Stereochemistry of Chitin Hydrolysis by a Plant Chitinase/Lysozyme and X-ray Structure of a Complex with Allosamidin: Evidence for Substrate Assisted Catalysis‡

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ABSTRACT: The plant enzyme hevamine has both chitinase and lysozyme activity. HPLC analysis of the products of the hydrolysis of chitopentaose shows that hevamine acts with retention of the configuration, despite the absence of a nucleophilic or stabilizing carboxylate. To analyze the stabilization of a putative oxocarbonium ion intermediate, the X-ray structure of hevamine complexed with the inhibitor allosamidin was determined at 1.85 Å resolution. This structure supports the role of Glu127 as a proton donor. The allosamizoline group binds in the center of the active site, mimicking a reaction intermediate in which a positive charge at C1 is stabilized intramolecularly by the carbonyl oxygen of the *N*-acetyl group at C2.

Chitin, a linear polysaccharide of $\beta(1,4)$-linked *N*-acetylglucosamine residues, is a major constituent of the cell wall of fungi and the exoskeleton of insects and crustaceans. It can be hydrolyzed by chitinases, enzymes that are produced not only by fungi, insects, and crustaceans, for which they have an essential function during growth and molting (Kramer et al., 1993, Sahai & Manocha, 1993), but also by bacteria, which degrade chitin for use as a carbon and energy source (Morgavi et al., 1994), and plants, which produce chitinases to defend themselves against pathogenic fungi (Collinge et al., 1993).

On the basis of their amino acid sequence, chitinases have been divided into two unrelated families, families 18 and 19 of the glycosyl hydrolases (Henrissat, 1991). Family 18 chitinases have been found in a wide range of species, including bacteria, fungi, plants, insects, mammals, and viruses (Henrissat, 1990; Hakala et al., 1993; Kramer et al., 1993; Ayres et al., 1994). Within this family, the prokaryotic chitinases show only two short sequence motifs that are homologous to the eukaryotic enzymes (~10% overall identical residues). Nevertheless, the chitinases from both classes possess a similar catalytic $(\beta\alpha)_8$-barrel domain as revealed by the X-ray structures of the bacterial *Serratia marcescens* chitinase A (Perrakis et al., 1994) and hevamine, a plant chitinase from *Hevea brasiliensis* (Terwisscha van Scheltinga et al., 1994). In both enzymes the substrate binding cleft is located at the carboxy-terminal ends of the β-strands in the $(\beta\alpha)_8$-barrel, and the proton-donating catalytic glutamic acid residue has an equivalent position. On the other hand, family 19 chitinases have been found solely in plants so far. They show a lysozyme-like fold that consists of a bilobal $\alpha+\beta$ folding motif, as exemplified by the X-ray structure of the chitinase from *Hordeum vulgare* L. seeds (Hart et al., 1995). In analogy to HEW[1] lysozyme, the substrate binding cleft is thought to be between the two lobes.

Hydrolysis of polysaccharides can, in principle, yield two different products, with the anomeric oxygen at C1 being either in an axial or in an equatorial position (yielding the α or β configuration at C1, respectively). As the *N*-acetylglucosamine units in chitin are $\beta(1,4)$-linked, a product with an axial hydroxyl group at C1 would require a catalytic mechanism with inversion of the configuration, whereas an equatorial hydroxyl group implies retention of the configuration. The stereochemistry of chitin hydrolysis has been determined for several chitinases. Two family 18 bacterial chitinases from *Bacillus circulans* WL-12 and *Streptomyces griseus* were shown to retain the configuration at C1 (Armand et al., 1994). Retention of configuration was also proposed for the *S. marcescens* chitinase A (Perrakis et al., 1994), possibly with stabilization of the oxocarbonium ion by Asp391. Model building of a chitohexaose substrate in the active site of hevamine suggested that the hydrolysis catalyzed by hevamine could proceed with inversion of the configuration, especially as no carboxylate was evident to stabilize a putative oxocarbonium intermediate (Terwisscha van Scheltinga et al., 1994).

To investigate whether the proposed difference in stereochemistry between chitinases of prokaryotic and eukaryotic origin indeed exists, we have now determined the stereochemistry of chitin hydrolysis by hevamine. We show that hevamine, like the bacterial chitinases, acts by retention of configuration, despite the absence of a stabilizing carboxylate

‡ Atomic coordinates for the complex have been deposited with the Brookhaven Protein Data Bank (file name 1LLO).

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[1] Abbreviations: NAG, *N*-acetylglucosamine; chitobiose, *N,N'*-diacetylchitobiose; chitotriose, *N,N',N''*-triacetylchitotriose; chitotetraose, *N,N',N'',N'''*-tetraacetylchitotetraose; chitopentaose, *N,N',N'',N''',N''''*-pentaacetylchitopentaose; chitohexaose, *N,N',N'',N''',N'''',N'''''*-hexaacetylchitohexaose; HEW, hen egg white; NMR, nuclear magnetic resonance; HPLC, high-performance liquid chromatography; F_o, observed structure factor amplitude; F_c, calculated structure factor amplitude; RMS, root mean square.

