X-ray structure of Glu 53 human lysozyme

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Abstract

The three-dimensional structure of a modified human lysozyme (HL), Glu 53 HL, in which Asp 53 was replaced by Glu, has been determined at 1.77 Å resolution by X-ray analysis. The backbone structure of Glu 53 HL is essentially the same as the structure of wild-type HL. The root mean square difference for the superposition of equivalent C α atoms is 0.141 Å. Except for the Glu 53 residue, the structure of the active site region is largely conserved between Glu 53 HL and wild-type HL. However, the hydrogen bond network differs because of the small shift or rotation of side chain groups. The carboxyl group of Glu 53 points to the carboxyl group of Glu 35 with a distance of 4.7 Å between the nearest carboxyl oxygen atoms. A water molecule links these carboxyl groups by a hydrogen bond bridge. The active site structure explains well the fact that the binding ability for substrates does not significantly differ between Glu 53 HL and wild-type HL. On the other hand, the positional and orientational change of the carboxyl group of the residue 53 caused by the mutation is considered to be responsible for the low catalytic activity (ca. 1%) of Glu 53 HL. The requirement of precise positioning for the carboxyl group suggests the possibility that the Glu 53 residue contributes more than a simple electrostatic stabilization of the intermediate in the catalysis reaction.

Keywords: enzyme mechanism; human lysozyme; mutant structure; protein structure; X-ray structure

Human lysozyme (EC 3.2.1.17, abbreviated to HL), consisting of 130 amino acid residues, is one of the chicken-type lysozymes that hydrolyzes polysaccharides of bacterial cell walls. Since the three-dimensional structure of chicken lysozyme (CL) has been revealed by X-ray analysis (Blake et al., 1965), the catalytic properties of the enzyme have been extensively studied in relation to the geometry of the active site and substrate binding (Imoto et al., 1972; Jollès & Jollès, 1984; Kirby, 1987). The active site of the CLs consists of six subsites for sugar binding, which are designated A–F. The active center is located in between the D and E subsites. In HL the carboxyl groups in the two amino acid residues, Glu 35 and Asp 53, which is Asp 52 in CL, have been considered to be directly involved in the catalysis reaction. The carboxyl group of Glu 35 has been suggested to act as a proton donor that produces an oxo-carbonium ion as a reaction intermediate by protonation of the substrate saccharides (Blake et al., 1967; Vernon, 1967; Chipman & Sharon, 1969). The role of Asp 53 remains controversial, and some models for the reaction

mechanism have been discussed. The ionized carboxyl group of the Asp residue has been suggested to act as a counter ion for the electrostatic stabilization of the intermediate oxo-carbonium ion. In CL, the modification of Asp 52 to Asn, which has a neutral side chain group, has been found to reduce activity to a few percent (Kuroki et al., 1986; Malcolm et al., 1989). This interpretation for the role of the Asp residue has been widely accepted. However, another model has suggested the possibility that the carboxyl group acts as a nucleophile to form a covalent intermediate (Kirby, 1987). Although there has been no direct evidence for the intermediate compound, the proposed models have stressed the importance of the ionized carboxyl group of the Asp residue. On the other hand, we have found that the mutation of Asp 53 to Glu in HL also reduces activity to ca. 1% of the wild-type HL (Muraki et al., 1987). Because the side chain of Glu is longer by one methylene group than the side chain of Asp, the mutation changes the position and orientation of the carboxyl group of residue 53. The low enzyme activity of Glu 53 HL has been ascribed to the displacement of the carboxyl group. However, it is also possible that the larger side chain group of Glu 53 induces a structural change in the

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Fig. 1. The backbone structure of Glu 53 HL. The side chain of the Glu 53 residue is shown by thin lines.

active site cleft. Therefore, knowledge of the precise geometry of the active site region is required for the evaluation of the catalytic property of Glu 53 HL. In a previous paper, we discussed the possible mechanism of enzyme action based on biochemical and physicochemical data including preliminary X-ray data (Muraki et al., 1991). In this paper, we report details of the refined structure of Glu 53 HL and discuss the role of residue 53.

