Crystal Structure of *Tapes japonica* Lysozyme with Substrate Analogue

**STRUCTURAL BASIS OF THE CATALYTIC MECHANISM AND MANIFESTATION OF ITS CHITINASE ACTIVITY ACCOMPANIED BY QUATERNARY STRUCTURAL CHANGE**

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*Tapes japonica* lysozyme (TJL) is classified as a member of the recently established i-type lysozyme family. In this study, we solved the crystal structure of TJL complexed with a trimer of N-acetylglucosamine to 1.6 Å resolution. Based on structure and mutation analyses, we demonstrated that Glu-18 and Asp-30 are the catalytic residues of TJL. Furthermore, the present findings suggest that the catalytic mechanism of TJL is a retaining mechanism that proceeds through a covalent sugar-enzyme intermediate. On the other hand, the quaternary structure in the crystal revealed a dimer formed by the electrostatic interactions of catalytic residues (Glu-18 and Asp-30) in one molecule with the positive residues at the C terminus in helix 6 of the other molecule. Gel chromatography analysis revealed that the TJL dimer remained intact under low salt conditions but that it dissociated to TJL monomers under high salt conditions. With increasing salt concentrations, the chitinase activity of TJL dramatically increased. Therefore, this study provides novel evidence that the lysozyme activity of TJL is modulated by its quaternary structure.

A known bacteriolytic enzyme, lysozyme (EC 3.2.1.17), is widely distributed throughout the animal and plant kingdom. Several types of lysozyme have been described to date as follows: chicken, goose, bacteria, plant, and phage. Recently, there has been increasing interest in a new type of lysozyme, i.e. invertebrate-type (i-type) lysozyme. The existence of this new type of lysozyme was proposed as early as 1975 (1). A lysozyme composed of 123 amino acids (13.8 kDa) was recently isolated from the marine bivalve *Tapes japonica* and was shown to be an i-type lysozyme (2). This was the first lysozyme to be identified as an i-type lysozyme based on determination of the complete amino acid sequence at the protein level. Furthermore, several lysozymes have been identified, including those from the following organisms: two coastal bivalves belonging to the genus *Mytilus* (3), four deep-sea bivalves belonging to the genera *Bathymodiolus* and *Calyptogena* (3), a bivalve belonging to *Chlamys islandica* (4, 5), and a starfish belonging to *Asterias rubens* (6). In 2002, alignment and phylogenic analyses using six bivalve lysozymes suggested that i-type lysozymes form a monophyletic family (7). The tertiary structures of representative lysozymes from chickens (8), geese (9), bacteria (10), plants (11), and phages (12) have already been determined but that of the i-type lysozymes has not. Because the primary sequences of i-type lysozymes are homologous to those of any other types of lysozyme, the tertiary structure cannot be constructed.

Interestingly, the lytic activity of bivalve lysozymes has been shown to be very sensitive to the ionic strength of a solution. With increasing salt concentrations, oyster and blue mussel lysozyme activity against *Micrococcus lysodeikticus* was activated and increased until exposure to a 0.1 M salt solution had been reached; at higher salt concentrations, lysozymatic activity then decreased (13–15). The latter, decreasing activity under higher salt conditions, might have been introduced by the weakness of the electrostatic interactions between negative charges on the bacterial cell surface and the positive charges of the lysozyme. However, the mechanism of salt-dependent activation up to a 0.1 M concentration remains unclear.

