Structural and functional effect of Trp-62 → Gly and Asp-101 → Gly substitutions on substrate-binding modes of mutant hen egg-white lysozymes

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In order to clarify the structural role of subsite B of hen egg-white lysozyme in hydrolytic activity towards a carbohydrate substrate, we analysed the structures of Trp-62 → Gly and Asp-101 → Gly mutant hen lysozymes, which have no side chain at positions 62 or 101, complexed with a substrate analogue, (*N*-acetyl-D-glucosamine)₃ [(GlcNAc)₃], using X-ray crystallography. The overall protein structures in the mutant lysozyme complexes were almost identical to those in the wild type. In the crystals of all the mutant complexes, the (GlcNAc)₃ molecule, which is an inhibitor of wild-type lysozyme, had no inhibitory effect, but was hydrolysed as a substrate. One of the products,

(GlcNAc)₂, the reducing end of which is an α -anomer, was bound in an unproductive binding mode, protruding from the active-site cleft, and was able to act as an inhibitor. Hydrolysis of the synthetic substrate by the mutants occurred in a β -anomer-retaining manner, and so the α -anomer product was converted from the β -anomer product. Thus the interactions of Asp-101 and Trp-62 in subsite B are not essential for the catalytic mechanism, but co-operatively enhance the affinity of the substrate in the productive binding mode, other than the inhibitor in the unproductive mode.

INTRODUCTION

Transition-state theory suggests that, in its transition state, an enzyme should exhibit increased affinity for its substrate [1,2]. Therefore, it is likely that in the transition state, the active site of an enzyme is closed to strengthen the interactions between the enzyme and the substrate, and once the reaction has been completed the active site opens to release the product. Song et al. [3] provided an excellent example of such a reaction pathway by trapping some intermediate states of human lysozyme, and showed that the active-site cleft changed markedly, depending on the product (carbohydrate)-binding mode, suggesting that the cleft was closed to improve interaction with the substrate and opened to promote release of the product. Structural changes in any subsite structure upon substrate and product binding would maximize the efficiency of the enzymic reaction.

Hen egg-white lysozyme catalyses the hydrolysis of β -1,4 glycosidic bonds of alternating copolymers of GlcNAc (*N*-acetyl-D-glucosamine) and MurNAc (*N*-acetylmuramic acid) in bacterial cell walls, or of the homopolymer of GlcNAc, chitin [4]. Hen lysozyme has been extensively studied using functional and structural analyses [5,6]. It has six binding subsites for the sugar residues, A–F, and recognizes the GlcNAc residue at both sides of subsite B in the active-site cleft especially. Subsite B consists of only two amino acids: (i) Trp-62 from the left side; and (ii) Asp-101 from the right side, along the conventional direction of the active-site cleft. Subsite B in hen lysozyme is that which

shows the greatest degree of dynamic change upon recognition of the substrate sugar molecule. Upon binding to the substrate sugar, Trp-62 and Asp-101 undergo induced fitting to the substrate, and their temperature factors are changed so that their structural order is increased [7,8]. A. Kidera and A. Koike (unpublished work) have applied the normal-mode refinement [9] to X-ray diffraction data for the hen egg-white lysozyme–(GlcNAc)₃ complex, and clearly demonstrated that the substrate moves co-operatively with the aromatic ring at position 62 and the main-chain atoms around position 101. On the other hand, in site B there are two atomic features generally observed in protein–carbohydrate interactions [10–12]: (i) the ‘co-operative’ hydrogen bond between the O δ atom of Asp-101 and the O6 atom of the GlcNAc residue in site B; and (ii) the stacking interactions between the aromatic ring of Trp-62 and the non-polar surface of the GlcNAc residue. In our previous study [13,14] we found that Trp-62 → Gly and Asp-101 → Gly mutants showed a shift of their substrate-binding modes, and that the Trp-62 → Gly mutant exhibited little hydrolytic activity towards the GlcNAc oligomer.

