Origin of Carbohydrate Recognition Specificity of Human Lysozyme Revealed by Affinity Labeling[†,‡]

Michiro Muraki* and Kazuaki Harata

Biomolecules Department, National Institute of Bioscience and Human Technology, Tsukuba, Ibaraki 305, Japan

Naoki Sugita and Ken-ichi Sato

Faculty of Engineering, Kanagawa University, Yokohama, Kanagawa 221, Japan

Received June 4, 1996; Revised Manuscript Received August 6, 1996[⊗]

ABSTRACT: In order to reveal the origin of carbohydrate recognition specificity of human lysozyme by clarifying the difference in the binding mode of ligands in the active site, the inactivation of human lysozyme by 2′,3′-epoxypropyl β-glycoside derivatives of the disaccharides, *N*,*N*′-diacetylchitobiose [GlcNAc-β-(1→4)-GlcNAc] and *N*-acetyllactosamine [Gal-β-(1→4)-GlcNAc], was investigated and the three-dimensional structures of the affinity-labeled enzymes were determined by X-ray crystallography at 1.7 Å resolution. Under the conditions comprising 2.0×10^{-3} M labeling reagent and 1.0×10^{-5} M human lysozyme at pH 5.4, 37 °C, the reaction time required to reduce the lytic activity against *Micrococcus luteus* cells to 50% of its initial activity was lengthened by 3.7 times through the substitution of the nonreducing end sugar residue, GlcNAc to Gal. The refined structure of human lysozyme labeled by 2′,3′-epoxypropyl β-glycoside derivatives of *N*,*N*′-diacetylchitobiose (HL/NAG-NAG-EPO complex) indicated that the interaction mode of the *N*,*N*′-diacetylchitobiose moiety in substites B and C in this study was essentially the same as in the case of the complex of human lysozyme with the free ligand. On the other hand, the hydrogen-bonding pattern and the stacking interaction at subsite B were remarkably different between the HL/NAG-NAG-EPO complex and human lysozyme labeled by the 2′,3′-epoxypropyl β-glycoside of *N*-acetyllactosamine (HL/GAL-NAG-EPO complex). The reduced number of possible hydrogen bonds as well as the less favorable stacking between the side chain of Tyr63 in human lysozyme and the galactose residue in the HL/GAL-NAG-EPO complex reasonably explained the less efficient ability of the 2′,3′-epoxypropyl β-glycoside of *N*-acetyllactosamine as compared to that of *N*,*N*′-diacetylchitobiose as an affinity labeling reagent toward human lysozyme.

Residue-specific recognition of carbohydrate by proteins plays a key role in many biologically essential phenomena for the maintenance of homeostasis of living animals and plants, which include the defense action against microorganisms, cell to cell communication, and so on (Quiocho, 1986; Vyas, 1991). Human lysozyme (HL)[1] is an enzyme found in a variety of secretory human body fluids, especially in tears (Fleming, 1922). HL shows high affinity against either the β-1,4-linked homopolymer of *N*-acetylglucosamine [chitin; [GlcNAc-β-(1→4)-GlcNAc]$_n$] or the β-1,4-linked alternative copolymer composed of *N*-acetylmuramic acid and *N*-acetylglucosamine [bacterial cell wall polysaccharide; [Mur-NAc-β-(1→4)-GlcNAc]$_n$]. In the catalysis performed by the vertebrate lysozyme such as HL a substrate molecule is hydrolyzed in the active cleft composed of several subsites, in which each subsite recognizes the monomeric sugar residue unit (Imoto et al., 1972). The physiological role of human lysozyme has not yet been clarified (Jollès & Jollès, 1984); therefore, it is biochemically worthwhile to probe the possibility of interaction with the other saccharide sequences existing in biological sources, which have similarity in the chemical structure to that of the ordinary substrates. The *N*-acetyllactosamine sequence [Gal-β-(1→4)-GlcNAc] is often found as a component of several kinds of biologically important substances, such as N-linked and O-linked glycans in various glycoproteins (Marshall, 1972; Hokke et al., 1994) and the ABH type II blood group immunogenic determinant of some glycolipids (Lloyd et al., 1968). In spite of the overall similarity in chemical structure (Figure 1), *N*-acetyllactosamine is recognized by hen egg white lysozyme (HEWL) less strongly than *N*,*N*′-diacetylchitobiose, and its inhibitory effect on the lytic activity against *Micrococcus luteus* cells was reported to be the same level as the *N*-acetylglucosamine monomer (Shinitzky et al., 1966).

One of the most promising methods to introduce the substrate-type ligand molecule into the active site of the enzyme is the chemical modification by the affinity labeling reagent (Colman, 1990). The irreversible inhibition of the lytic activity of HEWL by the 2′,3′-epoxypropyl β-glycoside

[†] This work was supported by a grant from the Agency of Industrial Science and Technology, MITI, Japan.