In this study, we report the first x-ray crystallographic structure of an i-type lysozyme, *T. japonica* lysozyme (T JL), complexed with a substrate analogue, a trimer of N-acetylglucosamine. Based on crystallographic results and biochemical analyses, we determined the active residues and the catalytic mechanism of TJL. Moreover, we demonstrated that TJL activity is salt concentration-dependent, and this dependence reflected changes in the quaternary structure of TJL.
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EXPERIMENTAL PROCEDURES

Preparation and Crystallization—TJL was overproduced and purified as described in our previous paper (16). Lyophilized TJL (6.6 mg/ml) was dissolved in 100 mM sodium acetate containing 3.3 mg/ml of trimer of N-acetylglucosamine (NAG)₃. The conditions for crystallizations of the complex were screened using screen kits, such as Crystal Screen I and II (Hampton Research) and Wizard I and II (Emerald Biostructure). Finally, a crystal suitable for crystallographic analysis was obtained by vapor diffusion using the hanging drop method. A 1-µl droplet of protein was mixed with an equal volume of reservoir solution and incubated at 20 °C over 3 days. The reservoir solution consisted of 0.8 M citric buffer (pH 5) containing 0.5 M ammonium sulfate and 0.5 mM n-heptyl-β-D-glucoside. The reservoir volume was 200 µl.

Data Collection, Structure Determination, and Refinement—For preparation of heavy metal derivatives, crystals of TJL were soaked for 10 min at room temperature in the reservoir solution containing 100 µM K₂PtCl₄ or 10 µM K₂OsO₄. The crystals were immediately transferred to cryoprotectant solution (reservoir solution containing 10% (v/v) glucose). Native crystals were also soaked in the cryoprotectant solution. X-ray diffraction data of platinum derivative was collected at 100 K using the synchrotron radiation (λ = 0.979041) at the BL38B1 station of SPring-8 (Hyogo, Japan) and Jupiter 210. X-ray diffraction data of native and osmium derivatives were collected using PROTEUM R (Bruker). Data were processed using HKL2000 (17). The platinum locations were directly determined by the program SnB (18), and the osmium locations were determined by the program SOLVE (19). Platinum and osmium parameters were refined using the program SOLVE, and phases were improved with the program RESOLVE (20). Because data quality of platinum data is better than native data, we use platinum data for structural refinement. The initial model was built by the auto model building programs ARP/wARP (21) and LAFIRE (22). Further manual model building was accomplished using the program O (23) and COOT (24). The refinement was done with the program Refmac5 (25). The coordinates of (NAG)₃ were taken from PDB code 1HEW (26). The data collections and refinement statistics are summarized in Table 1 and Table 2, respectively. The coordinates of the complex have been deposited in the Protein Data Bank under code 2DQA.

| TABLE 1 | Crystallographic data of native and heavy atom derivatives |
|----------|------------------------------------------------------------|
| X-ray source | Native | K₂PtCl₄ | K₂OsO₄ |
| Wavelength (Å) | PROTEUMR | 1.5418 | Spring8-BL38 | 0.97904 |
| Space group | P2₁ | P2₁ | P2₁ |
| Unit cell dimensions (Å) | a | 43.0 | 42.7 | 42.8 |
| | b | 89.2 | 88.8 | 88.5 |
| | c | 43.5 | 43.6 | 43.5 |
| | β (°) | 117.3 | 116.0 | 115.6 |
| | Diffraction limit (Å) | 50.0 to 3.1 | 50.00 to 1.6 | 50.00 to 3.30 |
| | Total reflections | 18,675 | 128,543 | 15,031 |
| | Unique reflections | 5,299 | 36,411 | 4,316 |
| | Completeness (%) | 98.8 (77.7) | 94.8 (78.4) | 99.4 (99.8) |
| | Redundancy | 3.5 (3.5) | 3.5 (2.7) | 3.5 (3.6) |
| | R_merge (%) | 10.3 (18.4) | 6.2 (30.8) | 15.8 (23.0) |

* R_merge = Σ(h)/(m(m−1))½Σ(Σ∆Fobs−Fcalc)/ΣFcalc, where ∆Fobs is the mean intensities of symmetry-equivalent reflections, and m is redundancy.

