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Catalytic Reaction Mechanism of Goose Egg-white Lysozyme by Molecular Modelling of Enzyme–Substrate Complex

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Despite the low similarity between their amino acid sequences, the core structures of the fold between chicken-type and goose-type lysozymes are conserved. However, their enzymatic activities are quite different. Both of them exhibit hydrolytic activities, but the goose-type lysozyme does not exhibit transglycosylation activity. The chicken-type lysozyme has a retaining-type reaction mechanism, while the reaction mechanism of the goose-type lysozyme has not been clarified. To clarify the latter mechanism, goose egg-white lysozyme (GEL)–*N*-acetyl-D-glucosamine (GlcNAc)6 complexes were modelled and compared with hen egg-white lysozyme (HEL)–(GlcNAc)6 complexes. By systematic conformational search, 48 GEL–(GlcNAc)6 complexes were modelled. The right and left side, and the amino acid residues in subsites E–G were identified in GEL. The GlcNAc residue D could bind towards the right side without distortion and there was enough room for a water molecule to attack the C1 carbon of GlcNAc residue D from α-side in the right side and not for acceptor molecule. The result of molecular dynamics simulation suggests that GEL would be an inverting enzyme, and Asp97 would act as a second carboxylate and that the narrow space of the binding cleft at subsites E–G in GEL may prohibit the sugar chain to bind alternative site that might be essential for transglycosylation.

Key words: binding simulation, goose-type lysozyme, inverting enzyme, molecular dynamics simulation, systematic conformational search.

Abbreviations: GEL, goose egg-white lysozyme; HEL, hen egg-white lysozyme; GlcNAc, *N*-acetyl-D-glucosamine; MD, molecular dynamics.

INTRODUCTION

Lysozymes hydrolyze the β-(1,4)-glycosidic linkage of *N*-acetyl-D-glucosamine (GlcNAc) oligomer. In the chicken-type lysozymes, the X-ray crystallographic structure of hen egg-white lysozyme (HEL) was first determined (*1*). In previous studies, HEL was shown to have six subsites, called sites A–F, that can accommodate a (GlcNAc)6 oligomer, and two distinct binding modes (right- and left-side binding modes) in the binding cleft (*2*, *3*). When the (GlcNAc)6 oligomer binds to the left side, the complex is non-productive, and when the (GlcNAc)6 oligomer shifts to the right side, the complex is productive (*4–6*). In this step, the GlcNAc residue D is thought to be distorted, and Glu35 acts as a proton donor. Asp52 acts as a second carboxylate to stabilize the oxocarbenium ion (*7*). On the right side, the hydrolytic reaction is thought to occur via two mechanisms, i.e. through an oxocarbenium ion intermediate [called the 'Phillips mechanism' (*7*)] or through a covalent enzyme–product intermediate [the 'Koshland mechanism' (*3*)]. In the Phillips mechanism, a water molecule replaces the GlcNAc residue E and attacks the oxocarbenium ion, creating a product GlcNAc residue with the same anomeric configuration (β). Thus HEL is considered to be a retaining enzyme (*8*). In our previous study, we modelled the complexes between HEL and (GlcNAc)6 oligomer that could support this reaction scheme (*9*).

HEL also exhibits transglycosylation activity (*10*), and its reaction mechanism has been estimated to be as follows. After the substrate bound to the right side, the acceptor molecule (GlcNAc homopolymer) bound to the left side and catalysed the transglycosylation. The subsites E and F are considered to be related to the binding of the acceptor (*10*). Thus the two binding modes, the right- and left-side modes, are considered to be related to the transglycosylation mechanism.

In the goose-type lysozymes, the X-ray crystallographic structures of goose egg-white lysozyme (GEL) were first determined in the free state and in a complex with (GlcNAc)3 (PDB id: 153L and 154L) (*11*). Honda and Fukamizo (*12*) first identified the six subsites in GEL, which they called the B–G sites. GEL hydrolyzes the GlcNAc homopolymer less effectively than HEL (*12*, *13*). Although the lengths of the amino acid sequences of HEL and GEL are 129 and 185, respectively, and the sequences of GEL and HEL have no apparent similarity (28.6% amino acid similarity), the core structures of the lysozyme fold are conserved. GEL also hydrolyzes the β-(1,4)-glycosidic linkage of GlcNAc oligomer;

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however, importantly, GEL does not show the transglycosylation activity (*14*). It is unknown whether or not GEL has two binding modes, such as right and left sides. In addition, it is unknown whether or not the conformation of the GlcNAc residue D is distorted and why GEL lacks transglycosylation activity. To understand the catalytic reaction mechanism of GEL, it is important to estimate the binding modes and to explore the amino acid residues interacting with the substrate. The amino acid residues related to the substrate binding have been identified at subsites B–D by the X-ray structure of GEL with (GlcNAc)3 oligomer (*11*); while, the amino acid residues related to the subsites E–G have not been identified.

Here we used the GlcNAc oligomer as a substrate. The GlcNAc residues bound to the subsites B–G were called GlcNAc residues B–G, respectively. Complexes between GEL and (GlcNAc)6 oligomer were modelled and molecular dynamics (MD) simulation was performed to investigate the binding modes and to identify the amino acid residues interacting with the GlcNAc oligomers.

