47′G lysozyme). These results suggest that the boundary of the left-side binding site lies in the β-sheet composed of Ala42–Ser60 located in the left side of the active-site cleft of lysozyme. That is, Asn46 and Thr47 are in the left-side substrate binding site at the lower subsites and the boundary is between Thr47 and Asp48.

We observed also that the mutation of right-side Asn37 to Gly does not influence the equilibrium binding of either $(NAG)_3$ or chitin to lysozyme but increases its GC activity (Table II). These observations indicate that the side chain of Asn37, a right-side residue, is not involved in the initial binding of the substrate but increases the energy level of the transition state. The side chain of Asn37 may unfavorably interact with the substrate in the transition state. The participation of the right-side residues in the transition state may be supported by the results reported by Muraki et al. (1989), who have indicated that the mutation of Arg115 (corresponding to Arg114 in chicken lysozyme), a right-side residue, affects the cleavage patterns of $(NAG)_6$.

Banerjee et al. (1975) and Holler et al. (1975) have shown that the hydrolysis of $(NAG)_6$ catalyzed by lysozyme proceeds by the formation of the initial nonproductive (α-complex) and productive (β-complex) complexes between lysozyme and $(NAG)_6$, followed by a change from the β-complex to the second productive complex (γ-complex) from which catalytic hydrolysis occurs. As mentioned earlier, in the case of glycol chitin, a solubilized high polymer of *N*-acetyl-D-glucosamine, the formation of nonproductive α-complex can be neglected because of the low concentration of the terminal end of the substrate compared with that of internal residues. On the other hand, Pincus and Scheraga (1979) have predicted on the basis of conformational energy calculations that there are two distinct binding modes between lysozyme and $(NAG)_n$, "left-sided" and "right-sided" modes, and that the former is more stable than the latter (Figure 1). Together with these earlier results and predictions, the results presented in this paper provide more detailed mechanistic features of the lysozyme reaction. That is, in the case of lysozyme-catalyzed hydrolysis of glycol chitin, the substrate binds first to lysozyme at the energetically favorable but catalytically inactive left-sided binding site involving such residues as Asn46 and Thr47 (β-complex), rearranges to the energetically unfavorable but catalytically active right-sided binding site involving such the residues as Asn37 and Arg114 (γ-complex), and then undergoes catalytic hydrolysis.[2]

Recently, the structure of the complex between chicken lysozyme and NAM-NAG-NAM, a β(1–4)-linked trisaccharide of *N*-acetyl-D-muramic acid (NAM) and NAG, which binds to subsites B, C, and D out of six saccharide binding subsites, has been determined by an X-ray crystallographic study (Strynadka & James, 1991). The resulting structure indicates that the NAM residue in site D is distorted from the full-chair conformation to approximately half-chair conformation; that is, the conformation of the NAM residue bound in subsite D resembles the geometry required for the oxocarbonium ion transition state. Moreover, it has been shown that Asn46 participated in substrate binding to NAM at site D. However, as mentioned in their paper, unlike the case for a NAM residue, it is possible to position an undistorted NAG residue into subsite D so that there are no unfavorable contacts because the atoms that comprise the lactyl group are not present in a NAG residue. Thus, for the fundamental lytic activity, in which lysozyme hydrolyzes the glycosidic bonds of a copolymer of NAG and NAM, it is possible that a dominant substrate binding mode resembles the transition state (γ-complex). For simple substrates such as glycol chitin and $(NAG)_6$, another binding mode (β-complex) might become dominant.

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[2] According to the notation of Banerjee et al. (1975), we called both the "left-sided" complex (β-complex) and "right-sided" complex (γ-complex) "productive" complexes. However, the "left-sided" complex would be rather regarded as a "nonproductive" one because it is catalytically inactive.

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