| TABLE 2 | X-ray refinement statistics |
|----------|-----------------------------|
| Resolution (Å) | A | 33.8 to 1.6 | B | 17.5/21.0 |
| Rmerge (%) | A | B | 7.1/4.9 | 7.1/4.9 |
| No. of atoms/average B-factor (Å²) | Protein | 954/18.7 | 954/19.7 |
| | (NAG)₃ | 43/16.3 | 43/16.4 |
| | Platinum | 8/29.8 |
| | Glucose | 2/55.3 |
| | Water | 354/40.0 |
| Root mean square deviations | Bond length (Å) | 0.006 | 0.006 |
| | Bond angles (°) | 1.1 | 1.1 |
| Ramachandran analysis (%) | Most favored | 91.0 | 93.0 |
| | Additionaly allowed | 8.0 | 7.0 |
| | Generously allowed | 1.0 | 0.0 |
| | Disallowed | 0.0 | 0.0 |

* Because two TJL molecules existed in the asymmetric unit, these molecules were named A and B.

Mutant Proteins—Site-directed mutagenesis was performed as described in our previous report (27). The resulting mutants were purified using the same protocol as used for the wild-type TJL described in our previous paper (16). The yields of E18A and D30A were 1.5 and 2.1 mg/liter, respectively. The tertiary structure of mutant proteins was checked by CD spectrometry before the experiments. The molar ellipticity of mutant was identical to that of wild-type TJL.

Determination of Configuration of the Catalytic Products—Ten microliters of 50 µM (NAG)₃ (n = 3–6) was mixed with 90 µl of 7 µM lysozyme solution, and the mixture was incubated at 25 °C for each incubation time. Aliquots (50 µl) are analyzed by high performance liquid chromatography (HPLC) using a Mightysil RP-18 GP column (4.6 × 250 mm, Kanto Chemical Co., Inc.) with a 0.02% trifluoroacetic acid eluent at a flow rate of 0.4 ml/min. The elution of oligomer of NAG was detected by absorbance at 210 nm. The ratio of anomers of NAG was determined by calculating the area of eluted peaks.

Analysis of Lysozyme Catalytic Products—Lysozymes (0.7 µM) and hexamers of NAG (5 µM) were incubated with 20 mM glycine buffer at pH 3.0 containing 100 mM NaCl and 37 °C for 4 h. The solutions (0.5 µl) were mixed with 1 µl of matrix solutions (saturated sinapinic acid in 0.05% trifluoroacetic acid and 50% acetonitrile). The mixture was analyzed by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) (Voyager RP spectrometer, PerSeptive Biosystems).

Chitinase Activity—Lysozymes were incubated at 40°C for 1 h under various conditions. We mixed 45 µl of p-nitrophenyl penta-N-acetyl-β-chitopentaoside (PNP-(NAG)₅) (1 mM) dissolved in aqueous solution with 15 µl of lysozyme solution. Aliquots (10 µl) were withdrawn from the reaction mixture at the appropriate incubation intervals and analyzed by HPLC using a YMC-Pack A-014 column (6 × 300 mm) with an acetonitrile/water (80:20) eluent at a flow rate of 1 ml/min. The elution of PNP-(NAG)₅ was detected by absorbance at 300 nm. The concentration of PNP-(NAG)₅ was determined by calculating the area of an eluted peak.
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Gel Chromatography—Lysozymes (3.5 mg/ml) were incubated with the eluted buffer (50 mM KH₂PO₄, pH 7.0, containing 83 or 500 mM NaCl). Forty-five μl of lysozyme solutions were loaded onto a column (7.5 × 600 mm, TSK-GEL G3000SWXL; TOSOH). The lysozymes were eluted with 50 mM KH₂PO₄ (pH 7.0) containing 83 or 500 mM NaCl at a flow rate of 0.5 ml/min. The eluted proteins were monitored at 280 nm absorbance.