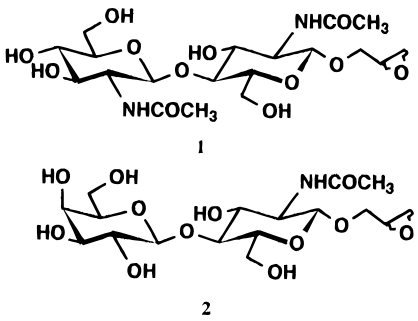
[‡] Coordinates have been deposited in the Brookhaven Protein Data Bank (filenames 1REX for native HL, 1REY for the HL/NAG-NAG-EPO complex, and 1REZ for the HL/GAL-NAG-EPO complex).

* To whom correspondence should be addressed. E-mail: muraki@nibh.go.jp. FAX: 81 298-54-6194.

[⊗] Abstract published in *Advance ACS Abstracts,* October 1, 1996.

[1] Abbreviations: HL, wild-type human lysozyme; GlcNAc, 2-acetamido-2-deoxy-D-glucopyranose; Gal, D-galactopyranose; Man, D-mannopyranose; *N*,*N*′-diacetylchitobiose, β-1,4-linked dimer of 2-acetamido-2-deoxy-D-glucopyranose; *N*-acetyllactosamine, 2-acetamido-2-deoxy-4-(β-D-galactopyranosyl)-D-glucopyranose; HL/NAG-NAG-EPO complex, HL affinity labeled by the 2′,3′-epoxypropyl β-glycoside of *N*,*N*′-diacetylchitobiose; HL/GAL-NAG-EPO complex, HL affinity labeled by the 2′,3′-epoxypropyl β-glycoside of *N*-acetyllactosamine; HEWL, hen egg white lysozyme; rms, root mean square.

S0006-2960(96)01318-9 CCC: $12.00



1

2

FIGURE 1: Chemical structure of the ligands used in this study: **1**, NAG-NAG-EPO; **2**, GAL-NAG-EPO. Detailed names of the compounds are described in the text.

of *N*-acetyl-D-glucosamine oligomers (Thomas et al., 1969) and the identification of the attachment site of the affinity label by X-ray crystallography (Moult et al., 1973) have been reported; however, no refined structure of the affinity-labeled enzyme using the high-resolution data is available. So far, the X-ray structure of the bacterial cell wall trisaccharide, GlcNAc-β-(1→4)-MurNAc-β-(1→4)-GlcNAc, bound in subsites B, C, and D of HEWL at 2.5 Å resolution has been reported (Kelly et al., 1979). The saccharide binding modes in human lysozyme cocrystallized with hexa-*N*-acetylchitohexaose, which can serve as good control data to verify the binding mode of the *N*,*N*′-diacetylchitobiose moiety in the subsites B and C, were recently presented at 1.6 Å resolution (Song et al., 1994). Our previous study pointed out that the contact between the planar side-chain group of Tyr63 and the sugar residue at subsite B was a major determinant of binding specificity toward an electrostatically neutral substrate in the catalytic action of human lysozyme (Muraki et al., 1992). In the present study, in order to clarify the difference in the interaction mode between *N*,*N*′-diacetylchitobiose and *N*-acetyllactosamine in the active site of HL, the efficiency of the 2′,3′-epoxypropyl β-glycoside of *N*-acetyllactosamine as an affinity labeling reagent was compared with that of *N*,*N*′-diacetylchitobiose, and the three-dimensional structures of the affinity-labeled enzymes were determined at 1.7 Å resolution. The results provided the fundamental information on the origin of the carbohydrate recognition specificity of HL.

EXPERIMENTAL PROCEDURES

Materials. Human lysozyme was purchased from Green Cross Co. or from Sigma Chemical Co. and further purified by a cation-exchange column (Mono S, Pharmacia) as described previously (Jigami et al., 1986). The concentration was determined on the basis of the absorption coefficient ($A^{1\%}_{280nm} = 25.65$ cm^{-1}) (Parry et al., 1969). Lyophilized *M. luteus* cells were from Sigma. *N*,*N*′-Diacetylchitobiose (>95% purity) were from Seikagakukogyo Co. The affinity labeling reagents, the 2′,3′-epoxypropyl β-glycoside of *N*,*N*′-diacetylchitobiose and the 2′,3′-epoxypropyl β-glycoside of *N*-acetyllactosamine, were synthesized organochemically, basically according to the method Thomas (1970) with some modifications as a mixture of the (2*R*) and the (2*S*) stereoisomers concerning the epoxide group. The details of the synthesis will be described elsewhere. The purity of the samples was checked by ^{1}H NMR and proved to be essentially pure. ^{1}H NMR analysis indicated that the ratio of the stereoisomers was 4:1 in excess of the same stereoisomer for both compounds. All other chemical reagents were of biochemical or analytical grade.

