A Covalent Enzyme-Substrate Adduct in a Mutant Hen Egg White Lysozyme (D52E)*

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A mutant hen egg white lysozyme, D52E, was designed to correspond to the structure of the mutant T4 lysozyme T26E (Kuroki, R., Weaver, L. H., and Matthews B. W. (1993) Science 262, 2030–2033) to investigate the role of the catalytic residue on the α-side of the saccharide in these enzymes. The D52E mutant forms a covalent enzyme-substrate adduct, which was detected by electron ion spray mass spectrometry. X-ray crystallographic analysis showed that the covalent adduct was formed between Glu-52 and the C-1 carbon of the N-acetylgalcosamine residue in subsite D of the saccharide binding site. It suggests that the catalytic mechanism of D52E mutant lysozyme proceeds through a covalent enzyme-substrate intermediate indicating a different catalytic mechanism from the wild type hen egg white lysozyme. It was confirmed that the substitution of Asp-52 with Glu is structurally and functionally equivalent to the substitution of Thr-26 with Glu in T4 lysozyme. Although the position of the catalytic residue on the β-side of the saccharide is quite conserved among hen egg white lysozyme, goose egg white lysozyme, and T4 phage lysozyme, the adaptability of the side chain on the α-side of the saccharide is considered to be responsible for the functional variation in their glycosidase and transglycosidase activities.

It is known that the catalytic sites of most glycosidases have common features. For example, β-glycosidases have a glutamate on the β-side of the saccharide which acts as an acid to donate a proton to glycosidic oxygen (O-4) to initiate the catalytic reaction (1–4). Lysozyme is one of the β-glycosidases which cleaves the glycosidic linkage between N-acetylgalcosamine and N-acetylmuramic acid in the bacterial cell wall. There are three lysozymes in which the tertiary structures have been solved by x-ray crystallography (5–8). The catalytic mechanisms of these lysozymes have been discussed from the structural point of view. Hen egg white lysozyme is one of the enzymes in which the catalytic mechanism has been extensively investigated (9, 10). The catalytic mechanism of HEWL* is considered to proceed through an oxocarbonium ion intermediate as originally proposed by D. C. Phillips (9). Even though the configurations of the catalytic residues are somewhat similar in both HEWL and T4L (8), the catalytic mechanism of T4L was found to proceed via a single displacement mechanism (11, 12), indicating a different mechanism from that of HEWL.

Moreover, it is known that the kind of catalytic residue located on the α-side of the saccharide seems more adaptable because the acidic residue corresponding to Asp-52 of HEWL was not found on the α-side of the saccharide in GEWL (13), mutant HEWL (14), or mutant T4L (15–17). Recently, we have found that the mutant T4L, Thr-26→Glu, located on the α-side of the saccharide, resulted in the enzyme reacting directly with a saccharide to an enzyme-substrate adduct (11). To clarify the structural and functional features of this residue located on the α-side of the saccharide, we chose to modify HEWL and focused on the residue Asp-52. We mutated Asp-52 to Glu, because in HEWL Asp-52 is located on the α-side of the bound saccharide in a position similar to that of Thr-26 in T4L. It is shown here that this mutation (i.e. Asp-52→Glu) in HEWL does in fact, also lead to the production of a covalent enzyme-substrate adduct. This further demonstrates the overall relationship between the active sites and the mechanism of action of T4L and HEWL.

EXPERIMENTAL PROCEDURES

Materials—The wild type and D52E mutant lysozymes were prepared by expression and secretion from yeast as described previously (18). A (1,4)-linked hexamer of N-acetylgalactosamine [(NAG)₆] was purchased from Seikagaku Kogyo (Japan). All other chemicals were analytical grade for biochemical use.

Analysis of the Reaction Mixture of the D52E Mutant Lysozyme with [(NAG)₆]—The D52E mutant lysozyme (0.07 mM) was allowed to react with 0.5 mM of [(NAG)₆] at pH 5.0 and 25 °C. The reaction mixture was analyzed using reversed phase HPLC with a column of µ-Bondashe C18 (3.9 × 150 mm, 300 A, 5 μm) (Waters). The protein was eluted with a gradient from 1 to 80% acetonitrile containing 0.05% trifluoroacetic acid for 40 min at a flow rate of 0.5 ml/min. The protein elution was monitored by the absorbance at 215 nm. Molecular mass determination of the proteins eluted from the reverse phase column was carried out on a Finnigan TSQ7000 mass spectrometer (Finnigan MAT, San Jose, CA) with an ion-spray interface and a quadruple mass analyzer with an upper mass limit of m/z = 4000 Da.