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in the active site. To explain these results, we determined the X-ray structure of hevamine complexed with the chitinase inhibitor allosamidin. This suggests that allosamidin mimics a transition state in which the oxocarbonium intermediate is stabilized by the carbonyl oxygen of the *N*-acetyl group adjacent to the scissile bond.

MATERIAL AND METHODS

Determination of the Reaction Stereochemistry. Chitooligosaccharides were prepared as described previously (Armand et al., 1994). Hevamine was dissolved to a concentration of 1 mg/mL in a 4 mM acetate buffer (pH 4.0). To 40 μL of a saturated solution of chitopentaose in water, 5 μL of the hevamine solution was added. After incubation during 5 min at 40 °C, 20 μL of the reaction mixture was analyzed by HPLC using a C18 Nucleosil column (5 μm, Interchim), equipped with a refractometric detector and eluted with water. The remaining 25 μL was kept on ice until the analysis was finished. Then 20 μL of a solution of 1 mg/mL chitotetraose in water was added. Twenty microliters of the solution was analyzed by HPLC. The remaining solution was kept at room temperature for 18 h and then also analyzed by HPLC. Controls were carried out using 20 μL of solutions of 2 mg/mL *N*-acetylglucosamine, 1 mg/mL chitotetraose, saturated chitopentaose, and 0.1 mg/mL hevamine, respectively.

Structure Determination of the Hevamine–Allosamidin Complex. Crystals of hevamine were grown as described previously (Rozeboom et al., 1990), with space group $P2_12_12_1$ and cell dimensions a = 52.30 Å, b = 57.72 Å, and c = 82.05 Å. A soaking solution was prepared containing 20 mg/mL allosamidin, 100 mM acetate buffer (pH 4.0), and 20% (w/v) NaCl. A hevamine crystal was soaked in this solution for 24 h. Data were collected to a resolution of 1.85 Å on the X31 beamline at DESY, Hamburg. As a result of the soaking experiment, the cell dimensions changed slightly to a = 52.33 Å, b = 57.99 Å, and c = 82.25 Å. A σ_A-weighted $|F_o| - |F_c|$ map (Read, 1986) was calculated in the resolution range of 15.0–1.85 Å; the highest peak was found in the substrate binding cleft at subsite D. A model for allosamidin, built and energy-minimized using BIOGRAF, could easily be fitted in the electron density at subsites B, C, and D, with the allosamizoline bound at subsite D (Figure 3A).

Crystallographic refinement of hevamine complexed with allosamidin was performed with the program TNT (Tronrud et al., 1987). To calculate a free R factor (Brünger, 1992), 10% of the data collected was left out of the refinement. After the refinement, the R factor (8.0–1.85 Å) had improved from 0.236 to 0.146, and R_{free} had dropped to 0.200.

RESULTS AND DISCUSSION

Stereochemistry of the Hydrolysis Reaction Catalyzed by Hevamine. HPLC analysis is a powerful method to separate the α- and β-anomers of chitooligosaccharides, as can be seen from Figure 1. Previous experiments, using both reversed-phase HPLC and ^{1}H NMR (Armand et al., 1994), have shown that the peaks with the shorter retention times correspond to the β-anomers; for all chitooligosaccharides studied [NAG, $(NAG)_2$, $(NAG)_3$, $(NAG)_4$, and $(NAG)_5$] the equilibrium ratio between the α- and β-anomers is approximately 3:2. To analyze the products of the reaction