In the present study, we focused on the induced fitting of both Trp-62 and Asp-101 in subsite B, which show the general features of protein–carbohydrate interactions, and their movement together with the substrate. Their roles in the hydrolytic activity towards the carbohydrate were investigated by X-ray crystallographic analysis of the Trp-62 → Gly mutant–substrate and Asp-101 → Gly mutant–substrate complexes, and discussed on the basis of functional analysis.

Abbreviations used: GlcNAc, *N*-acetyl-D-glucosamine; MurNAc, *N*-acetylmuramic acid; WT0, wild-type hen egg-white lysozyme; WT3, wild-type hen egg-white lysozyme–(GlcNAc)₃ complex; WT4, wild-type lysozyme–(GlcNAc)₄ complex; W62G3, Trp-62 → Gly mutant hen egg-white lysozyme–(GlcNAc)₃ complex; D101G3, Asp-101 → Gly mutant hen egg-white lysozyme–(GlcNAc)₃ complex.

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EXPERIMENTAL

Preparation and crystallization of the mutant lysozymes

The mutant hen lysozymes were expressed in *Saccharomyces cerevisiae* AH22 and purified by cation-exchange chromatography twice, as described previously [13]. The mutations were confirmed by DNA sequencing and ¹H-NMR spectroscopy [13–15; K. Maenaka, G. Kawai, K. Watanabe, and I. Kumagai, unpublished work].

The crystallization condition of the mutant lysozymes with a substrate analogue, (GlcNAc)₃, was followed as described previously [12]. The concentrations of the mutant proteins were 5–10 mg/ml. On the basis of the binding constants of the mutants towards (GlcNAc)₃ [14,15], the amount of (GlcNAc)₃ in the crystallization solution was five times that of the protein in the complex of the Asp-101 → Gly mutant and ten times that in the complex of the Trp-62 → Gly mutant. The crystallization solution was buffered by sodium acetate (pH 4.7) in the presence of 3.3% sodium chloride (w/v) and allowed to stand over the reservoir solution [10–15% sodium chloride (w/v), sodium-acetate buffer (pH 4.7)] at 20 °C.

Data collection and structural determination

The crystal data summarized in Table 1 were similar to those of the wild-type enzyme [12]. Diffraction data were collected at 4 °C by an automated oscillation-camera system (DIP320), equipped with a cylindrical 'imaging plate' [3,16,17] on a rotating anode generator (M18X), operated at 50 kV, 90 mA with CuK α radiation. The crystals were oscillated by 2.0 degrees for each frame around an appropriate diagonal axis in the (*h*, *k*, 0) plane. For each crystal, intensity data were collected for a total of 50 frames with negligible radiation damage during the experiment. The exposure time per frame was 50 min. The diffraction intensities were evaluated with the WELMS program system [18] on a SUN spark workstation, and processed using the PROTEIN program package [19] in a VAX 8810 system.

Structural refinement

We used the independently refined protein co-ordinates of the wild-type hen lysozyme [12] as an initial model for structural determination. After several cycles of refinement, (2Fo – Fc) and (Fo – Fc) maps were calculated with the PROTEIN program, where Fo and Fc are the observed and calculated structure factor amplitudes respectively. Each mutation site was replaced and

fitted to the respective electron densities on an Evans and Sutherland PS-390 graphics system using the FRODO software package [20]. The total atomic parameters were refined using the PROTEIN/CONEXN/PROLSQ program package [21,22] in a FACOM VP2600 vector computer, after the substrate saccharide had been fitted carefully to the electron densities. These atomic parameters were included in the structure model.

NMR analysis

¹H-NMR experiments were performed at 500 MHz on a Bruker AMX 500 spectrometer at 37 °C as described previously [14]. The wild-type lysozyme sample was used at a concentration of 4 mM in a volume of 400 μ l at pH 4. Preliminary titration experiments were performed by adding the (GlcNAc)₃ to the protein solution.