Results

The backbone structure of Glu 53 HL is shown in Figure 1 and Kinemage 1. This structure is nearly identical to that of wild-type HL (Artymiuk & Blake, 1981). The calculation for least-squares superposition of equivalent C α atoms gives a root mean square (rms) difference of 0.141 Å, which is less than the estimated coordinate error (ca. 0.2 Å). This indicates that the replacement of residue 53 from Asp to Glu does not affect the backbone structure of HL. The Glu 53 residue is located in the β -strand of Arg 50–Gly 55 (Fig. 1), which forms an antiparallel β -sheet with two strands, Ala 42–Asn 46 and Gln 58–Ser 61 (Kinemage 2). The carboxyethyl group of Glu 53 was determined on the $F_o - F_c$ map as shown in Figure 2. The well-shaped electron density confirmed the side chain group of Glu 53. The temperature factor averaged for the peptide group and side chain group is shown in Figure 3. The average

temperature factor is 15.4 Å² for all protein atoms, 12.2 Å² for the peptide groups, and 18.6 Å² for the side chain groups. The carboxyethyl group of Glu 53 gives a temperature factor of 26.7 Å². A relatively high temperature factor is observed in the regions Asn 46–Arg 50, Pro 71–Asn 75, Val 100–Gly 105, and the C-terminal region. The Asn 46–Arg 50 region includes a β -turn connecting two β -strands. The Pro 71–Asn 75 region is part of a loop. The Val 100–Val 105 region connects a long α -helix of Ile 89–Val 99 and a short 3_{10} -helix of Gln 104–Trp 109. In contrast, three long α -helix regions, Glu 4–Gly 16, Leu 25–Gly 37, and Ile 89–Val 99, exhibit a relatively low temperature factor.

Intramolecular contacts of side chain groups less than 3.2 Å are given in Table 1. There are 41 intramolecular contacts, most of which are considered to be hydrogen bonds or salt linkages. Side chain groups of 21 residues form hydrogen bonds with main chain peptide groups. Side chain groups of nine residues are involved in the intermolecular contacts with six neighboring molecules. The structure model includes 112 water molecules. Most of the water molecules are bound on the surface of the protein molecule. There are 29 water molecules that form at least two hydrogen bonds with the protein, and 8 water molecules that are involved in the formation of a hydrogen bond bridge linking two protein molecules.

Fig. 2. An $F_o - F_c$ map showing the electron density of the side chain of Glu 53. F_c and phases were calculated without the contribution of the side chain of residue 53.

Discussion

indicated that the conformation of flexible regions or side chains located at the molecular surface is affected by the conditions of crystallization, such as pH, temperature, inorganic salts for precipitation, etc. (Moult et al., 1976; Berthou et al., 1983; Hogle & Sundaralingam, 1983). In the case of wild-type HL, the cell dimensions significantly differ between the crystal obtained from the 7 M ammonium nitrate solution at pH 4.5 (Blake et al., 1983) and that from the 2.5 M sodium chloride solution at pH 6.0 (Inaka et al., 1991). The crystal form of Glu 53 HL is isomorphous with that of wild-type HL obtained at pH 4.5, and the rms difference (0.141 Å) between the equivalent C α atoms is less than the estimated coordinate error (ca. 0.2 Å). As shown in Table 1, most of intermolecular contacts less than 3.2 Å in Glu 53 HL are also found in the structure of wild-type HL. Therefore, we discuss the difference between Glu 53 HL and wild-type HL only in relation to the mutation at residue 53, although the structural model might also be affected by the resolution and quality of reflection data and the refinement procedure.

In CLs, two residues carrying a carboxyl group, Glu 35 and Asp 52, have been suggested to be directly involved in the catalytic reaction. Glu 35 has been considered to act as a proton donor because of its high pK_a value. On the other hand, the role of residue 53 remains controversial. To elucidate the role of this residue, modification of its side chain has been intensively investigated. Asp 52 of CL has been specifically esterified (Parsons & Raftery, 1969), treated with carbodiimide (Lin & Koshland, 1969), and converted to homoserine (Eshdat et al., 1974) or Asn (Kuroki et al., 1986; Malcolm et al., 1989). The altered CLs have shown that substrate-binding ability is only slightly affected by this modification, but the catalytic activity of CL is reduced to a few percent of that of wild-type CL. Kuroki et al. (1986) have reported that the dissociation constant for the binding of hexa-*N*-ace-