Large Scale Preparation of the D52E Mutant with a Covalent Adduct for Crystallization—One mg of D52E mutant was allowed to react with 1 mg of [(NAG)₆] at pH 5.0 and 40 °C for 2 days. To remove the excess saccharide, the D52E mutant with a covalent adduct was purified by gel filtration using a column of Sephadex G-25 (1.3 × 65 cm). The eluent containing protein was collected and lyophilized to store. The lyophilized sample of D52E mutant with saccharide adduct was stable after being purified for at least 1 month at 4 °C. If the sample was kept with extra [(NAG)₆], the hydrolysis reaction proceeded, and the covalent adduct was decomposed within 2 weeks.

Crystallization and Tertiary Structure Analysis of the D52E Mutant Lysozyme with and without Saccharide Adduct—Crystallization of the apo-D52E mutant and the D52E mutant with a covalent adduct were performed using approximately the same method as that of the wild type reported previously (6, 19). Five μl of 200 mM sodium acetate buffer containing 15 mg/ml of protein was mixed with 5 μl of reservoir solution (200 mM sodium acetate buffer containing 4.0% NaCl (w/v), pH 4.5) and allowed to stand over the reservoir solution at 15 °C. In the case of the crystallization of D52E mutant with a covalent adduct, no extra [(NAG)₆] was used.
Catalytic Mechanism of the Mutant Lysozyme

RESULTS

The Mutation Design to Trap the Covalent Adduct in Hen Egg White Lysozyme—The structures of the catalytic sites of the T4L and HEWL were compared based on the location of the residues that interact with the saccharides in subsite C and D. For this comparison, the OE2 atom of Glu-11 in T4L and the OE1 atom of Glu-35 in HEWL were chosen as equivalent atoms because these residues act as a general acid to hydrolyze the glycosyl bonds. Two pairs of atoms, NH of Leu-32 and CO of Phe-104 in T4L and NH of Asn-60 and CO of Ala-107 in HEWL, were chosen because they interacted with the N-acetyl group of NAG bound to subsite C. The residues chosen are summarized in Table I. Although Asp-20 in T4L has been considered to correspond to Asp-52 in HEWL (8), these residues were excluded from the structural comparison because the catalytic role of these aspartic acids were recently proposed to be different (11, 12). From this comparison, it seems that the location of Thr-26 in T4L is structurally equivalent to the location of that of Asp-52 in HEWL. In view of this correspondence, Asp-52 was replaced with glutamate to test for the formation of a covalent adduct, as was observed in the mutant Thr-26 → Glu in T4L.

Analysis of the Reaction Mixture of Mutant Lysozymes with (NAG)₆—The reaction mixture of mutant lysozymes with (NAG)₆ at pH 5.0 and 25 °C was analyzed by reversed phase HPLC. The elution pattern of the reaction mixture from D52E mutant and (NAG)₆ after 1 day of reaction is shown in Fig. 1c with the elution pattern of D52E by itself (shown in Fig. 1a). A new peak (A) was eluted before the D52E peak, and the yield of peak A was increased up to more than 90% when the reaction was allowed to continue for 2 days at 25 °C. To identify peak A, the molecular mass of the peak was determined by electron ion spray-mass spectrometry. The mass spectra of peak A and the D52E mutant lysozyme eluted from the reversed phase HPLC are shown in Fig. 2, a and b, respectively. From the analysis, the mass of peak A was determined to be 15,130 daltons, which is 815 daltons larger than that of D52E mutant lysozyme (14,315 daltons). The excess molecular mass (815 daltons) was equal (within error) to that of (NAG)₆ (830.84 daltons) after subtracting the molecular mass of one water molecule (18 daltons). Thus, the excess molecular mass can be attributed to the saccharide adduct covalently linked to the mutant lysozyme.

X-ray Structure of D52E Mutant Lysozyme in Three Different Forms—Three conditions were created for the structural analysis of D52E mutant lysozyme. One was the D52E mutant lysozyme by itself (apo-form) as a reference. The second was the co-crystallization with (NAG)₆ expecting the slow reaction to form a covalent adduct during crystallization. The third was the crystallization of the purified sample by gel filtration after the covalent complex was formed. The covalent adduct was stable in 50 mM sodium acetate buffer at 4 °C at least 2 weeks after being purified. All three samples were crystallized isomorphously to the wild type lysozyme (6, 19). Tertiary structures of the D52E mutant lysozyme under these conditions were determined at 1.9, 1.8, and 2.2 Å resolutions, respectively, by x-ray crystallography. The crystallographic parameters and refinement statistics are summarized in Table II.