RESULTS

Overall Structure and Interaction between TJL and (NAG)₃—The crystal structure of TJL was determined at 1.6 Å resolution. The structure was refined with good bond geometry and crystallographic quality statistics. The Ramachandran plot produced by PROCHECK (28) revealed that 92–93% of the residues are in the most favored regions and 7% are in additional allowed regions. The structure refinement statistics are shown in Table 2. The crystal structure of TJL is characterized by six α-helices and one β-sheet (Fig. 1). This enzyme is a unique protein that contains 14 cysteines in the small molecule (123 amino acid residues). This was the first study to reveal that all of the cysteines in TJL form disulfide bonds (Fig. 1).

In the crystal structure, the $F_o - F_c$ omit electron density map of (NAG)₃ was clearly observed (Fig. 2). As expected, the sugar moiety in (NAG)₃ is fixed in the large cleft in TJL by several hydrogen bonds (Fig. 2 and supplemental Table 1). The involvement of hydrogen bonding in the interaction with the substrate analogue has also been observed in the goose-type lysozyme complex with (NAG)₃ (9). Moreover, the sugar moiety was fixed by van der Waals forces and hydrophobic interactions involving Pro-44, Tyr-45, Tyr-77, His-94, and Pro-98 in TJL. The residues involved in hydrogen bonding and hydrophobic interaction with substrate were conserved among other i-type lysozymes (Fig. 3). Therefore, these residues may play an important role in the interaction with the substrate in i-type lysozymes.

Structural Comparisons with Other Lysozymes—A structural similarity search, performed with the DALI server (30) using the coordinates of the TJL monomeric structure, indicated that the tertiary structure is homologous to that of other types of lysozyme, despite the low level primary sequence identity between TJL and the other lysozymes (Table 3). According to the DALI search results, we compared the TJL and hen egg white lysozyme (HEL, PDB code 1HEW) structures, as these structures are representative lysozyme folds. The overall structure in HEL and TJL were similar (Fig. 4A). The secondary structural arrangement of α2-, α3-, α4-helices, and β-sheet in HEL were consistent with that of α1-, α3-, and α4-helices and β-sheet in TJL, respectively (Figs. 3 and 4B). A previous report suggested that the chitinase and lysozyme families have a conserved fold containing catalytic residues (31). In this conserved fold, the secondary structure arrangements of the two families are similar, and the hydrophobic interactions between each of the elements in the secondary structures are very similar as well (31).

In both lysozymes, several residues contributed to hydrogen bonding and hydrophobic interactions against (NAG)₃. In particular, His-94 in TJL was overlaid on Trp-108 in HEL, which is conserved among c-type lysozymes, based on a comparison of the tertiary structures of TJL and HEL (Fig. 4B). Phillips and co-workers (32) demonstrated that HEL has six subsets for the binding of an oligomer of N-acetylgalcosamine, and they designated these subsites A–F. (NAG)₃ is reported to bind to subsites A–C in HEL (32). Similarly, in this study, it was found that (NAG)₃ bound to subsites A–C in TJL. In both lysozymes, (NAG)₃ was located in the large cleft, which is formed by commonly conserved folds (Fig. 4B) (31). Thus, the structure of the i-type lysozymes appears to possess a conserved fold in the lysozyme family.