Table 1. Intra- and intermolecular contacts ($d < 3.2$ Å) involving side chains

Distance (Å)		Distance (Å)	
Intramolecular contacts ^a			
N(Lys 1)---OG1(Thr 40)	2.7*	OD2(Asp 49)---OG(Ser 51)	3.1*
NE(Arg 41)	2.9	OG(Ser 51)---ND2(Asn 60)	3.1*
OD1(Asp 87)	3.2*	N(Thr 52)---OG(Ser 61)	2.9*
NZ(Lys 1)---OE2(Glu 7)	3.0*	OH(Tyr 54)---OD2(Asp 67)	2.8*
NH1(Arg 5)---O(Gln 126)	3.1*	O(Gly 55)---NE2(Gln 58)	2.9*
NH2(Arg 5)---O(Tyr 124)	3.2*	O(Phe 57)---NE1(Trp 109)	2.9*
O(Gln 126)	3.0*	OG(Ser 61)---OG1(Ser 70)	2.8*
O(Glu 7)---OG1(Thr 11)	2.8*	OD1(Asp 67)---N(Lys 69)	3.1*
NH2(Arg 10)---O(Cys 128)	3.1*	OG1(Thr 70)	2.8*
O(Leu 15)---NZ(Lys 97)	2.8*	OD1(Asn 75)---N(Leu 79)	3.0
O(Met 17)---NE1(Trp 28)	3.0	O(Leu 79)	3.1*
OH(Tyr 20)---NE(Arg 101)	3.0	O(Ala 76)---ND1(His 78)	2.7
OG(Ser 24)---ND2(Asn 27)	2.9*	O(Ala 83)---NE2(Gln 86)	2.9*
OD1(Asn 27)---NE1(Trp 112)	3.1*	NE2(Gln 86)---OD1(Asp 91)	3.0*
O(Ala 32)---OG(Ser 36)	3.1*	NH2(Arg 98)---OD2(Asp 102)	3.0*
NE1(Trp 34)---OH(Tyr 124)	3.0*	O(Arg 107)---NE(Arg 113)	2.9
OG(Ser 36)---O(Ile 56)	2.7*	NH1(Arg 113)	2.9
O(Ala 42)---NE2(Gln 58)	3.1*	O(Asn 114)---NH1(Arg 119)	2.9*
ND2(Asn 44)---OD1(Asn 46)	2.8	O(Asp 120)---OE1(Gln 123)	3.0
O(Asp 49)---NH1(Arg 62)	2.8*	NE2(Gln 123)	2.9
OD1(Asp 49)---OG(Ser 51)	3.0*		
Distance (Å)		Symmetry operator	
Intermolecular contacts			
NH2(Arg 10)---O(Arg 14)	3.1	$\frac{1}{2} - x,$	$1 - y, -\frac{1}{2} + z$
NH2(Arg 41)---N(Asp 120)	3.0	$-\frac{1}{2} + x,$	$\frac{1}{2} - y, 1 - z$
O(Ala 47)---NH1(Arg 119)	2.8	$-\frac{1}{2} + x,$	$-y, \frac{1}{2} + z$
NH2(Arg 119)	3.2	$-\frac{1}{2} + x,$	$-y, \frac{1}{2} + z$
O(Glu 48)---NH1(Arg 119)	3.0	$-\frac{1}{2} + x,$	$-y, \frac{1}{2} + z$
NH1(Arg 62)---OD1(Asn 114)	2.9	$-\frac{1}{2} + x,$	$-y, \frac{1}{2} + z$
NH2(Arg 62)---OD1(Asn 114)	2.8	$-\frac{1}{2} + x,$	$-y, \frac{1}{2} + z$
ND2(Asn 114)	3.0	$-\frac{1}{2} + x,$	$-y, \frac{1}{2} + z$

^a An asterisk indicates the contact found in the structure of wild-type HL.

tylchitohexaose is similar between wild-type CL and the Asn 52 CL prepared by chemical modification. In contrast, Malcolm et al. (1989) have prepared Asn 52 CL by site-directed mutagenesis. They pointed out the possibility of conformational change because the dissociation constant for the tri-*N*-acetylchitotriose complex of recombinant Asn 52 CL has been found to be smaller than that of wild-type CL. These results have confirmed that the carboxyl group is essential for the catalytic reaction. On the other hand, Muraki et al. (1987) have demonstrated that replacement of the Asp 53 of HL to Glu reduces enzyme activity to ca. 1% of wild-type HL in spite of the presence of the carboxyl group. Glu 53 HL and wild-type HL have been found to have almost the same binding ability for oligosaccharides. The dissociation constants for the complexes with tri-*N*-acetylchitotriose and hexa-*N*-acetylchito-hexaose do not significantly differ between Glu 53 HL and wild-type HL (Muraki et al., 1991).