The structure of D52E mutant (apo-form) was refined to R = 16.8% at 1.9 Å resolution. The overall structure of the D52E mutant was almost identical to that of the wild type. The root-mean-square differences (RMSD) of Cα positions from the wild type and D52E mutant lysozymes were 0.160 Å. The mutation of Asp-52 to glutamate did not affect the overall structure of hen egg white lysozyme.

The D52E mutant structure co-crystallized with (NAG)₆ was determined at 1.8 Å resolution with an R value of 16.4%. The 2Fo–Fc density map contoured at 1σ value is shown in Fig. 3A. The final refined structure is superposed on the map. It was found that the oligomer of N-acetylglucosamine was observed

TABLE I

| Equivalent residues between HEWL, GEWL, and T4L from the structural and mutation analysis |
|---------------------------------|-----------------|-----------------|-----------------|-----------------|-----------------|
| HEWL                          | T4L             | GEWL            | Interacting atom | Subsite*        |
| Asn-59 (N–H)                   | Leu-32 (N–H)    | Asp-97 (N–H)    | NAG (C=O)        | C               |
| Phe-104 (C=O)                  | Ala-107 (C=O)   | Tyr-147 (C=O)   | NA (N–H)         | C               |
| Asp-52                         | Gly-11          | Glu-73          | O-6, (O)         | D               |
| Glu-35                         | Glu-11          | Glu-11          | O-6, (O)         | D               |
| Asn-46 (OD2)                   | Asp-20 (OD2)    | None            | NAG (N–H)        | D               |
| Thr-26                         | Gly-90          | α-Side of C-1   | D               |

* Corresponding to the subsites of the hen egg white lysozyme (4, 7).

FIG. 1. Elution pattern of the reversed phase HPLC of the D52E mutant lysozyme. a, before adding (NAG)₆; b, after reacting with (NAG)₆ for 1 h and 25 °C; c, after a 1-day reaction.
to be bound from A to C subsites in the mutant lysozyme. No saccharide was observed in the subsite D in this condition. Although the saccharide model was not built into the weak electron densities outside of subsite A, the bound oligomer of the N-acetylglucosamine was considered to be a tetramer because D52E mutant lysozyme cleaved \((\text{NAG})_6\) to \((\text{NAG})_4\) and \((\text{NAG})_2\) (see Fig. 2). The interaction between the bound saccharide and the D52E mutant lysozyme observed in the subsite A, B, and C was essentially the same as those observed in other lysozyme-saccharide complexes reported previously (21–24). The bound saccharide at subsite C revealed a \(\beta\)-anomer, although more \(\alpha\)-anomer is found (59%) in solution (25). The hydrogen bonding interaction (3.4 Å) observed between OE1 of Glu-52 and the O-1 of NAG bound at subsite C may cause the \(\beta\)-anomer to be at subsite C. The overall structure of this complex was quite similar to those both in the wild type (RMSD = 0.201 Å) and in the apo-D52E mutant (RMSD = 0.150 Å), respectively.

The D52E mutant having a covalent adduct was determined at 2.2 Å resolution. The 2\(F_o\)–\(F_c\) density map contoured at 1\(\sigma\) is shown in Fig. 3B. Fig. 3C shows the omit map \((F_{o(D52E)}-F_{e(D52E)})\) from the observed data of the D52E mutant with adduct, model data of D52E, and phases calculated from the D52E model with the Glu-52 side chain atoms omitted, contoured at a 2\(\sigma\). The final refined structure of the D52E mutant with adduct is superimposed on the map. The tertiary structure of the D52E mutant with a covalent adduct was

![Fig. 2. Mass spectra of the proteins eluted from the reversed phase HPLC (see Fig. 1c), a, from peak A; b, from D52E mutant.](image)

### Table II

Data collection and refinement statistics of the D52E mutant lysozymes in the several conditions