Reaction of Lysozyme with Affinity Labeling Reagent. In the experiment to follow the time-dependent inactivation, HL (1.0×10^{-5} M) or HEWL (1.3×10^{-5} M) was incubated with an affinity reagent (2.0×10^{-3} M) in 0.2 M sodium acetate buffer (pH 5.4) at 37 °C. At a certain interval an aliquot of the reaction mixture was taken out and diluted by 50 times with the same buffer. The diluted sample was immediately frozen by liquid N_2 and kept frozen at −60 °C until 100 μL of the sample was used for the assay of lytic activity. As to the preparation of the sample of the affinity-labeled enzyme for crystallization, HL (20 mg) and the affinity labeling reagent (25 mg) were incubated in 1.5 mL of 0.2 M sodium acetate buffer (pH 5.4) at 37 °C. After the reaction proceeded until the reaction mixture showed less than 1% of the initial lytic activity, the reaction mixture was separated by a cation-exchange chromatography (Mono S). The main peak detected at 280 nm contained the affinity-labeled enzyme.

Assay of Lytic Activity. The lytic activity against *M. luteus* cells was determined spectrophotometrically basically according to the turbidimetric method (Locquet et al., 1968). The relative activity was calculated from the initial decrease of the absorbance at 650 nm within 0.75 min (for HL) or 2.0 min (for HEWL) in 4 times diluted McIlvaine's buffer (pH 6.0) (McIlvaine, 1921) at 30 °C.

Crystallization and Structural Determination of the Affinity-Labeled HL. Crystallization of the chromatographically purified samples was carried out as described previously (Muraki et al., 1991) using the repeated seeding procedure (Thaller et al., 1985). Typically the prisms of 0.3 mm × 0.3 mm × 0.6 mm for the HL/NAG-NAG-EPO complex and 0.3 mm × 0.2 mm × 0.2 mm for the HL/GAL-NAG-EPO complex were obtained. The space group of both affinity-labeled HLs was orthorhombic ($P2_12_12_1$) with a little larger *a*-axis length as compared to native HL (Osserman et al., 1969). Diffraction data were collected on an Enraf-Nonius FAST diffractometer with a FR571 generator (40 kV, 50 mA, and focal spot size of 0.2 × 2 mm). Crystallographic refinement was carried out using a software package, X-PLOR version 3.1 (Brünger et al., 1992) alternating with manual intervention using TURBO-FRODO version 5.0 (Bio-Graphics Co., 1994). A set of protein coordinates of the native HL determined by Artymiuk and Blake (1981) (Protein Data Bank, entry code 1LZ1) was used as an initial model for the refinement. The structure of native HL was also redetermined and used as the reference coordinates to assess the atomic displacements that occurred in the labeling reaction. The final structure of native HL in this study was essentially the same as that determined by Artymiuk and Blake (1981); however, the average temperature factor was somewhat smaller. Some data collection statistics and refinement parameters were summarized in Table 1. The coordinate errors were estimated to be less than 0.2 Å for the HL/NAG-NAG-EPO complex and native HL and less than 0.25 Å for the HL/GAL-NAG-EPO complex as judged by the method of Luzzati (1952). All stereochemical parameters of the final models checked by PROCHECK (Laskowski et al., 1993) were either within inside or better than the average value ranges obtained from the good quality models. Water molecules which could form at least one hydrogen bond with a protein atom or an already existing

Table 1: Data Collection Statistics and Refinement Parameters[a]

	protein		
	native HL (no ligand)	HL/NAG-NAG-EPO	HL/GAL-NAG-EPO
(1) data collection			
space group	$P2_12_12_1$	$P2_12_12_1$	$P2_12_12_1$
cell dimensions			
a (Å)	57.14	58.63	58.41
b (Å)	61.02	60.76	60.71
c (Å)	33.10	33.12	32.84
resolution range (Å)	21.6−1.4	14.7−1.6	14.7−1.6
no. of unique reflections	22434	14427	12070
R_{merge} (%)	6.9	4.6	4.9
completeness of data (%)	79.3	90.8	76.9
(2) refinement			
resolution range (Å)	8.0−1.5	8.0−1.7	8.0−1.7
no. of reflections used ($\|F\| > 3\sigma_F$)	16901	8997	8072
no. of protein atoms	1029	1029	1029
no. of ligand atoms	0	33	30
no. of solvent molecules	106	129	115
final *R*-factor (%)	19.1	16.9	16.9
final free *R*-factor (%)	20.8	22.4	21.5
rms deviations from ideality			
bond lengths (Å)	0.008	0.009	0.009
bond angles (deg)	1.466	1.537	1.539

[a] $R_{merge} = (\sum\sum|I_j(hkl) - \langle I(hkl)\rangle|)/(\sum\sum\langle I(hkl)\rangle)$; *R*-factor $= \sum||F_o| - |F_c||/\sum|F_o|$.