Monzingo *et al*. (*8*) assumed that GEL would be an inverting enzyme by inferring the location of the second carboxylate, but they found no structural evidence for this assumption. To determine whether GEL is an inverting or a retaining enzyme, we searched for a water molecule that could attack from the α-side of the substrate using the complexes modelled in this study. Finally, we discuss the possible reasons why GEL does not catalyse the transglycosylation and propose the catalytic reaction mechanisms of GEL and HEL.

MATERIALS AND METHODS

Obtaining the Initial Structure for the Systematic Conformational Search—Coordinates for the X-ray crystallographic structure of the complex between GEL and (GlcNAc)3 were obtained from the Protein Data Bank (PDB code: 154L) (*11*). The structure of the complex was determined at high resolution (1.6 Å). Coordinates for the X-ray structure of GlcNAc were obtained from CSD (Cambridge Structure Database, Accession number: BCHITT10) to add to the reducing end of the GlcNAc oligomer in the systematic conformational search.

Modelling of GEL–(GlcNAc)6 Complexes by Systematic Conformational Search—Hydrogen atoms were added to the heavy atoms in the 154L structure using the LEaP module of the AMBER 7 software package (*15*). In 154L, (GlcNAc)3 bound to the subsites B–D. The glycosidic bond between the GlcNAc residues D and E would be hydrolysed. The conformation of the GlcNAc residue D in 154L was in the chair configuration (β-anomeric conformation). According to the reaction mechanism of HEL, the GlcNAc residue D in GEL would be distorted to the half-chair configuration in the intermediate state. Therefore the systematic conformational search was initially performed for the GlcNAc residue D. First, the GlcNAc residue D in 154L was removed, and the GlcNAc residue in the CSD database was added to the reducing end of (GlcNAc)2 by removing an oxygen atom and a hydrogen atom at the β-(1,4) glycosidic bond. To investigate whether or not the GlcNAc residue D would be distorted, a systematic conformational search was performed for two cases: the chair and half-chair forms for the GlcNAc residue D. The former complex is called the 'chair model', and the latter one is called 'half-chair model'.

We modelled GEL–(GlcNAc)6 complexes using the same systematic conformational search method as performed to model HEL–(GlcNAc)6 complexes in our previous study (*9*).

MD Simulation of GEL–(GlcNAc)6 Complexes—MD simulations were performed for the GEL–(GlcNAc)6 complexes by means of the procedures used in our previous study (*9*). The system was solvated in a periodic cubic box and filled with the TIP3 water molecules. MD simulation was performed at 300 K for 500 ps. During the MD simulation, the last 200 conformations were sampled at 1 ps intervals, and then used for the structural analysis. The interaction energies of the hydrogen bonding, van der Waals bonding and Coulombic attraction in the complex between GEL and (GlcNAc)6 were calculated using the ANAL module of the AMBER 7 software package (*15*).

RESULTS

Modelling of GEL–(GlcNAc)6 Complexes by Systematic Conformational Search—We modelled GEL–(GlcNAc)6 complexes by using a systematic conformational search. The GlcNAc residue D was considered to be distorted in the transition state in HEL. Therefore, we modelled the GEL–(GlcNAc)3 complexes in the cases of both the chair and half-chair models for the GlcNAc residue D. In the half-chair model, the GEL–(GlcNAc)3 complexes could not be modelled, because the distorted GlcNAc residue D had a steric hindrance with the side chain of Asn148. This result corresponded to the previous study in which Weaver *et al*. (*11*) described that the GlcNAc residue D could be rotated at several degrees deeper into the cleft without distortion. Therefore, we modelled GEL–(GlcNAc)6 complexes in only the chair model.

The numbers of complexes were 50, 32 and 24 for the complexes GEL–(GlcNAc)3, GEL–(GlcNAc)4 and GEL–(GlcNAc)5, respectively. The complexes were classified based on the cluster analysis using their coordinates (*9*). The numbers of clusters were 3, 7 and 11 for the complexes GEL–(GlcNAc)3, GEL–(GlcNAc)4 and GEL–(GlcNAc)5, respectively. Finally, we modelled 48 GEL–(GlcNAc)6 complexes and classified them into 11 clusters. The complex with the lowest energy in each cluster was named C1–C11 and was selected for applying the MD simulation.

MD Simulation of GEL–(GlcNAc)6 Complexes—MD simulations were performed to explore the amino acid residues interacting with (GlcNAc)6 oligomer in the complexes C1–C11. The number of the hydrogen bonds between the amino acid residues and (GlcNAc)6 oligomer in 200 complexes sampled for each complex (C1–C11) were calculated. The structural information of the 11 complexes (C1–C11) is shown in Table 1.