S–S bond formations in TJL were conserved in i-type lysozyme family but differed markedly from those in HEL (Fig. 3). Furthermore, the secondary structure arrangement in the C-terminal region (residues 79–123) differed significantly from those of HEL (Fig. 4A). The hydrophobic residues in the C-ter-
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FIGURE 3. Secondary structure and sequence alignment of i-type lysozymes and hen egg white lysozyme. T. japonica, C. islandica (4, 5), Mytilus edulis (3), Hirudo medicinalis (29), and A. rubens (6) are abbreviated as Tja, Cis, Med, Hme, and Aru, respectively. These sequences were aligned by ClustalW. The conserved residues in i-type lysozymes are shown in red. The red boxes indicate the identified catalytic residues. The residues participating in electrostatic interaction surface in the dimer structure are indicated as yellow lines. The sequences of TJL and HEL are aligned according to the DALI search results. The residues from Arg-20 to Met-105 in HEL are shown. The residues in HEL and TJL, which have structural coincidence suggested by DALI, are conserved in the other i-type lysozymes (Fig. 3). Moreover, the amino acid residues corresponding to Asp-30 in TJL were conserved in the other i-type lysozymes (Fig. 3). For the location of acidic residue, Asp-30 in TJL is located in β1, whereas Asp-52 in HEL is located in β2 (Fig. 3 and 4B). Therefore, we prepared mutant TJLs in which Asp-30 was mutated to Ala, and we examined the activities of the E18A mutants against PNP-(NAG)₅ at 40 °C. We found that the E18A mutants exhibited no lysozyme activity (Table 4). These results demonstrate that Glu-18 is a catalytic residue in TJL. In the crystal structure of TJL, Asp-30 was proximal to Glu-18 (Fig. 5A). Moreover, the amino acid residues corresponding to Asp-30 in TJL were conserved in the other i-type lysozymes (Fig. 3). However, for the location of acidic residue, Asp-30 in TJL is located in β1, whereas Asp-52 in HEL is located in β2 (Fig. 3 and 4B). Therefore, we prepared mutant TJL in which Asp-30 was mutated to Ala, and we examined the activity of the D30A mutant on PNP-(NAG)₅ at 40 °C. In fact, the D30A mutation did not show any lysozyme activity (Table 4), indicating that Asp-30 plays a pivotal role in the catalytic mechanism of TJL.

Catalytic mechanisms have been proposed for members of the lysozyme family (Fig. 5B) (33, 34). An inverting mechanism (Fig. 5B, upper panel) and two retaining mechanisms that proceed through either an oxocarbonium ion intermediate (Fig. 5B, middle panel) or a covalent enzyme-substrate intermediate (Fig. 5B, lower panel) were proposed previously. The inverting mechanism and the retaining mechanism led to hydrolysis of the NAG oligomer to yield two different anomers, i.e. the α-anomer or β-anomer, respectively (Fig. 5B). To determine the catalytic mechanism of TJL, we analyzed the anomerization of sugar produced by TJL. In this study, we observed that the α- and β-anomers derived from a trimer to a hexamer of authentic NAG could be completely separated by reverse phase-HPLC (Fig. 5C). Fig. 5C also shows the HPLC profile of authentic (NAG)₄. A previous paper reported that the ratio of the α-anomer to the β-anomer derived from the authentic NAG was 6:4, as based on the HPLC separation results (35). The ratio of the former peak to the latter in structures derived from (NAG)₄ anomers was 4:6; therefore, we determined that the β-anomer was eluted more rapidly upon HPLC separation. Hydrolysis of (NAG)₄ by HEL gave the β-anomer of (NAG)₄ (36). After

TABLE 3

| Proteins                          | Z-score | r.m.s.d.⁺ | C-a⁻ | Sequence identity |
|----------------------------------|---------|-----------|------|------------------|
| Soluble lytic transglucosylase   | 6.9     | 2.3       | 75 (164) | 12.0 |
| (SI70) (phage-type)              |         |           |      |                  |
| Hen egg-white lysozyme (HEL)     | 6.5     | 2.5       | 84 (129) | 18.0 |
| (chicken-type)                   |         |           |      |                  |
| Goose egg-white lysozyme (goose-type) | 6.0 | 3.2       | 101 (185) | 13.0 |
| Bacteriofage A lysozyme (phage-type) | 5.3 | 3.5       | 98 (154) | 9.0 |
| Chitosanase from Streptomyces    | 2.2     | 4.3       | 86 (238) | 9.0 |
| sp. (plant-type)                 |         |           |      |                  |

⁺ r.m.s.d. indicates root mean square deviation.
⁻ The number indicated the number used in the overlay. The total number of C-a atoms in each molecule is presented in parentheses.

minal helical region were conserved in the i-type lysozyme family (Fig. 3). Therefore, these S-S bond formations and the structure of the C-terminal region in TJL were also found to be unique among lysozyme families.