Table 2: Inhibition of Lytic Activity of Human Lysozyme by *N*,*N*′-Diacetylchitobiose (NAG-NAG) and *N*-Acetyllactosamine (GAL-NAG)

inhibitor	remaining activity (%)[a]
NAG-NAG (1.0 mM)	64
NAG-NAG (10 mM)	50
GAL-NAG (1.0 mM)	98
GAL-NAG (10 mM)	89

[a] Expressed as the percentage of decrease in absorbance at 650 nm without inhibitor, measured at 30 °C (pH 6.0). Concentration of *M. luteus* cells: 0.25 mg/mL. Enzyme concentration: 0.3 μg/mL.

water molecule are included only if the final *B*-factor is less than 60 Å^2. All the graphical drawings of three-dimensional structures were produced using TURBO-FRODO. The coordinates have been deposited with the Protein Data Bank (Bernstein et al., 1977), Brookhaven National Laboratory.

RESULTS

Inactivation of Lysozyme by Affinity-Labeling Reagent. *N*-Acetyllactosamine showed a positive but significantly less effective inhibition on the lysis of *M. luteus* cells by HL than *N*,*N*′-diacetylchitobiose (Table 2). This suggests that the interaction of *N*-acetyllactosamine with the active site residues of HL is weaker than that of *N*,*N*′-diacetylchitobiose. Figure 2 demonstrates the change in the remaining lytic activity during the affinity labeling reaction. Under the reaction condition comprising 2.0×10^{-3} M labeling reagent and 1.0×10^{-5} M HL at pH 5.4, 37 °C, the reaction time required to reduce the lytic activity against *M. luteus* cells to 50% of its initial activity was 7.6 and 28 h for NAG-NAG-EPO and GAL-NAG-EPO, respectively, indicating the half-life of the lytic activity was lengthened by 3.7 times through the substitution of the nonreducing end sugar residue, GlcNAc to Gal. The activity of GAL-NAG-EPO as an irreversible inhibitor was much stronger toward HEWL compared to HL (Figure 3), which was also the case for NAG-NAG-EPO (Sharon & Eshdat, 1974) and the corresponding derivative of Man-β-(1→4)-GlcNAc (Muraki et al., unpublished experiments). This suggests the generality regarding either the lower affinity of the active site of HL or the weaker reactivity with the side-chain carboxylate group of Asp53 in HL against this kind of affinity labeling reagent

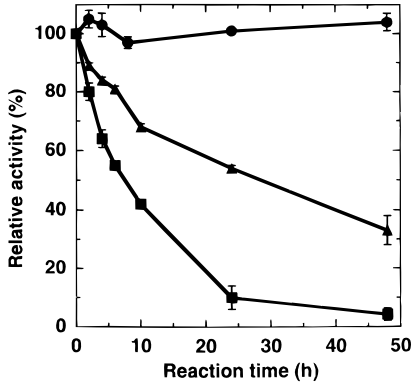


FIGURE 2: Inactivation of HL by NAG-NAG-EPO and GAL-NAG-EPO. HL (1.0×101^{-5} M) was incubated with NAG-NAG-EPO (2.0×10^{-3} M, square), GAL-NAG-EPO (2.0×10^{-3} M, triangle), or buffer only (circle) at 37 °C, pH 5.4.

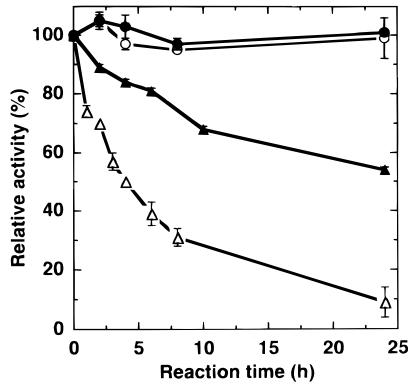


FIGURE 3: Comparison of inactivation of HL and HEWL by GAL-NAG-EPO. HL (1.0×10^{-5} M) or HEWL (1.3×10^{-5} M) was incubated with GAL-NAG-EPO (2.0×10^{-3} M, closed triangle for HL and open triangle for HEWL) or buffer only (closed circle for HL and open circle for HEWL) at 37 °C, pH 5.4.