The present X-ray structure of Glu 53 HL shows that the conversion of Asp 53 to Glu does not significantly change the backbone structure of the enzyme. The geometry of the substrate binding region is largely conserved between Glu 53 HL and wild-type HL as shown in Figure 5 and Kinemage 3. Except for residue 53, the side chain with the catalytically important residues, Glu 35, Tyr 63, Trp 64, etc., is in the same orientation as that observed in the wild-type HL. This result indicates that the replacement of residue 53 does not induce a large structural change in the active site region. Some changes are observed in the formation of hydrogen bonds involving the residue 53. In wild-type HL, Asp 53 forms hydrogen bonds with Asn 44, Asn 46, and Asn 60. Asn 46 and Asn 60 are also hydrogen-bonded to Ser 51. In Glu 53 HL, the side chain groups of Asn 46 and Asn 60 have a conformation similar to that found in wild-type HL, and the hydrogen bonds with Ser 51 are conserved. The carboxyl group of Glu 53

Fig. 5. A local structure including the Glu 53 residue. The structure of wild-type HL (Artymiuk & Blake, 1981) is superimposed and shown by thin lines.

cannot form hydrogen bonds with Asn 46 and Asn 60, but the side chain of Asn 44 changes its conformation so as to form a hydrogen bond with Glu 53. The replacement of residue 53 also changes the geometrical relationship with the carboxyl group of Glu 35, which is another reactive group in the active center. In wild-type HL, the distance between the C δ of Glu 35 and C γ of Asp 53 is 7.2 Å. On the other hand, in Glu 53 HL, the corresponding distance is 6.5 Å, which is shorter by 0.7 Å. However, the shift in the carboxyl group of residue 53 does not seem to affect the carboxyl group of residue 35. The effect of the replacement on the pK_a value of the carboxyl group of Glu 35 has been estimated to be small by theoretical calculation based on the preliminary structure of Glu 53 HL (Muraki et al., 1991). Therefore, the low enzymatic activity of Glu 53 HL can be ascribed to the change in the interaction of the carboxyl group of residue 53 with the substrate.

The importance of the precise disposition of the carboxyl group at the active center has also been demonstrated in site-directed mutagenesis studies of other enzymes. A drastic change in enzyme activity caused by the mutation between Glu and Asp has been observed in triosephosphate isomerase (Straus et al., 1985) and *Escherichia coli* maltodextrin phosphorylase (Schinzel & Palm, 1990). In those enzymes, the catalytic activity reduces to less than 0.5% when Glu is replaced by Asp.

In the reaction mechanism that has been proposed based on the X-ray structure of CL and its complex with tri-*N*-acetylchitotriose (Blake et al., 1967; Chipman & Sharon, 1969), the carboxyl group of Asp 52 has been suggested to play a role in stabilizing the reaction intermediate. In the transition state, a sugar residue bound to the D subsite has been suggested to be in a sofa form (Ford et al., 1974; Strynadka & James, 1991). If residue 53 in HL participates in stabilizing the intermediate oxo-carbonium ion electrostatically, the positional change of the carboxyl group caused by the mutation affects the contribution toward stabilization. The change in the geometric arrangements of the carboxyl group and the C-1 and O-5 atoms of the sugar residue may reduce the electrostatic effect to stabilize the transition state, resulting in the apparent low enzyme activity.

The structural model of the substrate complex of CL

suggests that a carboxyl oxygen atom of Asp 52 is ca. 3 Å from the C-1 atom of the substrate (Blake et al., 1967). The crystal structure of the CL complex with a transition-state analogue, the δ -lactone of tetra-*N*-acetylchitotetraose, has shown that the distance between the carboxyl group of Asp 52 and the C-1 atom of the sugar residue located at the D subsite is 2.4 Å (Ford et al., 1974). Recently, Strynadka and James (1991) have reported the refined structure of the CL complex with *N*-acetylmuramyl-*N*-acetylglucosaminyl-*N*-acetylmuramic acid, which occupies the B, C, and D subsites. In that structure, the carboxyl group of Asp 52 is located 3.2 Å from the C-1 atom of the *N*-acetylmuramic acid residue. If the same binding geometry can be assumed for HL, the replacement of Asp 53 with Glu causes unfavorably short contact with the substrate molecule. The carboxyl group of residue 53 points toward the pyranose ring located at the D subsite. The model-building study indicates that the distance between the carboxyl group of Glu 53 and the sugar residue will be less than 2 Å. Such unfavorable close contact may block the accommodation of the sugar residue at the D subsite or may induce a shift of the substrate molecule and/or conformational change of the side chain group of Glu 53. Even if binding to the D subsite is blocked, oligosaccharides can be bound by occupying mainly the A, B, and C subsites. If such nonproductive binding dominates the complex formation, the *k*_{cat} value for the hydrolysis of oligosaccharides could be decreased despite having a similar *K*_m value.