To investigate whether or not the complex is productive, the distance between the β-(1,4)-glycosidic oxygen (O4) atom linking GlcNAc residues D and E, and the proton bonded to the $O^{\varepsilon 2}$ atom ($H^{\varepsilon 2}$ atom) of Glu73 as a catalytic site were calculated against all of the structures sampled in each MD simulation. As shown in Table 1,

Table 1. **Structural data of the 200 complexes sampled in each MD simulation.**

Model	Name	Distance (Å)		Interaction energy (kcal mol^{-1})[c]			Number of hydrogen bonds					
		Glu73[a]	Gly90[b]	LL	LP	Total	GlcNAc residue					
		($H^{\varepsilon 2}$)	(H^{α})				B	C	D	E	F	G
Chair	C1	3.4	6.6	181.3	−162.5	18.9	2	460	165	135	43	1
	C2	3.2	7.3	177.2	−141.6	35.6	4	459	120	164	32	52
	C3	4.1	7.2	179.9	−149.0	30.9	15	481	92	156	1	14
	C4	2.8	6.0	178.7	−190.9	−12.2	1	285	101	203	131	183
	C5	6.7	5.9	178.1	−173.1	5.0	1	353	244	258	94	32
	C6	4.3	7.3	179.2	−148.3	30.9	8	446	60	146	57	3
	C7	7.7	7.1	179.5	−146.6	32.8	10	270	110	52	75	9
	C8	3.7	7.1	181.4	−151.3	30.1	0	436	41	161	20	159
	C9	3.8	6.8	189.7	−224.4	−34.6	1	427	118	186	324	323
	C10	3.6	7.1	183.5	−178.7	4.8	1	464	138	269	74	19
	C11	4.0	5.6	178.7	−163.1	15.6	2	510	237	109	59	9

[a]The distance between the proton ($H^{\varepsilon 2}$ atom) of Glu73 and the β-(1,4)-glycosidic oxygen (O4) atom linking GlcNAc residues D and E. [b]The distance between the hydrogen atom (H^{α}) of Gly90 and the β-(1,4)-glycosidic oxygen (O4) atom linking GlcNAc residues D and E. [c]LL: intermolecular energy (kcal mol^{-1}); LP: intramolecular energy (kcal mol^{-1}).

the distances ranged from 2.8–7.7 Å. As described above, we modelled the complexes in only a chair model. This result indicates that the GlcNAc residue D came close enough to the catalytic residue (Glu73) without distorting the sugar ring. The distances between the β-(1,4)-glycosidic oxygen (O4) atom linking GlcNAc residues D and E, and the Cα atom in Gly90 which corresponded to Asp52 in HEL ranged from 5.6–7.3 Å, too great for any interactions to occur. Thus there was no amino acid residue corresponding to Asp52 which would act to stabilize the oxocarbenum ion in GEL. The average numbers of hydrogen bonds in the GlcNAc residues B–G in the 200 sampled structures were 4, 417, 130, 167, 83 and 73, respectively. In GEL, the (GlcNAc)6 oligomer is hydrolysed between the GlcNAc residues D and E, and the GlcNAc residue C binds most strongly with the amino acid residues at subsite C in GEL. On the other hand, in HEL, the average numbers of hydrogen bonds in GlcNAc residues A–F were 182, 104, 426, 120, 95 and 26, respectively (*9*). In HEL, the (GlcNAc)6 oligomer is hydrolysed between GlcNAc residues D and E. The GlcNAc residue C interacts most strongly with the amino acid residues at subsite C in HEL. We considered that the GlcNAc residue C tightly binds to the amino acid residues at subsite C in HEL and GEL to stabilize the intermediate complexes.

Exploration of Amino Acid Residues Interacting with Substrate—Table 2 shows the amino acid residues interacting with each GlcNAc residue and the number of hydrogen bonds in the GEL–(GlcNAc)6 complexes.

The GlcNAc residue B interacted infrequently with Ser100, His101, Ala151 and Gly152 by hydrogen bonding among all of the complexes. The GlcNAc residue C frequently interacted with Asp97, His101 and Tyr147 among all of the complexes. As described above, the interaction between the GlcNAc residue C and the amino acid residues in subsite C was strongest among all of the GlcNAc residues. Glu73 as a catalytic residue for a proton donor interacted with GlnNAc residues D and E. Gly90, which corresponded to the Asp52 that stabilizes the oxocarbenium ion in HEL, did not interact with GlcNAc residues D and E frequently. The GlcNAc residues B–D interacted with the amino acid residues commonly in most of the complexes C1–C11. However, the GlcNAc residues E–G interacted with a variety of amino acid residues, which means that there are some binding modes in subsites E–G.

In Table 3, we compare the amino acid residues which were predicted to interact with the substrate by each method.

In the previous studies, the amino acid residues interacting with HEL were identified (*1–6*). To investigate the structurally conserved amino acid residues in the binding clefts between GEL and HEL, the main-chain atoms of Glu73 and Asp97 in GEL and Glu35 and Asn59 in HEL, which are located around the catalytic site, were superimposed. If the distance between the Cα atoms in each amino acid residue of GEL and HEL was <2.0 Å, they were assumed to be structurally corresponded. The corresponding amino acid residues are listed in the same row in this table. Trp62 (subsites B and C), Asn59 (subsite C), Trp63 (subsite C), Ala107 (subsite C), Glu35 (subsites D and E_R), Asp52 (subsite D), Gln57 (subsite E_L), Phe34 (subsite F_R) and Arg45 (subsite F_L) in HEL correspond to Ser100 (subsites B and C), Asp97 (subsite C), His101 (subsite C), Tyr147 (subsite C), Glu73 (subsites D and E_R), Gly90 (subsite D), Gln95 (subsite E), Arg72 (subsite F_R) and Arg87 (subsite F_L) in GEL, respectively. We refer to these amino acid residues as 'structurally conserved amino acid residues'.