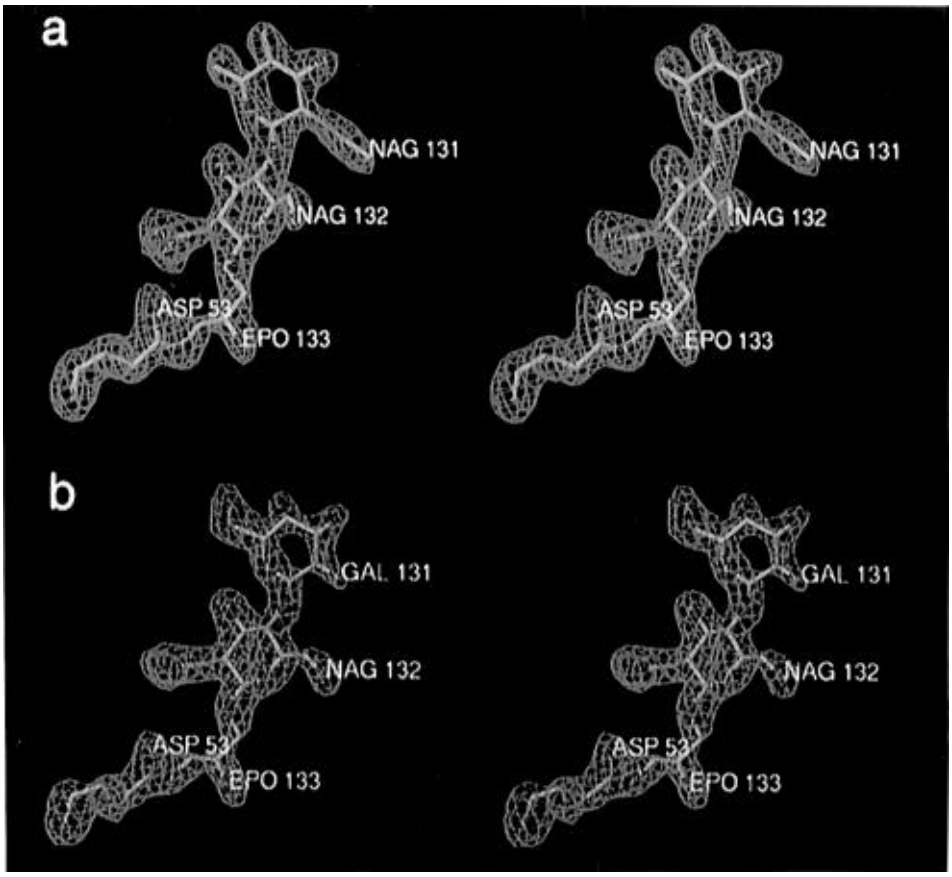


FIGURE 4: Stereoview of the difference ($F_o - F_c$) electron density map (contoured at 3σ) shown with the refined position of the ligand and Asp53 superimposed: (a) NAG-NAG-EPO complex; (b) GAL-NAG-EPO complex.

Table 3: Geometry of Glycosidic Linkage[a]

linkage	ϕ (deg)	ψ (deg)	ψ_H (deg)	O5−O3′ (Å)
NAG131−NAG132	−84	−120	35	3.0
GAL131−NAG132	−69	−119	53	3.0
NAGB−NAGC[b]	−87 (−82)	−120 (−120)	34 (37)	3.1 (3.0)

[a] Values of ϕ and ψ are the torsion angles about C1−O1(O4′) and O1(O4′)−C4 defined by O5−C1−O1(O4′)−C4′ and C1−O1(O4′)−C4′−C5′, respectively. The value of ψ_H is the helical twist parameter defined as the average of the pseudorotation angles ψ_1 = O5−C1−C4′−C3′ and ψ_2 = C2−C1−C4′−C5′ (Mo & Jensen, 1978). [b] Values are those in the structure determined by Song et al. (1994).

as compared to the corresponding structural elements of HEWL.

Structures of the HL/NAG-NAG-EPO Complex and the HL/GAL-NAG-EPO Complex. In Figure 4, ($F_o - F_c$) electron density maps of the bound ligand including the connection region between the protein part and the ligand part are shown. The well-shaped and strong electron densities in the maps confirmed the binding of the ligand in high occupancy and the existence of a covalent bond between HL and the affinity reagent. Both disaccharide moieties of the labeling reagent in this study occupied subsites B and C in essentially the same manner as in the case of free ligand (Song et al., 1994). However, the difference of about 20° in the helical twist parameter of glycosidic linkage was observed between the HL/NAG-NAG-EPO complex and the HL/GAL-NAG-EPO complex (Table 3). Concomitant with the difference in the geometry of glycosidic linkage, both the hydrogen-bonding pattern and the stacking interaction at subsite B were remarkably different between the HL/NAG-NAG-EPO complex and the HL/GAL-NAG-EPO complex. The possible hydrogen-bonding network around the ligand part is depicted in Figure 5. The number of the possible hydrogen bonds concerning the nonreducing end saccharide residue including the water-mediated one was proved to be smaller in the HL/GAL-NAG-EPO complex than in the HL/NAG-NAG-EPO complex. In contrast to the involvement of the OD1 atom of Asp102 in the hydrogen bond to the OH-6 group of the NAG131 residue in the HL/NAG-NAG-EPO complex, the same atom of HL participates in the hydrogen bond to the OH-4 group of the GAL131 residue in the HL/GAL-NAG-EPO complex. In the HL/GAL-NAG-EPO complex, the galactose residue no more made a direct hydrogen bond with the side-chain NH_2 group of Gln104 due to the lack of an acetamide group at position 2 (Figure 5). Instead, it moved slightly in different orientation sacrificing the stacking interactions with Tyr63 (Figure 6) to enable the axial OH-4 group to form a water molecule mediated hydrogen bond with the side-chain NH_2 group of Gln104 (Figure 5). The movement of the GAL131 residue in the HL/GAL-NAG-EPO complex relative to the NAG131 residue in the NAG-NAG-EPO complex suggested that the acetamide group of NAG in subsite B played an important role in the greater affinity of *N*,*N*′-diacetylchitobiose than *N*-acetyllactosamine for HL.