Measurement of lysozyme hydrolysis

The reduced saccharide fragment having two units of GlcNAc-MurNAc, together with D-Ala-D-Gln-diamino pimelic acid, was prepared as described by Kuroki et al. [23,24] and used as a substrate in this study. The substrate (4.4×10^{-5} M) was digested by the wild-type (2.1×10^{-5} M) and mutant lysozymes (Trp-62 → Gly, 1.1×10^{-5} M; Asp-101 → Gly, 5.8×10^{-6} M) in 50 μ l of 50 mM sodium acetate buffer, pH 5.0. The reaction mixture was analysed by reversed-phase HPLC as described by Kuroki et al. [23,24].

RESULTS

Crystal structure refinement

The crystal data and X-ray-intensity processing statistics are summarized in Table 1. The crystals of the complexes were isomorphous with those of the wild-type enzyme. The final refinement parameters and statistics are summarized in Table 2. All diffraction data for the mutant complexes were limited to at least 1.8 Å resolution, and their *R* merge values were less than 7%. The crystallographic *R* factors of all refined models were about 17%. Each of the refined models included more

Table 1 Crystal data and X-ray-diffraction processing statistics for the mutant lysozyme complexes

	W62G3	D101G3
Space group	P4 ₃ 2 ₁ 2	P4 ₃ 2 ₁ 2
Cell constants (Å)		
a, b	78.87	79.05
c	38.17	38.01
Resolution (Å)	1.5	1.75
Number of measured reflection intensities	34 645	41 118
Number of independent reflections	11 281	10 296
Completeness of data (%)	56.8	81.4
<i>R</i> merge ^a (%)	6.77	6.57

^a *R* merge (%) = $\frac{\sum |I - \{I\}|}{\sum \{I\}} \times 100$, where *I* = measured intensity and $\{I\}$ = average intensity.

Table 2 Final refinement statistics for the mutant lysozyme complexes

	W62G3	D101G3
Number of total atoms	1124	1123
Number of solvent atoms	104	97
Number of parameters	4497	4493
Resolution range in refinement (Å)	6.0–1.8	6.0–1.8
Number of reflections used	8184	9312
<i>R</i> -factor ^a (%)	17.2	17.1
r.m.s., deviation from ideal values, root-mean-square error		
Distance (Å)		
Bond length	0.013	0.011
Angle related	0.033	0.034
Planar	0.044	0.041
Planar groups (Å)	0.011	0.011
Chiral volumes (Å ³)	0.139	0.141
Non-bonded contacts (Å)		
Single torsion	0.166	0.166
Multiple torsion	0.186	0.176
Possible H-bond	0.224	0.199

^a *R*-factor (%) = $\frac{\sum ||F_o| - |F_c||}{\sum |F_o|} \times 100$

Figure 1 Structural comparisons in the region around the mutation site, position 101

WT0 (white) and D101G3 (black). This figure was drawn using the MOLSCRIPT program [29].

than 90 water molecules. No peaks exceeding $0.3 \text{ e}\text{\AA}^{-3}$ were found in the final $|F_o| - |F_c|$ maps.

Protein structures of the mutant lysozymes

The overall structures of the mutant proteins were almost identical with that of the wild-type protein, determined previously by our group [12]. The root-mean-square deviations of the main-chain atoms (N, C α , C and O) were 0.17 Å between WT0 (wild-type hen egg-white lysozyme) and W62G3 [Trp-62 → Gly mutant-(GlcNAc)₃ complex], and 0.24 Å between WT0 and D101G3 [Asp-101 → Gly mutant-(GlcNAc)₃ complex].

In W62G3, the indole ring was deleted only from the position in the structure of WT0 and was found without significant structural changes around position 62 (results not shown). Therefore, the replacement of Trp-62 did not affect significantly overall or local folding of the protein. On the other hand, one conformational change was found in the loop region around position 101, which is the mutation site in D101G3 (Figure 1). The peptide-bond plane of CO (residue 101)–NH (residue 102) in the Asp-101 → Gly mutant was rotated, and the main-chain carbonyl oxygen of Gly-101 was oriented towards the interior of the protein and formed some hydrogen bonds towards the main-chain nitrogen of Asn-103 (3.2 Å) and that of Gly-104 (3.3 Å). The conformation of the loop region became similar to the type I β -turn structure that is also found in turkey lysozyme, which belongs to the same c-type lysozyme as hen lysozyme, and has a glycine at position 101 [25] (Figure 1, Table 3).