According to the superimposition, Asp86 in GEL corresponds to Arg45 in HEL. Arg45 is considered to be one of the amino acid residues on the left side of the binding cleft in HEL. Thus, we suggest that there are two binding modes in GEL as in HEL. Judging from the locations of the amino acid residues, Asp86 and Arg87 might contribute to the substrate binding on the left side (subsite F_L); while, Glu24, Arg72, Glu73, His75, Gln95 and His166 might contribute to the substrate binding on the right side in GEL (subsite F_R). According to the binding modes of C1–C11, the complexes C1, C3–C7 and

Table 2. **Numbers of the hydrogen bonds between amino acid residues and (GlcNAc)6 oligomers in the 200 sampled structures.**

GlcNAc	Amino acid residue	Complex										
		C1	C2	C3	C4	C5	C6	C7	C8	C9	C10	C11
B	Ser100	0	4	1	0	0	0	6	0	0	0	1
	His101	0	0	1	1	0	0	4	0	0	1	0
	Ala151	2	0	1	0	1	8	0	0	1	0	1
	Gly152	0	0	12	0	0	0	0	0	0	0	0
C	Asp97	200	200	200	93	159	200	110	200	200	225	208
	Arg99	0	0	1	0	0	0	3	0	0	1	1
	Ser100	0	0	0	0	0	0	0	0	0	1	2
	His101	60	59	80	91	52	46	43	67	68	25	99
	Tyr147	200	200	200	101	142	200	114	169	145	200	200
	Asn148	0	0	0	0	0	0	0	0	14	12	0
D	Glu73	18	67	30	64	107	6	42	13	15	31	6
	Arg87	0	0	0	0	0	0	0	0	2	0	0
	Asn89	60	0	14	0	61	0	0	0	1	73	200
	Gly90	0	0	0	0	0	0	0	0	0	0	1
	Gln95	0	0	1	0	0	0	0	0	0	0	0
	Asp97	0	0	1	4	0	0	0	1	4	3	1
	Arg99	66	5	3	15	69	0	3	1	3	6	5
	Tyr147	3	4	2	3	0	1	0	2	2	3	1
	Asn148	17	43	39	13	7	20	25	23	88	18	22
	Thr165	0	0	1	0	0	33	39	0	2	3	0
	Tyr169	1	1	1	2	0	0	1	1	1	1	1
E	Arg72	0	0	0	0	0	58	28	93	79	0	0
	Glu73	23	53	20	22	0	77	4	23	31	8	0
	Gly85	0	0	0	0	0	0	0	0	0	1	0
	Asp86	4	102	1	4	0	0	0	0	0	0	0
	Arg87	94	0	123	101	67	0	1	0	0	97	31
	Gly88	0	0	0	0	52	0	0	0	0	98	0
	Asn89	2	0	0	0	20	0	0	0	0	9	0
	Gly90	0	0	0	0	7	0	1	0	1	1	2
	Gln95	11	9	12	75	112	1	2	8	63	54	76
	Asn148	0	0	0	1	0	8	0	5	5	0	0
	Thr165	1	0	0	0	0	2	1	21	7	1	0
	His166	0	0	0	0	0	0	15	11	0	0	0
F	Glu24	0	0	0	0	0	0	0	20	0	0	0
	Arg72	0	0	0	76	0	0	0	0	132	0	0
	Glu73	0	1	0	1	0	0	0	0	0	0	0
	His75	0	8	0	8	0	0	1	0	0	0	0
	Asp86	42	0	0	0	93	39	71	0	126	72	58
	Arg87	1	0	0	7	1	15	3	0	32	2	1
	Gln95	0	0	0	0	0	0	0	0	34	0	0
	His166	0	23	1	39	0	3	0	0	0	0	0
G	Pro23	0	48	0	17	0	3	0	155	0	1	0
	Glu24	0	4	0	112	24	0	0	4	172	0	0
	His75	0	0	0	54	0	0	0	0	3	7	0
	Lys78	0	0	0	0	0	0	0	0	37	0	0
	Asp86	1	0	14	0	8	0	9	0	111	11	9

C9–C11 were considered to be left-sided complexes, and the complexes C2 and C8 were considered to be right-sided complexes. Importantly, we newly found that Pro23, Glu24, His75, Lys78 and Asp86 were related to the subsite G. In addition, Pro23, Glu24, His75 and Lys78 were related to the right side (subsite G$_R$) and Asp86 was related to the left side (subsite G$_L$).

Is GEL a Retaining or Inverting Enzyme?—We next investigated whether GEL is a retaining or inverting enzyme using the complexes modelled in this study. As mentioned above, there is no second carboxylate in GEL that corresponds to Asp52 in HEL. Retaining enzymes require a carboxylate very near to GlcNAc residue D to stabilize the oxocarbenium ion. In HEL, this is provided

Table 3. **Comparison of the amino acid residues predicted to interact with the substrate by the following methods.**