<table>
<thead>
<tr>
<th>Data Collection</th>
<th>D52E</th>
<th>Complex</th>
<th>Covalent adduct</th>
</tr>
</thead>
<tbody>
<tr>
<td>Space group</td>
<td>P4,2,2</td>
<td>P4,2,2</td>
<td>P4,2,2</td>
</tr>
<tr>
<td>Cell dimensions (Å)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(a)</td>
<td>79.4</td>
<td>79.2</td>
<td>78.9</td>
</tr>
<tr>
<td>(c)</td>
<td>37.9</td>
<td>38.2</td>
<td>38.4</td>
</tr>
<tr>
<td>Total reflections</td>
<td>15,556</td>
<td>18,178</td>
<td>37,617</td>
</tr>
<tr>
<td>Independent reflections</td>
<td>11,055</td>
<td>9,244</td>
<td>7,152</td>
</tr>
<tr>
<td>(R_{merge})</td>
<td>6.84</td>
<td>4.29</td>
<td>4.42</td>
</tr>
<tr>
<td>Refinement</td>
<td>20–1.90</td>
<td>20–1.80</td>
<td>20–2.20</td>
</tr>
<tr>
<td>Reflections included</td>
<td>9536</td>
<td>9236</td>
<td>5969</td>
</tr>
<tr>
<td>Data completeness (%)</td>
<td>95</td>
<td>78</td>
<td>91</td>
</tr>
<tr>
<td>(\Delta) bond length (Å)</td>
<td>0.014</td>
<td>0.010</td>
<td>0.013</td>
</tr>
<tr>
<td>(\Delta) bond angle (°)</td>
<td>2.60</td>
<td>2.52</td>
<td>2.69</td>
</tr>
<tr>
<td>(\Delta) planes</td>
<td>0.013</td>
<td>0.010</td>
<td>0.010</td>
</tr>
<tr>
<td>(R) factor (%)</td>
<td>16.8</td>
<td>16.6</td>
<td>17.2</td>
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</table>
FIG. 3. Difference Fourier map (2Fo-Fc map) of D52E mutant-saccharide complex (A) and D52E mutant with covalent adduct (B). Each map was contoured at a 1σ value. The Fc-F map density map (C) from the observed data of D52E mutant with adduct, calculated data, and phases from D52E model with Glu-52 side chain atoms omitted, contoured at a 2σ value. The final refined structures are superimposed on the map.
Catalytic Mechanism of the Mutant Lysozyme

Finally refined to an R value of 16.6%. The mass spectrometry data showed that a tetrasaccharide was covalently bound to the protein. The electron density, however, showed strong density in the sites B and C, and weaker density in sites A and D. This is in agreement with prior studies showing that saccharides in the A site show weak density, probably due to the weak energy of interaction (10). Based on refinement and on difference electron density maps, the occupancy of the saccharide appears to be approximately 100% in A, B, and C subsites but only about 50% in subsite D. It seems that the covalent adduct in D52E mutant was partially decomposed during crystallization. To allow for the possibility that the geometry of the saccharide in subsite D might be nonstandard (cf. Ref. 11), no guide values were applied to the angle parameters. The refinement indicated that C-1 of N-acetylglucosamine and OE1 of Glu-52 located on the α-side of the bound saccharide are covalently linked. In addition, the six-membered ring of NAG at subsite D was seen to be slightly distorted toward the sofa form but not as much as observed in the structure of T26E mutant T4L with a covalent adduct (11). Other interactions between the covalently bound saccharide and the mutant lysozyme at subsites A, B, and C were similar to those of the wild type and mutant lysozymes (21–24). The backbone structure (Cα positions) of the protein was also similar to that of the wild type (RMSD = 0.254 Å) and D52E mutant lysozymes (RMSD = 0.223 Å) and was most similar to the protein structure of the D52E mutant and (NAG)4 complex (RMSD = 0.174 Å).

**DISCUSSION**

Three catalytic mechanisms have been proposed for the mechanisms of glycosidases (1–3). They are an inverting mechanism (Fig. 4a) and two retaining mechanisms which proceed through either an oxocarbonium ion intermediate (Fig. 4b) or a covalent enzyme-substrate intermediate (Fig. 4c). HEWL is one of the retaining glycosidases (26). The catalytic mechanism has been considered to involve an oxocarbonium ion intermediate (Fig. 4b) that is stabilized by a negative charge of the carboxylic acid at Asp-52 (9, 10). The catalytic mechanism of T4 lysozyme was recently determined to have an inverting mechanism creating the α-anomer after hydrolyzing the β-glycosidic linkage (12). Although T4 phage and hen egg white lysozymes have distinct catalytic mechanisms (12), a similar glycosyl-enzyme intermediate was observed in mutants. The location of the covalent linkage was confirmed to be between OE1 of Glu-52 and C-1 of NAG by x-ray crystallography as shown above, similar to the linkage seen in the T26E mutant of T4L. This indicates that the glutamic acid replacing Asp-52 of HEWL or Thr-26 of T4L acts as a nucleophile to attack C-1 from the α-side of the saccharide in subsite D. The mutant D52E has already been made by at least two groups (18, 27) and was reported to retain less than 3% activity. The existence of the covalent enzyme-substrate adduct suggests that the catalytic action in D52E mutant HEWL proceeds through a covalent enzyme-substrate intermediate as shown in Fig. 4c. This also implies that the D52E mutant HEWL has a different catalytic mechanism from that proposed for the wild type HEWL. The structural comparison between the wild type and D52E mutant showed that the position of the carboxylate oxygen atom closest to the substrate binding site has shifted about 1.8 Å toward the inside of the cleft in the mutant. This positional shift of the oxygen atom may be responsible for the creation of the covalent adduct. Moreover, a minor species corresponding to the covalent enzyme-substrate adduct was observed in D52S mutant by electron ion spray mass spectrometry (28). If this minor species was created during the catalytic process, it would require a double displacement mechanism the same as that of the D52E mutant HEWL. The tertiary structure of the D52S mutant (24), however, does not seem to support this mechanism, because the position of the OG atom of Ser-52 is 2.6 Å further away from the substrate compared with the OE1 atom of Glu-52. This would place the OG atom of Ser-52 too far away to react with the saccharide molecule directly. To solve this discrepancy, further analysis of the catalytic mechanism of these mutant HEWL is needed.