Binding modes of GlcNAc residues

NMR data obtained by Lumb et al. [26] for wild-type lysozyme complexed with (GlcNAc)_{1–3} are basically consistent with the present data. In the present study, NMR [two dimensional NMR homonuclear Hartman–Hahn spectroscopy (NOESY and HOHAHA)] spectra for WT3 [wild-type hen egg-white lysozyme-(GlcNAc)₃ complex; the 1:1 protein–substrate complex], shown in Figure 2, indicated that the N1H resonances of Trp-108 were separated into two and showed chemical exchange on the NMR time scale, suggesting that WT3 had two major conformations. Some resonances were also separated (Asp-52, Tyr-53, Cys-64, Asp-66 and Cys-76) that make up the core structure of the

Table 3 Comparison of backbone ϕ and ψ values (degree) of turkey and the Asp-101 → Gly lysozymes for residues 100–104

Residue number		Turkey	Asp-101 → Gly
100	ϕ	−78	−76
	ψ	−7	−8
101	ϕ	−69	−70
	ψ	170	165
102	ϕ	−57	−59
	ψ	−35	−27
103	ϕ	−99	−93
	ψ	15	−1
104	ϕ	56	59
	ψ	−131	−142

active-site cleft, and the β -sheet and loop region around position 62. The other resonances that changed upon binding were also located around the active-site cleft. Therefore, the wild-type hen egg-white lysozyme bound to the (GlcNAc)₃ molecule along the active-site cleft in two major binding modes.

The final models of W62G3 and D101G3 based on the X-ray structural analyses showed that during the crystallization of each complex, the (GlcNAc)₃ molecule had already been hydrolysed. The bound (GlcNAc)₂ molecule, which was fitted into the strong electron densities (Figure 3), was in the α -form at its reducing end. The reducing-end residue occupied part of the D site of the wild-type lysozyme-(GlcNAc)₄ complex (WT4) and formed an ordered hydrogen-bonding network whose site will be referred to as 'site L1' hereafter (Figure 4). The non-reducing-end residue is positioned outside the active-site cleft and on the hydrogen-bonding network, the platform region, including the catalytic residue Asp-52 (this site is referred to as 'site L2') [7]. Since the (GlcNAc)₂ molecule is not bound in subsites A and B, the mutation sites there (Gly-62 and Gly-101) do not interact with the substrate analogue. A comparison of the enzyme–substrate interactions between WT4 and each mutant-(GlcNAc)₃ complex is shown in Figure 4. These results showed that most of the hydrogen bonds between the protein and the carbohydrate are commonly used in each complex. These are the hydrogen bonds between (i) the main-chain oxygen atom of Asp-107 and the nitrogen atom of the *N*-acetyl group of the reducing-end sugar residue in site L1, (ii) the main-chain nitrogen atom of Asn-59 and the O7 atom of the sugar in site L1, and (iii) the O6 ϵ atom of Glu-35 and the O1 (α -anomeric configuration) oxygen atom of the sugar residue in site L1.

The final model of the GlcNAc residues fits the strong electron densities, and the B factors of the reducing-end residues in all complexes (Table 4) are rather low (less than $28 \text{ \AA}^2$) for forming the stable and ordered complex state. The *N*-acetyl group of the reducing-end sugar residue is positioned at the same site that the *N*-acetyl group of the sugar residue in site C of the wild-type hen lysozyme is complexed with the GlcNAc oligomer (Figure 4). The B factors of its O7 and N atoms are the lowest of all the atoms of the GlcNAc residues (Table 4). Therefore, this recognition is one of the major factors determining the binding of hen lysozyme to the GlcNAc oligomer, in spite of the different binding modes.