Subsite	Method		
	X-ray structure of GEL–(GlcNAc)3[a]	Constructed structures of GEL–(GlcNAc)6[b]	Previous studies in HEL[c]
A	–	–	Asp101
B	–	–	Asp101
	–	Ser100	Trp62
	His101	His101	–
	(Ile119)	–	–
	–	Ala151	–
	–	Gly152	–
C	Asp97	Asp97	Asn59
	(Ser100)	Ser100	Trp62
	His101	His101	Trp63
	Tyr147	Tyr147	Ala107
	–	–	Trp108
	–	Arg99	–
	–	Asn148	–
D	Glu73	Glu73	Glu35
	–	Gly90	Asp52
	–	–	Gln57
	(Asn148)	Asn148	–
	–	Arg87	–
	–	Asn89	–
	–	Gln95	–
	–	Asp97	–
	–	Arg99	–
	–	Tyr147	–
	–	Thr165	–
	–	Tyr169	–
E	–	Glu73	Glu35(ER)
	–	–	Asn44(EL)
	–	Gln95	Gln57(EL)
	–	–	Val109(ER)
	–	Arg72	–
	–	Gly85	–
	–	Asp86	–
	–	Arg87	–
	–	Gly88	–
	–	Asn89	–
	–	Gly90	–
	–	Asn148	–
		Thr165	
	–	His166	–
F	–	Arg72 (FR)	Phe34(FR)
	–	–	Asn37(FR)
	–	–	Arg114(FR)
	–	Arg87 (FL)	Arg45(FL)
	–	–	Asn46(FL)
	–	–	Thr47(FL)
	–	Glu24 (FR)	–
	–	Glu73 (FR)	–
	–	His75 (FR)	–
	–	Asp86 (FL)	–
	–	Gln95 (FR)	–
	–	His166 (FR)	–
G	–	Pro23 (GR)	–
	–	Glu24 (GR)	–
	–	His75 (GR)	–
	–	Lys78 (GR)	–
	–	Asp86 (GL)	–
Total	7	24	17

The GlcNAc residues A–F correspond to the subsites A–F in HEL, and the GlcNAc residues B–G correspond to the subsites B–G in GEL, respectively.

[a]The amino acid residues interacting with the (GlcNAc)3 oligomer in GEL by using the X-ray structure of the GEL–(GlcNAc)3 complex (PDB id: 154L) (*11*). The amino acid residues in parenthesis interact with the (GlcNAc)3 oligomer by the solvent-mediated interactions. [b]The amino acid residues predicted to interact with (GlcNAc)6 oligomers in GEL by using the GEL–(GlcNAc)6 complexes modelled by the systematic conformational search and MD simulation. [c]The amino acid residues predicted to interact with (GlcNAc)6 oligomer in HEL by using the X-ray structure of the HEL–(GlcNAc)3 complex and the previously reported binding simulations of the HEL–(GlcNAc)6 complex (*1–6*).

by Asp52. In contrast, inverting enzymes may require a second carboxylate which is farther away from the substrate; this relatively large separation allows room for an attacking water molecule between the second carboxylate and the α-side of the substrate. Indeed, it has been observed that the distances between the acidic residue and the second carboxylate are larger for inverting enzymes than for retaining enzymes. Monzingo *et al*. showed the structural similarity among barley chitinase, bacterial chitosanase, and goose (GEL), hen (HEL) and bacteriophage (T4L) lysozymes. They indicated that Glu89 located on the first β-strand in barley chitinase, Asp40 located on a loop between the first and second β-strands in bacterial chitosanase and Asp20 located on the first β-strand in T4L were second carboxylate based on the studies by Andersen *et al*. (*16*), Hollis *et al*. (*17*), Boucher *et al*. (*18*) and Kuroki *et al*. (*19*). There is sufficient room to accommodate a water molecule between these amino acid residues and which attack the substrate from the α-side. They therefore suggested that barley chitinase, bacterial chitosanase and T4L were inverting enzymes. Kuroki *et al*. (*20*) proved the inverting mechanism of T4L by product analysis. Monzingo *et al*. (*8*) considered that, if GEL was an inverting enzyme, then the second carboxylate must be far enough away to accommodate the attacking water, and proposed that Asp86 and Asp97 in GEL could be candidates for the second carboxylate. Asp86 on the loop between the first and second β-strand appears to be well positioned to serve as the catalytic base for inverting hydrolytic mechanism. Asp97 is also a plausible candidate because it occupies a position similar to Ser120 of chitinase, which is also implicated in positioning the attacking water in that enzyme. Thus they assumed that GEL would be an inverting enzyme, although there was no structural evidence.

In the X-ray structure of GEL in the free state (PDB id: 153L) (*11*), both of the distances of Glu73:C^{α}–Asp86:C^{α} and Glu73: C^{α}–Asp97: C^{α} are 11.2 Å. These distances are too great to act as retaining enzyme. Marcotte *et al*. (*21*) analysed the X-ray structure of an anti-fungal chitosanase and proposed the energy-minimized model for the complex between chitosanase and hexaglucosamine. In addition, they proposed a model for the inverting mechanism. However, the model was built only using the complex constructed by energy minimization, and the structural evidence for the attacking water molecule was not mentioned (*21*). Here, we searched for water molecules within 3.5 Å of the C1 carbon in the GlcNAc residue D, and each of $O^{\delta 2}$ atom in Asp86 and Asp97 among the complexes C1–C11. We then found a water molecule in each structure of the complexes C2, C4, C7 and C9, respectively. The numbers of the complexes accommodating the water molecules were 32, 67, 4 and 6 in complexes C2, C4, C7 and C9, respectively. These results indicated that a water molecule has the potential to enter into the space between the $O^{\delta 2}$ atom in Asp97 and the C1 carbon in the GlcNAc residue D. In the other complexes (C1, C3, C5, C6, C8, C10 and C11), there was no water molecule between the $O^{\delta 2}$ atom in Asp97 and the C1 carbon in GlcNAc residue D. As shown in Table 1, in the complex C4, the average distance between the β-(*1*,*4*)-glycosidic oxygen (O4) atom linking GlcNAc D and E and the proton in Glu73 was close to 2.8 Å, which is enough to form a hydrogen bond (*2*). In addition, the water molecule is located on the α-side of GlcNAc residue D. According to these results, we consider that the complex C4 might be productive. Figure 1 shows the water molecule which is close to the $O^{\delta 2}$ atom in Asp97 and the C1 carbon in the GlcNAc residue D in the complex C4.