According to the binding of the affinity label, a significant but rather localized change in main-chain structure was observed for both complexes. The large shifts were mostly localized at the region from residue 101 to 113 for both complexes, and the maximal shifts from native HL were 1.96 and 1.45 Å for the HL/NAG-NAG-EPO complex and for the HL/GAL-NAG-EPO complex, respectively. The posi-

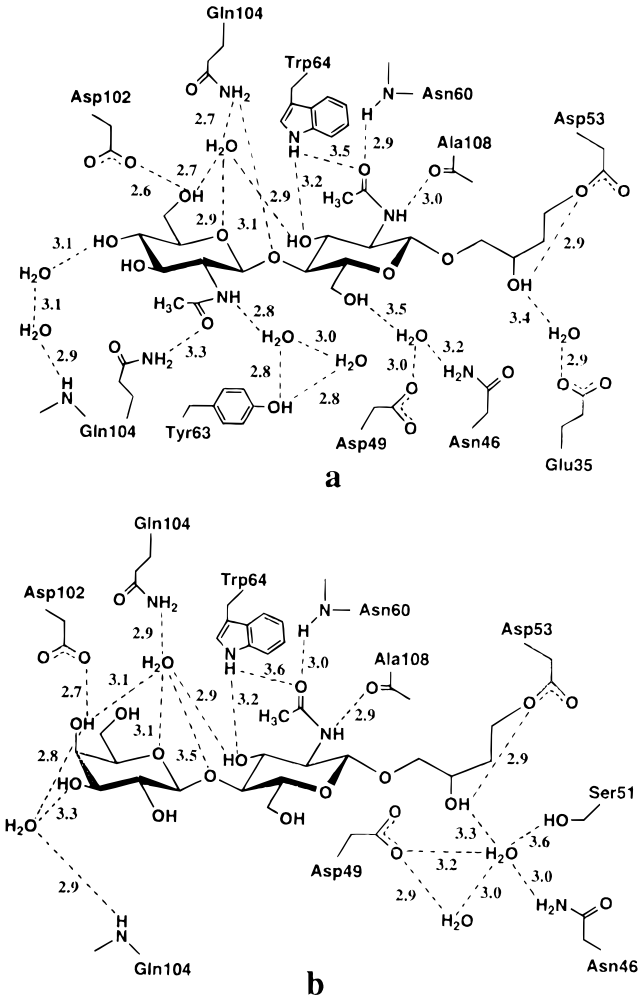


FIGURE 5: Possible hydrogen-bonding interactions between the protein part and the ligand part: (a) HL/NAG-NAG-EPO complex; (b) HL/GAL-NAG-EPO complex. Hydrogen bonds are shown with broken lines. The distance between the non-hydrogen atoms concerned are shown in angstrom units.

tional shifts should be ascribed as the ligand binding induced fit to enhance the interactions between the protein atoms and the saccharide atoms (Figure 7).

DISCUSSION

The geometrical features of the active site structures determine the substrate recognition specificity of enzymes. In the present study we have revealed the detailed interaction mode of the *N*-acetyllactosamine residue in the active site of a vertebrate lysozyme for the first time by X-ray crystallography. We also have demonstrated the effectiveness of the employment of affinity labeling to reveal the origin of the carbohydrate recognition specificity of HL by comparing the interaction modes of the ligand in the HL/NAG-NAG-EPO complex to that in the HL/GAL-NAG-EPO complex. The structural determination of a protein−ligand complex at atomic detail is useful for studying the structure and function relationships of enzymes, especially for the characterization of the topology of active sites and for the elucidation of the enzyme mechanism. However, the investigation on the recognition mechanism of the ligand exhibiting less strong affinity as compared to a natural ligand may encounter a hardship due to the difficulty in producing a fine complex crystal that contains the ligand molecule in high occupancy. In order to overcome this hardship, we have taken advantage of the covalent bond formation by the reaction of the epoxide group with a particular carboxylate group of the protein. Although this kind of affinity labeling has been applied for a variety of glycosidases, the information on the binding mode at an atomic level resolution is limited with the exceptions including *Bacillus* 1,3-1,4-β-glucanases determined at 2.0 Å resolution (Keitel et al., 1993).

When an irreversible labeling reagent has been employed as a ligand, it is important to examine the occurrence of the difference in the binding mode, which can be ascribed to the existence of the introduced covalent bond. For that purpose we have determined the structure of the HL/NAG-NAG-EPO complex and compared it with the structure of the complex of HL with a free ligand, hexa-*N*-acetylchitohexaose cleaved by HL (Song et al., 1994). In addition to the same patterns of hydrogen bonding and stacking interaction as in the case of the free ligand, the analysis of the geometry of the glycosidic linkage (Table 3) indicated the close similarity of the binding mode of the *N*,*N*′-diacetylchitobiose moiety in the HL/NAG-NAG-EPO complex to the sugar residues that occupied subsites B and C in the free ligand. Furthermore, the displacements of main-chain atoms accompanied by the attachment of the affinity label to HL (Figure 7) exhibited essentially the same characteristics as that observed in the binding of the free ligand. These results indicate the applicability of the irreversible, active site-directed inhibitors in this study as a probe for revealing the origin of carbohydrate recognition specificity of HL.