A reaction mechanism involving the formation of the enzyme-substrate covalent bond has also been discussed (Tsai et al., 1969; Craze et al., 1978). In this case, the carboxyl group of residue 53 should be located at such a position that it can attack the C-1 carbon atom of the substrate. Strynadka and James (1991) have shown that the carboxyl group of Asp 52 of CL can be 2.3 Å from the C-1 atom of the substrate but cannot approach more closely unless the hydrogen bond interactions with Asn 46 and Asn 59 are disrupted. They have concluded that covalent bond formation is unlikely because the lone-pair orbital of the carboxyl group is not favorably oriented with respect to the C-1 atom of the sugar residue. The shift of the carboxyl group caused by the mutation changes the geometrical relationship between the carboxyl group and

the substrate. This will severely impair covalent bond formation. Thus, the structure of the active site region of Glu 53 HL well explains why the Asp 53 to Glu mutation inhibits the catalytic reaction without affecting substrate binding. The markedly low enzyme activity of Glu 53 HL indicates that the position and orientation of the carboxyl group of the Asp 53 residue is essential for the catalytic reaction. The requirement for the precise positioning of the carboxyl group indicates that residue 53 contributes more than a simple electrostatic stabilization of the intermediate oxo-carbonium ion. The carboxyl group may be involved in a geometrically constrained interaction with the substrate, such as the formation of an incomplete covalent bond (Kirby, 1987).

Materials and methods

The preparation and purification of Glu 53 HL have been previously reported (Muraki et al., 1987). The lyophilized protein was dissolved in 3 M ammonium nitrate solution, pH 4.5 (25 mM sodium acetate buffer), to give a protein concentration of 20 mg/mL. Undissolved material was removed by centrifugation, and the supernatant was passed through a 0.45- μ m pore size filter. The sample solution was divided into a 50- μ L portion in a concave well and was equilibrated with 5 M ammonium nitrate solution. Crystals were grown at room temperature. Repeated seeding was required to obtain crystals of suitable size for X-ray experiments. The crystal was orthorhombic and the space group was $P2_12_12_1$ with $Z = 4$ ($V_m = 1.95 \text{ \AA}^3/\text{Da}$). Cell dimensions were $a = 57.13$, $b = 61.05$, and $c = 33.16 \text{ \AA}$. Diffraction data to a resolution of $1.77 \text{ \AA}$ were collected on an Enraf-Nonius FAST diffractometer with a GX21 generator (40 kV, 60 mA, and focal spot size of $0.3 \times 3 \text{ mm}$). Using two crystals, two data sets consisting of 38,963 and 36,299 reflections were collected and were reduced to 10,294 and 9,566 unique reflections with respective R -merge values of 0.080 and 0.084. The merge of the two data sets produced a data set of 10,597 reflections, which corresponds to 89.7% of the unique reflections with a resolution of $1.77 \text{ \AA}$.

The crystal form of Glu 53 HL was isomorphous with the crystal form of wild-type HL (Artymiuk & Blake, 1981). A set of protein coordinates of wild-type HL (Protein Data Bank, entry code 1LZ1) was used as an initial model for structural determination. After the preliminary refinement by a stereochemically constrained least-squares method, the side chain group of Glu 53 was determined from an $F_o - F_c$ map. The structure was refined by the simulated annealing method using the program X-PLOR (Brünger et al., 1987). A molecular dynamics simulation was run at 3,000 K for 1.0 ps with a time step of 1.0 fs. Then, the model system was cooled to 300 K, and the simulation was continued for 0.25 ps. Successive refinement of atomic coordinates and temperature factors by energy minimization gave an R -value of 0.246. Water mol-

ecules were picked from peaks on the $2F_o - F_c$ and $F_o - F_c$ maps. Energy minimization and map calculation were repeated twice, and 112 fully occupied water molecules were included in the final structural model. The refinement converged at an R -value of 0.179 for 9,566 reflections (81.7% of unique reflections) with $|F_o| > 3\sigma(F)$ in the resolution range of $10\text{--}1.77 \text{ \AA}$. The maximum values for positive and negative residual electron density were 0.38 and $-0.36 \text{ e \AA}^{-3}$, respectively, and no significant peaks were found on the $F_o - F_c$ map. The rms deviations of bond distances and angles from their ideal values were $0.012 \text{ \AA}$ and 2.75° , respectively. The coordinate error was estimated to be about $0.2 \text{ \AA}$ according to Luzzati (1952). The refined atomic parameters have been deposited in the Protein Data Bank, Brookhaven.

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