The distance between the water molecule and the $O^{\delta 2}$ atom in Asp97 was less than that between the water molecule and the C1 carbon in GlcNAc residue D. We showed the structural evidence indicating that there is enough room for a water molecule in GEL to accommodate and attack the C1 carbon from the α-side of the substrate. Consequently, we suggested that GEL would be an inverting enzyme.

Comparison of the Binding Clefts between GEL and HEL—We consider that the binding of the acceptor molecule towards the left side of the binding cleft would be related to the transglycosylation mechanism in HEL. Therefore, we compared the structural differences of the binding cleft in GEL and HEL by superimposing the main-chain atoms of the 'structurally conserved amino acid residues' in Fig. 2.

The solvent-accessible surfaces of GEL and HEL are shown in Fig. 2a and b, respectively. The red lines show the schematic form of the binding cleft around the subsites D–F in HEL and subsites E–G in GEL, respectively. Apparently, the width of the binding cleft of GEL was narrower than that of HEL. The ribbon models of GEL and HEL are shown in Fig. 2c and d, respectively. As shown in Fig. 2d, HEL has two structural domains, the α-domain involving residues 1–35 and 85–129 and the β-domain involving residues 34–84 (*22*). In GEL, the region corresponding to the α-domain of

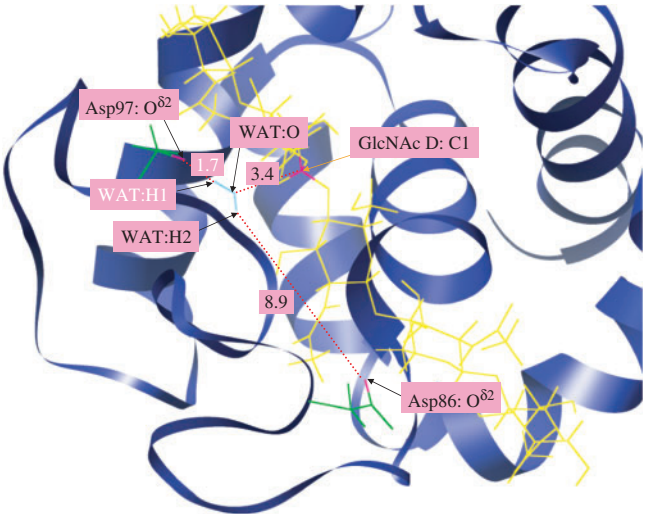

Fig. 1. **The water molecule which is close to the $O^{\delta 2}$ atom in the Asp97 and the C1 carbon in the GlcNAc residue D in the complex C4.** GEL and the (GlcNAc)6 oligomer are coloured with blue and yellow, respectively. The atoms coloured with pink are the C1 carbon in the GlcNAc residue D, the $O^{\delta 2}$ atom in Asp97 and the $O^{\delta 2}$ atom in Asp86. The water molecule (WAT) is coloured with sky blue. The numbers are the distances between each pair of atoms.

HEL is much larger than that of HEL. The hydrolytic activity of GEL for the GlcNAc oligomer is lower than that of HEL (*12*, *13*). In the hydrolytic reaction of HEL, the substrate binds towards the right side of the binding cleft. In GEL, the region corresponding to the α-domain of HEL is very large, and thus the binding cleft around subsites E–G of GEL would be narrower than that of HEL. Such a narrow binding cleft might decrease the efficiency of the substrate binding towards the right side and thereby the hydrolysis.

DISCUSSION

In this study, we performed a systematic conformational search to model the GEL–(GlcNAc)6 complexes because there was a high degree of freedom in (GlcNAc)6 oligomer. Pincus *et al*. (*4–6*) modelled HEL–(GlcNAc)6 complexes systematically, but the enzyme was held rigidly, and the substrate was allowed to move only within the binding cleft and to change conformation only during the energy minimization in order to reduce the computational time. We performed MD simulations for the GEL–(GlcNAc)6 complexes to identify the amino acid residues interacting with the (GlcNAc)6 oligomer precisely, because MD simulation gives much structural information that is difficult to obtain experimentally. Moreover, we found the amino acid residues related to subsites. In our future study, it is necessary to prove that by experimental method, such as site-directed mutagenesis. We also found two distinct binding modes corresponding to the right and left sides in HEL, and the GlcNAc residue D could bind towards the right side without distortion. The catalytic residue of GEL (Glu73) corresponding to that of HEL (Glu35) included in the α-domain is also