Hydrolytic activity

Since the product bound to the mutant proteins was the α -anomer, it is possible that the change in the catalytic mechanisms

Figure 2 Two-dimensional ^1H -NMR HOHAHA (A and B) and NOESY (C and D) spectra of the wild-type lysozyme (A and C) and its complex with $(\text{GlcNAc})_3$ (B and D)

The chemical exchange of the separated N1H resonances of the Trp-108 is confirmed by observation of the cross peak between the two resonances.

Figure 3 Electron-density map around the GlcNAc oligomer in binding sites L1-L2

(a)

(b)

(c)

Figure 4 Interactions in the region around site D between lysozyme and the bound GlcNAc oligomer

(a) WT4, (b) W62G3, (c) D101G3. In WT4, the (GlcNAc)₄ molecule was bound in sites A-B-C-D, showing sites C and D. In the other complexes (W62G3 and D101G3), the (GlcNAc)₂ molecule was bound in sites L1 and L2. This figure was produced using the MOLSCRIPT program [29].

Table 4 Average B factors ($\text{\AA}^2$) of the saccharide residues in the mutant lysozyme complexes

Saccharide residue	W62G3	D101G3
L1	27.9	25.9
(O7, N)	(25.9, 26.6)	(22.2, 24.2)
L2	33.7	33.7

caused the product to change from the β - to the α -anomer. In order to examine this possibility, the reduced substrate, including two units of GlcNAc-MurNAc, and D-Ala-D-Gln-diamino pimelic acid was used to identify the anomer of the product [23,24]. Both the mutants (Trp-62 → Gly and Asp-101 → Gly) exhibited anomer-retaining activity (β - to β -anomer), like that of wild-type hen egg-white lysozyme (results not shown). Therefore, it was concluded that the substitution at subsite B did not affect the catalytic mechanism and that the mutant proteins preferred the α -anomer product at sites L1-L2 on behalf of the loss of the subsite B structure.

DISCUSSION

Our aim in this study was to clarify the changes in molecular recognition between the substrate saccharide and mutant lysozyme species in which subsite B was mutated. Unexpectedly, however, we found that the mutant was complexed with the α -anomer of the hydrolysed product, which bound in an unproductive binding mode far from subsite B. Blake et al. [27] clarified the structure of the Trp-62 → oxindolealanine mutant lysozyme complex and obtained almost the same result as that obtained here. Interestingly, the oxindolealanine at position 62 seemed to shift toward Asp-101 and block the upper part of the site responsible for the productive binding mode (unfavourable contact), resulting in reduction of the activity, and bound the α -(GlcNAc)₂ product distant from subsite B. In the present study, less favourable interaction in subsite B produced a similar result: the Trp-62 → Gly and Asp-101 → Gly mutants, which have less interaction with the carbohydrate moiety in subsite B (for details, see below), had different binding modes in which the inhibitor molecule, (GlcNAc)₃, became a good substrate and was hydrolysed. Our previous study [12] showed that Trp-62 → Tyr and Trp-62 → Phe mutant lysozymes loosely bound the (GlcNAc)₃ molecule in the sites A-B-C and also B-C-D, while the wild-type enzyme bound it only in sites A-B-C. Since the present study of hydrolytic activity toward the reduced substrate showed that the mutant enzymes were, like the wild-type enzyme, anomer-retaining, the hydrolysis of (GlcNAc)₃ occurred through the same (much looser) productive binding modes as those of the wild-type protein (C-D-E or D-E-F), and later the product, β -(GlcNAc)₂, was converted to α -(GlcNAc)₂. This, in turn, was accepted in an unproductive binding mode, L1-L2 (near to site D), instead of placing the (GlcNAc)₃ molecule in part of the site responsible for the productive binding mode, sites B-C. Therefore, this unproductive binding mode normally appeared through steric hindrance or disruption of subsite B in the complex crystal.

Our previous X-ray crystallographic analysis [12] showed that substitution with the aromatic amino acids, Tyr or Phe, at position 62 slightly reduced but maintained the stacking interactions toward GlcNAc in site B of the mutant complex, and also altered the 'co-operative' hydrogen-bonding network with the O6 atom of GlcNAc in site C, thus inducing changes in the

substrate-binding modes while maintaining their total hydrolytic activities. Therefore, it is likely that loss of the ring in the Trp-62 → Gly mutant completely disrupts both the stacking interactions and the hydrogen-bonding network, thus considerably reducing the hydrolytic activity towards the GlcNAc oligomer.