Catalytic Residues and Catalytic Mechanism of TJL—The catalytic residues in HEL are Glu-35 in the α2-helix and Asp-52 in the β2-strand (32). Based on the folding similarity of HEL and TJL, Glu-35 in HEL corresponds to Glu-18 in TJL, respectively (Fig. 3 and 4B). The amino acid residues corresponding to Glu-18 in TJL are conserved in other i-type lysozymes (Fig. 3). Among all lysozymes and members of the chitinase family, the glutamate residue is conserved as a catalytic residue (31). To confirm whether Glu-18 is a catalytic residue, we prepared mutant TJLs in which Glu-18 was mutated to Ala, and we examined the activities of the E18A mutants against PNP-(NAG)₅ at 40 °C. We found that the E18A mutants exhibited no lysozyme activity (Table 4). These results demonstrate that Glu-18 is indeed a catalytic residue in TJL. In the crystal structure of TJL, Asp-30 was proximal to Glu-18 (Fig. 5A). Moreover, the amino acid residues corresponding to Asp-30 in TJL were conserved in the other i-type lysozymes (Fig. 3). However, for the location of acidic residue, Asp-30 in TJL is located in β1, whereas Asp-52 in HEL is located in β2 (Fig. 3 and 4B). Therefore, we prepared mutant TJL in which Asp-30 was mutated to Ala, and we examined the activity of the D30A mutant on PNP-(NAG)₅ at 40 °C. In fact, the D30A mutation did not show any lysozyme activity (Table 4), indicating that Asp-30 plays a pivotal role in the catalytic mechanism of TJL.
confirmed that the was injected onto an HPLC column. Because the ratio of the was primarily produced by TJL hydrolysis, which suggested that TJL is a retaining enzyme.

helix was mixed in 0.02 M glycine buffer (pH 3.0). The mass spectrum of showed a single mass ion peak of 14,324.3 (Fig. 5C, upper panel), which was derived from the HEL monomer (the estimated molecular weight based on the amino acid sequence was 14,313.1). On the other hand, the mass spectrum of TJL showed a monomer ion peak of 13,879.4 (the estimated molecular weight based on the amino acid sequence was 13,860.1), and another additional peak of 14,695.7 was also observed (Fig. 5D, lower panel). The difference between the two ion masses (816.3) approximately corresponded to the mass of the dehydrated (NAG)₆ (the estimated molecular weight of 818), and we found that the additional peak was derived from the covalent TJL-(NAG)₆ complex produced during the TJL hydrolysis of (NAG)₆. Based on the above results, we surmised that TJL is a retaining enzyme that proceeds through a covalent enzyme-sugar intermediate. This is the first example of a wild-type lysozyme forming a covalent bond intermediate in the course of hydrolysis during a catalytic reaction involving a natural substrate.

**Electrostatic Interactions Stabilizing the Dimer Interface** — In the crystal, two TJL molecules were found as a dimer in an asymmetric unit. We observed several electrostatic interactions in the crystal dimer structure (Fig. 6A and supplemental Table 2).

To determine whether or not the dimer structure was maintained in solution, we analyzed dimer formation using gel chromatography. Based on the quaternary structure in the crystal, the dimer structure would be stabilized by electrostatic interactions. Therefore, we carried out the gel chromatography analyses in the presence of high and low salt concentrations (Fig. 7, A–E). Because the retention time of the protein was influenced by the salt concentration (37), we used HEL as a control protein, as its molecular weight and net charges at pH 7 are similar to those of TJL (i.e., the net charges of HEL and TJL at pH 7 are +8 and +10, respectively). Moreover, as a dimer control, we used the HEL dimer cross-linked at Asp-101 by chemical modification (38). Under high salt conditions, the retention time of TJL was almost identical to that of HEL (Fig. 7, A and B). In contrast, under low salt conditions, TJL eluted more rapidly than HEL (Fig. 7, C and E). Furthermore, the retention time of TJL in the low salt solution was almost identical to that of the HEL dimer (Fig. 7, D and E). Therefore, TJL existed as a monomer under high salt conditions, because of the disruption of electrostatic interactions; however, TJL formed as a dimer in a low salt solution. These results strongly suggest that dimer for-
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FIGURE 6. Quaternary structure of TJL. A, electrostatic interactions at the dimer interface in the crystal. The side chains indicated in red and blue are derived from the A and B molecules, respectively. The name of residues derived from the B molecule were labeled by a single quotation mark. B, inhibitory structure in dimer formation. (NAG), and helix 6 from the neighboring molecule (B molecule, colored green) are represented in the TJL structure (A molecule). The catalytic residues (Glu-18 and Asp-30) are shown in red. Lys-108 and Lys-115 are shown in blue.