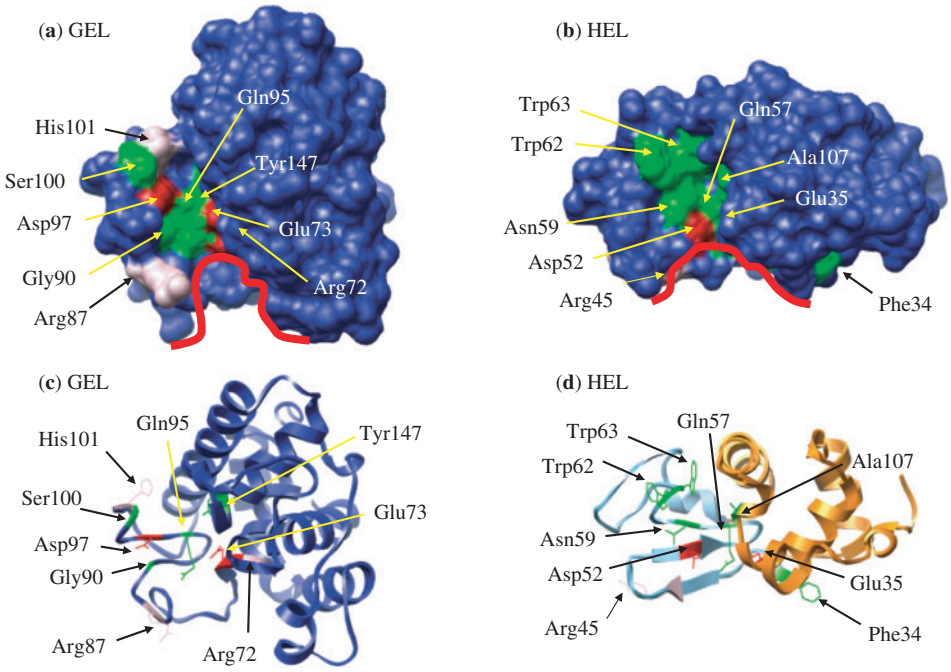

Fig. 2. **Structural differences of the binding clefts between GEL and HEL.** (a) The solvent-accessible surface of GEL (PDB id: 153L) (*11*). (b) The solvent-accessible surface of HEL (PDB id: 1LZC) (*27*). The amino acid residues coloured with red, pink and green are acidic, basic and neutral, respectively. (c) Ribbon model of GEL. (d) Ribbon model of HEL. The amino acid residues are 'structurally conserved'. The orange and sky blue ribbons in (d) indicate the α-domain and β-domain, respectively.

located on the same position in the right side (Fig. 2). If the catalytic reaction mechanisms of hydrolysis might be same between GEL and HEL, the different size of the α-domains might be affecting the hydrolysis for the GlcNAc oligomer.

To better understand the role of Asp86 and Asp97 in enzymatic activity, we investigated the conservation of Asp86, Asp97 and Glu73 in goose-type lysozyme. The 26 amino acid sequences of goose-type lysozymes were found in the public databases, UniProt (*23*) and pdbseqres (*24*). These amino acid sequences were from various organisms, such as tunicate, scallop, goose, swan, cassowary, rhea, ostrich, salmon, cod, flounder, perch, grouper, blowfish and carp. These sequences were aligned by CLUSTALW (*25*), and both Glu73 and Asp97 were found to be completely conserved (data not shown). On the other hand, Asp86 was mutated in three sequences of cod (into Pro), carp (into Pro) and tunicate (into Lys). Structurally, we identified that Glu73 acts as a proton donor (general acid), and that Asp86 and Asp97 are considered as candidates for the second carboxylate. Furthermore, we found a water molecule between Asp97 and GlcNAc residue D in the GEL–(GlcNAc)6 complexes. Therefore, the role of Asp97 might be more important for the catalytic reaction than that of Asp86.

To investigate the reason why GEL does not catalyse the transglycosylation, we focused on the width of the binding clefts in GEL and HEL. It is difficult to quantify the bulk of the binding cleft. Therefore, we calculated the distances between the oxygen (O1) atom at the reducing end among all pairs of the (GlcNAc)6 oligomers bound in the HEL and GEL to compare the width of the binding clefts. Figure 3 shows the distribution of

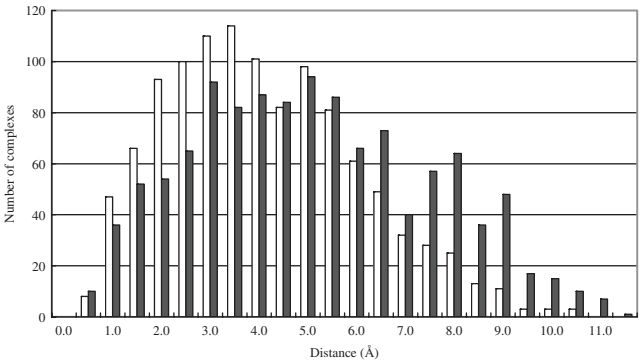

Fig. 3. **Distribution of the distances of the O1 atoms at the reducing end in the complexes HEL–(GlcNAc)6 and GEL–(GlcNAc)6.** The white and black bars indicate the number of GEL–(GlcNAc)6 and HEL–(GlcNAc)6 complexes, respectively.

the distances in the 48 GEL–(GlcNAc)6 complexes and 49 HEL–(GlcNAc)6 complexes (*9*).