Although no marked local conformational changes were found in the Trp-62 → Gly mutant, our previous NMR analysis [14] showed that chemical-shift changes in the loop region around Trp-62 and the β -sheet including Asp-52 were induced by the mutation Trp-62 → Gly. Because most of the chemical-shift changes were less than 0.1 ppm and their origins were uncertain, the effect of these subtle changes cannot be properly estimated.

On the other hand, the Asp-101 → Gly mutant exhibited a change in the substrate-binding modes [13,15]. The main-chain conformation around position 101 was changed by the mutation Asp-101 → Gly, and was similar to that of turkey lysozyme, which has a glycine residue at this position [25]. Harata and Muraki [25] have also pointed out that this loop region is flexible and its conformation depends on the crystal packing. Furthermore, if it is assumed that the binding of the GlcNAc oligomer to the Asp-101 → Gly mutant is the same as that of the wild-type, then the main-chain atoms around position 101 will be too distant to make direct contact with the GlcNAc oligomer. By comparison of the structures of human lysozyme complexed with (GlcNAc)₂-2',3'-epoxypropyl β -glucoside of *N*-acetylchitobiose and with Gal-GlcNAc-2',3'-epoxypropyl β -glucoside of *N*-acetylactosamine, Muraki et al. [28] clearly showed that the hydrogen-bonding network around the Gal residue in site B is changed markedly, reducing the stacking interaction between Tyr-63 (corresponding to Trp-62 in hen lysozyme) and Gal. Therefore, the destruction of the hydrogen-bonding network linking the GlcNAc residue in site B resulting from the Asp-101 → Gly mutation, rather than the conformational change in the loop region around position 101, reduces the stacking interaction of Trp-62. These co-operative effects reduced the affinity of subsite B to a greater extent than expected, so that the Asp-101 → Gly mutant was unable to maintain the GlcNAc oligomer in the active-site cleft, and exhibited a shift in the binding mode. These results are consistent with the co-operative movements of the substrate Trp-62 and Asp-101 revealed by normal mode analysis (A. Kidera and A. Koike, unpublished work).

The step of releasing the product molecule in an enzyme reaction is as important as that of binding the substrate molecule. Subsite B in the wild-type enzyme is thought to have more interaction than that of the mutants. By this interaction, the product molecule would be shifted from the position just after completion of catalysis towards site B to create some space for solvent molecules between the product molecule and the catalytic amino acid, Glu-35. Penetration by water molecules could be the first step in release of the product molecule from the protein molecule. In fact, the crystal structure of WT4 [12] shows that some water molecules are bound between the carbohydrate moiety of the product and the protein molecule in site D, and that the GlcNAc residue is bound slightly distant to the active site cleft. In the Trp-62 → Gly and Asp-101 → Gly mutants, the favourable interactions towards the substrate were much reduced, and so the differences between the lysozyme-substrate and the lysozyme-product interactions could hardly be compared. However, the co-operative movement and recognition of Trp-62 and Asp-101 for the substrate or product, pointed out above, are

likely to contribute to conversion from the Michaelis-Menten complex to the transition state, and to release of the product.

With regard to sites L1 and L2, in the wild-type lysozyme complexed with (GlcNAc)₃ [5], two binding modes are possible according to the reducing-end anomer involved: (i) B-C (β -anomer) and (ii) L1-L2 (α -anomer). In solution, the wild-type lysozyme binds (GlcNAc)₃ in at least two major modes (Lumb et al. [26] have also reported that WT3 has multiple binding modes including partial occupation of site D). Although the biological role of the L1-L2 sites is unknown, it is of considerable interest that wild-type lysozyme has anomer-dependent binding modes, including unproductive binding sites (L1-L2), for sugar molecules whose reducing-end is an α -anomer.

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