Some *N*-acetyllactosamine sequence binding proteins have been investigated by X-ray crystallography (Liao et al., 1994; Bourne et al., 1992, 1994). Among them, the structure of 14 kDa bovine spleen *S*-lectin has been determined as the complex with *N*-acetyllactosamine at 1.9 Å resolution, where the axial 4-OH of galactose, a main determinant of *S*-lectin specificity, forms two key hydrogen bonds (Liao et al., 1994). In relation with this, it is particularly interesting that the OD1 atom of Asp102 in HL altered the counterpart of the hydrogen bond from the O-6 atom of the *N*-acetylglucosamine residue to the O-4 atom of the galactose residue

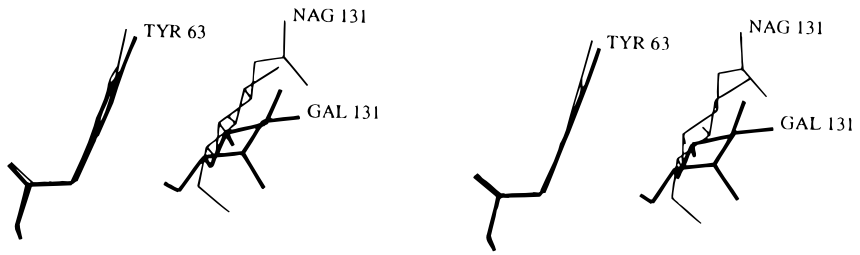


FIGURE 6: Stereoview of the conformations of Tyr63 residue and the saccharide residue bound at subsite B. The corresponding parts of the HL/NAG-NAG-EPO complex and the HL/GAL-NAG-EPO complex are drawn with thin lines and thick lines, respectively.

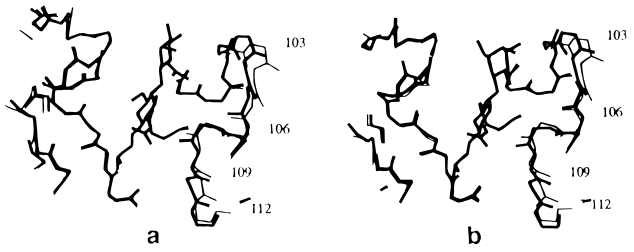


FIGURE 7: Conformational change of the main chain in the region of the active site cleft: (a) comparison of the HL/NAG-NAG-EPO complex to native HL with no ligand; (b) comparison of the HL/GAL-NAG-EPO complex to native HL with no ligand. The 13 Å sphere regions centered at the C4 atom of the saccharide residue bound at subsite C are shown. The ligand part atoms in each complex molecule are included. Native HL and the complex molecule are drawn with thin lines and thick lines, respectively.

among several changes in the hydrogen-bonding pattern between the HL/NAG-NAG-EPO complex and the HL/GAL-NAG-EPO complex (Figure 5). The stacking of aromatic residues against the faces of sugars is a feature of protein−carbohydrate interactions that contribute to the stability and specificity of the complex structures determined so far (Vyas, 1991). The importance of the stacking interaction between HL and the *p*-nitrophenyl chitopentaoside has been suggested by the structural and kinetic analysis of the mutants concerning Tyr63 in HL (Muraki et al., 1992). In addition to the reduction of the number of possible hydrogen bonds, the geometric interrelation between the side chain of Tyr63 and the galactose residue in the HL/GAL-NAG-EPO complex turned out to be less favorable for the stacking interaction than that between the side chain of Tyr63 and the *N*,*N*′-diacetylchitobiose in the HL/NAG-NAG-EPO complex (Figure 6). The above results indicate that the hydrogen-bonding interaction and the stacking interaction in the HL−carbohydrate ligand complex work cooperatively, which should take a crucial part in the carbohydrate recognition specificity of HL.

Recently, several studies on the engineering of carbohydrate binding specificity by use of amino acid residue replacement(s) of proteins have been reported (Vermersch et al., 1990; Drickermer, 1992; Declerck & Abelson, 1994). The detailed comparison of the protein structures complexed to various synthetic ligands with that complexed to natural ligands provides the comprehensive and fundamental principle that directs us to the strategical design of the ligand binding specificity. The use of the affinity labeling method should make a certain contribution to such study that will lead to the development of a carbohydrate binding protein with the desired recognition specificity.

REFERENCES

Artymiuk, P. J., & Blake, C. C. F. (1981) *J. Mol. Biol. 152*, 737−762.

Bernstein, F. C., Koetzle, T. F., Williams, G. J. B., Meyer, E. F., Jr., Brice, M. D., Rodgers, J. R., Kennard, O., Shimanouchi, T., & Tatsumi, M. (1977) *J. Mol. Biol. 112*, 535−542.