The average distances between the oxygen (O1) atom at the reducing end in the complexes of HEL–(GlcNAc)6 and GEL–(GlcNAc)6 were 4.8 Å (SD = 2.41) and 3.9 Å (SD = 1.98), respectively. The average distance of GEL was shorter than that of HEL, which indicated that the width of the cleft of GEL was narrower than that of HEL. We consider that this difference in the width of the binding clefts affected the binding process for the acceptor molecule in the transglycosylation. Based on these results, we propose the reaction schemes for HEL and GEL and infer the structural reason why GEL does not catalyse the transglycosylation in Fig. 4.

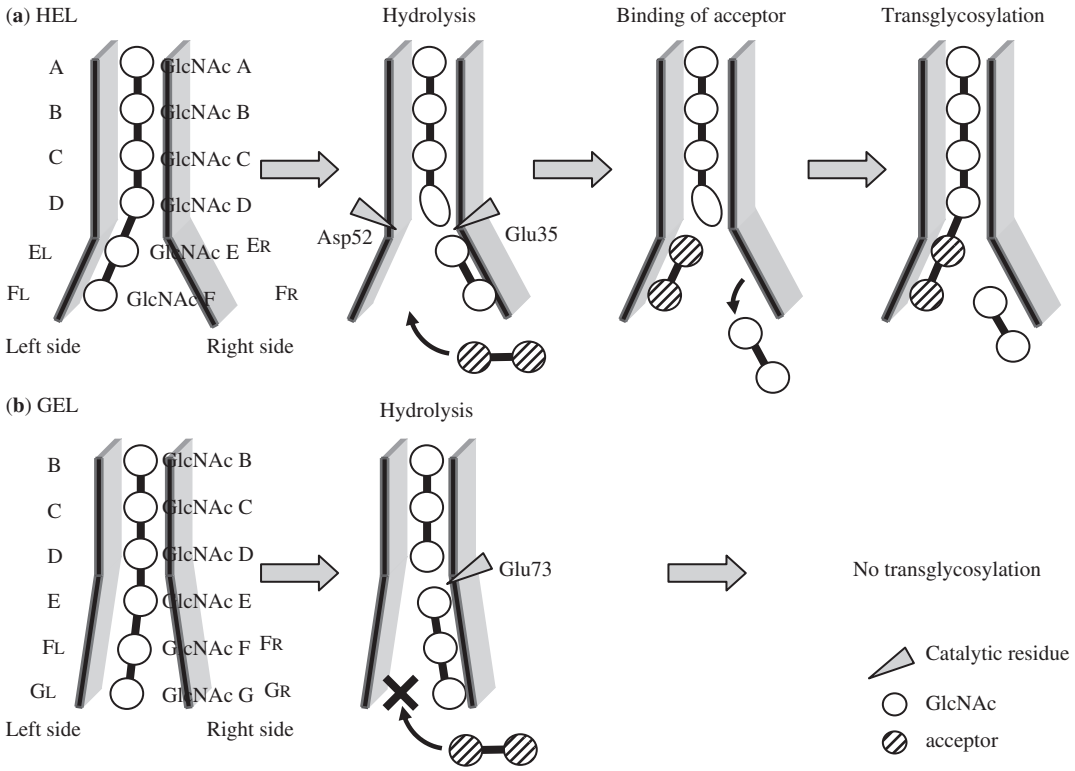

Fig. 4. **Inferred catalytic reaction scheme for HEL and GEL.** (a) Inferred catalytic reaction scheme for HEL. (b) Inferred catalytic reaction scheme for GEL. The thick grey lines represent the subsites of lysozymes. The white and lined circles represent the GlcNAc residue and acceptor molecule (GlcNAc oligomer), respectively.

As shown in Fig. 4a, the (GlcNAc)6 oligomer first binds to the left side of the binding cleft in HEL, rendering the complex non-productive. Next, the (GlcNAc)6 oligomer shifts to the right side to be productive. Glu35 would act as a proton donor, and Asp52 would act as a second carboxylate. In the catalytic reaction of the hydrolysis, a water molecule attacks the oxocarbenium ion from the β-side to create a product GlcNAc residue with the β-anomeric configuration. Therefore, HEL is a retaining enzyme. After the hydrolysis, the acceptor molecule would bind to the (GlcNAc)4 oligomer that remained in the binding cleft, thereby catalysing the transglycosylation (*26*). As shown in Fig. 4b, the (GlcNAc)6 oligomer would first bind to the left side of GEL. Next, the (GlcNAc)6 oligomer shifts to the right side. In the hydrolytic reaction, Glu73 would act as a proton donor, and a water molecule would attack the C1 carbon from the α-side to create a product GlcNAc residue with the α-anomeric configuration. At this time, Asp97 would act as a second carboxylate. Thus we considered that GEL would be an inverting enzyme. However, the room for the binding of the acceptor was not sufficient, and thus the acceptor molecule could not bind to the binding cleft (left side; subsites F_L and G_L). This might be one of the reasons why GEL does not catalyse the transglycosylation. We indicate the reaction scheme of GEL based on the modelled complexes and suggest that the room for the binding of the acceptor molecule is important for the efficiency of the catalysis of the transglycosylation.

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CONFLICT OF INTEREST

None declared.

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