Modulation of TJL Chitinase Activity by Dimer Formation—To examine the effects of dimer formation on TJL chitinase activity, we measured the chitinase activity of TJL in the presence of solutions with various salt concentrations (Fig. 7F). After the lysozyme was incubated with 0, 83, 133, 166, and 333 mM NaCl, chitinase activity was measured (Fig. 7F). In Fig. 7F, the activity of each lysozyme is defined as based on 100% in the absence of NaCl. As the salt concentration increased, the chitinase activity of HEL increased in a linear fashion (Fig. 7F). On the other hand, the chitinase activity of TJL markedly decreased in a linear fashion (Fig. 7F). As the salt concentration increased, the chitinase activity of HEL increased in a linear fashion (Fig. 7F). In contrast, with increases in the salt concentration, TJL dissociated and converted to the active monomer because of the disruption of electrostatic interactions.

Discussion

In this study, we determined the first crystal structure of i-type lysozyme, TJL, complexed with (NAG), and the active site residues based on the crystal structure. Moreover, we determined that TJL had a covalent enzyme-sugar intermediate in the course of hydrolysis during the catalytic reaction of the natural substrate.

Typically, a covalent enzyme-sugar intermediate would be detected under slow hydrolysis conditions, e.g. when mutant-impaired sugar hydrolysis or slow hydrolyzing substrates are used (39, 40). In this study, we were able to trap the substrate-enzyme intermediate by suppressing the hydrolysis of the sugar attached to TJL under acidic conditions (glycine buffer at pH 3.0). Meanwhile, the degradation of the covalent bond in the sugar-enzyme intermediate was accelerated under high pH conditions (41). Here we were unable to detect the intermediate with hydrolysis at pH 5.5 (data not shown). In addition to suppression of hydrolysis at pH 3, the rate of hydrolysis of sugar by TJL was slower than that of HEL, because the substrate, (NAG), remained in the mixture after hydrolysis by TJL for 1 h (Fig. 5C). The differing hydrolysis rates between these enzymes may have been due to formation of a covalent enzyme-sugar intermediate. On the other hand, the D52E mutant in HEL, where Asp-52 is mutated to Glu, was reported to form the covalent enzyme-sugar intermediate because the distance between catalytic residues was proximal. The distance between O-ε1 of Glu-35 and O-82 of Asp-52 of wild-type HEL was 6.29 Å, which was calculated by the HEL coordinate (PDB 1HEW). However, the distances between catalytic residues in HEL and TJL do not differ greatly. Although there was no substantial difference in the distances between the catalytic residues of both lysozymes, the formation of covalent intermediate occurred in only TJL.

We considered that the formation of covalent intermediate in TJL caused by an increase in the motion around the active cleft (hinge-bending motion, internal motion, etc.). By the mobile active cleft, nucleophilic acid may be proximal to oxygen of the sugar, which is crucial for the covalent enzyme-sugar intermediate. Consequently, together with the suppression of hydrolysis at pH 3, we were able to detect an intermediate composed of wild-type enzyme and natural substrate.