Bourne, Y., Rougé, P., & Cambillau, C. (1992) *J. Biol. Chem. 267*, 197−203.

Bourne, Y., Bolgiano, B., Nésa, M.-P., Penfold, P., Johnson, D., Feizi, T., & Cambillau, C. (1994) *J. Mol. Biol. 235*, 787−789.

Brünger, A. T., Kuriyan, J., & Karplus, M. (1987) *Science 35*, 458−460.

Colman, R. F. (1990) in *The Enzymes* (Sigman, D. S., & Boyer, P. D., Eds.) Vol. 19, pp 283−321, Academic Press, San Diego.

Declerck, N., & Abelson, J. (1994) *Protein Eng. 7*, 997−1004.

Drickamer, K. (1992) *Nature 360*, 183−186.

Fleming, A., (1922) *Proc. R. Soc. London, Ser. B 93*, 306−317.

Hokke, C. H., Roosenboom, M. J. H., Thomas-Oates, J. E., Kamerking, J. P., & Vliegenthart, J. F. G. (1994) *Glycoconjugate J. 11*, 35−41.

Imoto, T., Johnson, L. N., North, A. C. T., Phillips, D. C., & Rupley, J. A. (1972) in *The Enzymes* (Boyer, P. D., Ed.) Vol. 7, pp 665−868, Academic Press, New York.

Jigami, Y., Muraki, M., Harada, N., & Tanaka, H. (1986) *Gene 43*, 273−279.

Jollès, P., & Jollès, J. (1984) *Mol. Cell. Biochem. 63*, 165−189.

Keitel, T., Simon, O., Borris, R., & Heinemann, U. (1993) *Proc. Natl. Acad. Sci. U.S.A. 90*, 5287−5291.

Kelly, J. A., Sielecki, A. R., Sykes, B. D., James, M. N. G., & Phillips, D. C. (1979) *Nature 282*, 875−878.

Laskowski, R. A., MacArthur, M. W., Moss, D. S., & Thornton, J. M. (1993) *J. Appl. Crystallogr. 26*, 283−291.

Liao, D.-I., Kapadia, G., Ahmed, H., Vasta, G. R., & Herzberg, O. (1994) *Proc. Natl. Acad. Sci. U.S.A. 91*, 1428−1432.

Llyod, K. O., Kabat, E. A., & Licerio, E. (1968) *Biochemistry 7*, 2976−2990.

Locquet, J. P., Saint-Blancard, J., & Jollès, P. (1968) *Biochim. Biophys. Acta 167*, 150−153.

Luzzati, V. (1952) *Acta Crystallogr. 5*, 802−810.

Marshall, R. D. (1972) *Annu. Rev. Biochem. 41*, 673−702.

McIlvaine, T. C. (1921) *J. Biol. Chem. 49*, 183−186.

Mo, F., & Jensen, L. H. (1978) *Acta Crystallogr., Sect. B 34*, 1562−1569.

Moult, J., Eshdat, Y., & Sharon, N. (1973) *J. Mol. Biol. 75*, 1−4.

Muraki, M., Harata, K., Hayashi, Y., Machida, M., & Jigami, Y. (1991) *Biochim. Biophys. Acta 1079*, 229−237.

Muraki, M., Harata, K., & Jigami, Y. (1992) *Biochemistry 31*, 9212−9219.

Osserman, E. F., Cole, S. J., Swan, I. D. A., & Blake, C. C. F. (1969) *J. Mol. Biol. 46*, 211−212.

Parry, R. M., Jr., Chandan, R. C., & Shahani, K. M. (1969) *Arch. Biochem. Biophys. 103*, 59−65.

Quiocho, F. A. (1986) *Annu. Rev. Biochem. 55*, 287−315.

Sharon, N., & Eshdat, Y. (1974) in *Lysozyme* (Osserman, E. F., Canfield, R. E., & Beychok, S., Eds.) pp 195−218, Academic Press, New York.

Shinitzky, M., Grisaro, V., Chipman, D. M., & Sharon, N. (1966) *Arch. Biochem. Biophys. 115*, 232−233.

Song, H., Inaka, K., Maenaka, K., & Matsushima M. (1994) *J. Mol. Biol. 244*, 522−540.

Thaller, C., Eichele, G., Weaver, J. H., Wilson, E., Karlsson, R., & Jansonius, J. N. (1985) *Methods Enzymol. 114*, 132−135.

Thomas, E. W. (1970) *Carbohydr. Res. 13*, 225−228.

Thomas, E. W., McKelvy, J. F., & Sharon, N. (1969) *Nature 222*, 485−486.

Vermersch, P. S., Tesmer, J. J. G., Lemon, D. D., & Quiocho, F. A. (1990) *J. Biol. Chem. 27*, 16592−16603.

Vyas, N. K. (1991) *Curr. Opin. Struct. Biol. 1*, 732−740.

BI9613180