It is already known that there are features common to T4L, GEWL, and HEWL. The first is the location of glutamic acid (Glu-11, Glu-35, and Glu-73). It is well known that the acidic residue located on the α-side of the saccharide is usually glutamic acid (i.e. Glu-11 in T4L, Glu-35 in HEWL, and Glu-73 in GEWL), which has been shown to be essential for the activity by chemical modifications and mutation analysis (12, 14–16, 18, 29). The glutamate is considered to act as a general acid to donate a proton to the glycosidic oxygen (O-4). Another common feature is the location of main chain peptides that interact with the N-acetyl group of the saccharide bound at subsite C. The interactions in the saccharide binding sites corresponding to subsite C in HEWL are strongly conserved between GEWL, HEWL, and T4L as mentioned previously (8). In contrast, it was previously reported that the acidic residue located on the α-side of the saccharide is not always essential for the activity as seen in hen egg white lysozyme mutants (14, 18, 30, 31), T4 phage lysozyme mutants (15–17), and in the wild type lysozyme from goose egg white (13). The catalytic function of the Asp-52 in HEWL is considered to be stabilization of the oxocarbonium ion (mechanism in Fig. 4b), while Asp-20 in T4L is thought to act as a base to help activate the water molecule to attack the saccharide from the α-side of the saccharide (mechanism in Fig. 4a). It is now clear that the roles of the aspartic acids located on the α-side of the saccharide (Asp-20 in T4L, Asp-52 in HEWL) are different. The D52E mutant shows yet a third role in the catalytic function (mechanism in Fig. 4c), which has already been seen in other glycosidases (4, 32). The formation of the same covalent enzyme-substrate intermediate indicates that the locations of glutamic acid introduced into Asp-52 in HEWL and Thr-26 in T4L are functionally equivalent as shown in Table I.

A careful structure comparison of the catalytic sites from three lysozymes shows that Asp-52 is located at the position equivalent to Thr-26 in T4L and Gly-90 in GEWL. The similar Cα locations of Asp-52 in HEWL to Thr-26 in T4L and Gly-90 in GEWL suggest that the mutation of Asp-52 to a residue which has a short side chain such as Thr, Ser, Ala, or Gly will possibly result in changing the catalytic mechanism toward an inverting enzyme as observed for the wild type T4L. Indeed, a similar

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**FIG. 4.** Three catalytic mechanisms of the glycosidases. a, the mechanism of inverting enzyme; b, the mechanism of retaining enzyme that proceeds through an oxocarbonium ion intermediate; c, the mechanism of the retaining enzyme that proceeds through a covalent intermediate.
mutation has been made in β-glucosidase, and the formation of the α-anomer has been reported (32). Moreover, the D52S mutant of HEWL has been prepared, and the tertiary structure of the D52S mutant of HEWL with substrate was determined (24). The α-anomer seen in the crystal complex with the D52S mutant of HEWL is considered to be a product of hydrolysis. The α-anomer was interpreted as the result of mutarotation of product following a normal wild type HEWL retaining reaction. It would, however, be consistent with the D52S mutant of HEWL acting as an inverting enzyme as has been observed for wild type T4L. These findings support the hypothesis that the residue located on the α-side of the saccharide, such as Asp-52 in HEWL, Thr-26 in T4L, or probably Gly-90 in GEWL, is responsible for the differentiation of the catalytic mechanism. Moreover, the fact that T4L and GEWL, which have a short side chain on the α-side of the saccharide, do not catalyze transglycosylation, while HEWL, which has aspartate or glutamate (mutant) on the α-side of the saccharide and catalyzes transglycosylation, may indicate that this variability is also responsible for the functional difference between glycosidases and transglycosidases.

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