A retaining mechanism in which the reaction proceeds through a covalent enzyme-sugar intermediate has been seen in various β-glucosidase family members (40, 42, 43). In these enzymes, the nucleophilic acidic residue promotes a covalent bond in the enzyme-sugar intermediate. A glycosidase, Agrobacterium β-glucosidase, was found to exhibit a loss of
function by mutation of nucleophilic acidic residues (44). In TJL, Asp-30 is considered as a nucleophilic acidic residue because of the loss of lysozyme activity in the D30A mutant (Table 4). Furthermore, in the D30A mutant, no additional peak derived from the covalent TJL-(NAG)₄ complex appeared (data not shown). Considering these results, we propose that TJL is a retaining enzyme that proceeds through a covalent enzyme-sugar intermediate, in which Glu-18 acts as catalytic general acid and Asp-30 acts as nucleophilic residues to form a covalent bond to the sugar, respectively.

The crystal dimer structure suggests that helix 6 in one monomer is located in the active cleft of the other monomer, and the side chains of helix 6 protrude from the catalytic site. As is shown in Fig. 6B, (NAG)₃ occupies subsites A–C in TJL, and helix 6 occupies subsites D and E. Therefore, in the dimer structure, a substrate analogue such as PNP-(NAG)₅, which occupies subsites A–F, may not bind to the active site of TJL. TJL activity was found to depend on the salt concentration, resulting in quaternary structural changes in TJL. In the lysozyme and chitinase families, enzymes are expected to act as monomers, and until now, no report has shown lysozyme activity that is regulated by the quaternary structure of the enzyme. As similar with TJL activity, oyster and blue mussel lysozyme activities are modulated by salt concentration (14, 15, 45). Therefore, in the i-type lysozyme family, modulation of activity because of changes in the quaternary structure may be a common feature. On the other hand, the residues in dimer interfacial area, especially in helix 6, were not found to be highly conserved among i-type lysozymes (Fig. 3). Some i-type lysozymes have been shown to exhibit different salt-concentration sensitivities (14, 15, 45), which may reflect variations in dimer interactions between catalytic sites and helix 6, because of low sequence homology among enzymes at helix 6.

Bivalve lysozymes are believed to be involved in digestion (13) and are stocked in crystalline style. Crystalline style is rod-like organs in the bivalve stomach, where various digestive enzymes are stored and highly concentrated. TJL lysozyme may be rendered inactive by dimer formation under such highly concentrated conditions. Marine bivalves feed on marine bacteria as a nutrient source (13). When a bivalve feeds, it swallows the bacteria present in the seawater, which has a high salt concentration (about 500 mM). This water enters the stomach, and the inactive TJL dimer converts to the active TJL monomer, because of disruption of the electrostatic interactions at the dimer interface. Furthermore, because bivalve lysozyme is synthesized in hemocytes, it is involved in self-defense against pathogenic bacteria (46). When the body of a bivalve is injured, bacteria invade the bivalve together with the surrounding seawater. Thus, the lysozyme would rapidly respond to bacterial invasion via its conversion from a dimer to a monomer with an increase in the surrounding salt concentration. Thus, the quaternary structural changes that occur in TJL may play essential roles in both feeding and the self-defense of the living organism.

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**FIGURE 7.** TJL activity influenced by salt concentration. A–E, gel chromatography of HEL and TJL. The column (TSK-GEL G3000SWXL, TOSOH, 7.5 × 600 mm) was eluted with buffer (50 mM KH₂PO₄ (pH 7.0)) containing 500 mM (A and B) or 83 mM NaCl (C–E) at a flow rate of 0.5 ml/min. A and C, HEL; B and E, TJL; and D, chemically cross-linked HEL dimer. Each sample was dissolved in eluted buffer and incubated at 40 °C for 1 h before it was loaded on the column. F, dependence of chitinase activity of TJL (open circles) and HEL (closed squares) on salt concentration. After the lysozymes (5 μM) were incubated with 0, 83, 133, 166, and 333 mM NaCl (pH 7.0 and 40 °C), chitinase activity was measured.
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