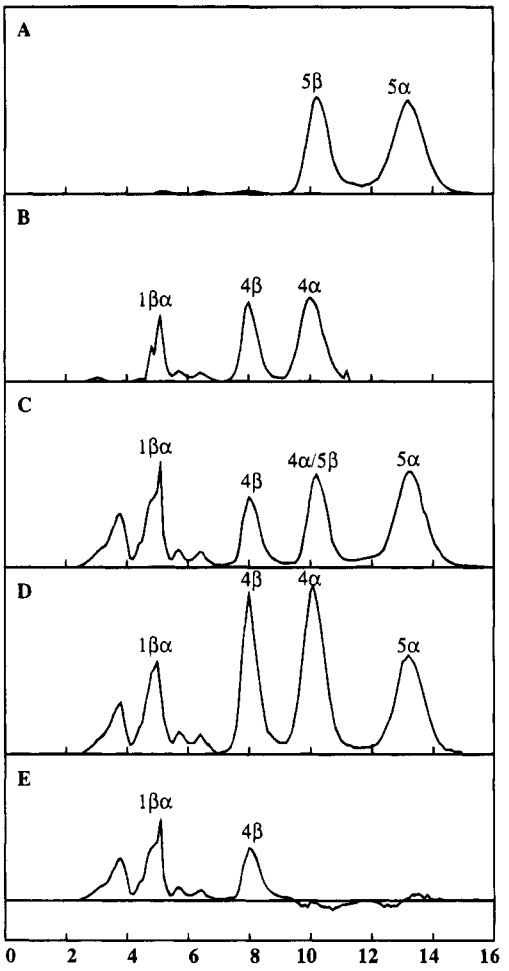

FIGURE 1: HPLC analysis of chitooligosaccharides. The x-axes show the retention time (minutes), and the y-axes show the signal measured by the refractometer. (A) Chitopentaose, the substrate. The peaks of the different anomers are clearly separated. (B) The combined elution profiles of *N*-acetylglucosamine and chitotetraose, the expected products of cleavage of chitopentaose by hevamine. (C) The elution profile of chitopentaose, incubated for 5 min at 40 °C with hevamine. The peak with an elution time shorter than the 1βα peaks is caused by the buffered protein solution. (D) The same as C, but with chitotetraose added, which clearly shows that the peaks corresponding to the α-anomer of chitotetraose and the β-anomer of chitopentaose overlap. (E) The same profile as C, but with the profile of chitopentaose as shown in A subtracted after scaling the peaks of the α-anomer.

catalyzed by hevamine, hevamine was first incubated with chitotetraose. However, no reaction occurred, and the chitotetraose remained intact. Next, we incubated hevamine with chitopentaose. After 5 min a large part of the chitopentaose had been degraded to chitotetraose and *N*-acetylglucosamine. Also small amounts of chitotriose and chitobiose had formed (Figure 1C). Adding chitotetraose to the reaction mixture unambiguously showed that the peaks of the α-anomer of chitotetraose and the β-anomer of chitopentaose overlap (Figure 1D). Subtracting the chitopentaose signal, after scaling the peaks of the chitopentaose α-anomer, clearly shows the amount of the β-anomer of chitotetraose to be much higher than the amount of the α-anomer (Figure 1E). After 18 h at room temperature, the reaction has completed, as no chitopentaose peaks are present anymore, and anomeric equilibrium has been reached for the chitotetraose formed, with the α- and the β-anomer present in the ratio 3:2.

Our results show that hevamine does not hydrolyze chitin fragments shorter than chitopentaose and that chitopentaose is cleaved mostly in chitotetraose and *N*-acetylglucosamine. This is consistent with our earlier results on the binding of chitotriose to hevamine (Terwisscha van Scheltinga et al., 1994). The substrate binding cleft of hevamine consists of six subsites, A−F. Chitotriose binds solely at subsites A−C, while cleavage takes place between subsites D and E. It appears that the first three subsites have the highest affinity for the substrate, so that small chitin fragments virtually do not bind at subsites D and E and are not cleaved by hevamine.

The HPLC elution profile of chitopentaose, incubated for 5 min with hevamine, clearly shows that the relative amount of the β-anomer of the formed chitotetraose is much higher than the α-anomer, whereas in the equilibrium state the amount of α-anomer is about 1.5 times that of the β-anomer. Therefore, we conclude that hevamine cleaves chitin with retention of the configuration at C1, like the family 18 bacterial chitinases (Armand et al., 1994). This result refutes our previous suggestion based on model building that hevamine acts by inversion of the configuration at C1.

Enzyme Mechanisms of Retaining Carbohydrate Hydrolases. The classical mechanism of glycoside hydrolysis with retention of configuration involves two carboxylates (McCarter & Withers, 1994). One donates a proton to the β-(1,4)-glycosidic oxygen, and aglycon departure is assisted by the second residue, which is thought either to make a covalent glycosyl intermediate or to provide an ion-pair stabilization to the oxocarbonium ion intermediate. After the aglycon has diffused away, a water molecule enters the active site and performs a second nucleophilic substitution at the carbohydrate C1. This results in an overall retention of the anomeric configuration (Koshland, 1953). In the case of hevamine, Glu127 is thought to be the proton donating amino acid residue (Terwisscha van Scheltinga et al., 1994). However, no residue is evident in the three-dimensional structure of hevamine that could serve to stabilize a putative oxocarbonium ion intermediate. This implies that the intermediate must be stabilised in an alternative way.

A possible way of intramolecular stabilization might occur through the *N*-acetyl group present in the substrate. Such a stabilization, called anchimeric assistance or neighboring group participation, is well known in organic chemistry. For instance, the spontaneous hydrolysis of methyl-2-acetamido-2-deoxy-β-D-glucopyranoside is 1000-fold enhanced over the hydrolysis of methyl β-D-glucopyranoside (Piszkiewicz & Bruice, 1968). Anchimeric assistance is also often used for the synthesis of 1,2-*trans*-glycopyranosides, such as β-D-derivatives of *N*-acetylglucosamine (Paulsen, 1982). This synthesis proceeds via a stable oxazoline intermediate, in which a covalent bond is present between the carbonyl oxygen of the *N*-acetyl group and the axial position of the C1 atom of the glucose residue (Figure 2B). The subsequent attack of a hydroxyl group on C1, through an S_N2 reaction, leads exclusively to the formation of a β-glycoside.

Anchimeric assistance by the *N*-acetyl group during the hevamine-catalyzed hydrolysis of chitin could elegantly explain why hevamine is active without a stabilizing carboxylate. Such a stabilization might occur either through a charge interaction between the carbonyl oxygen of the *N*-acetyl group or via an oxazoline intermediate with a covalent bond between C1 and the carbonyl oxygen.

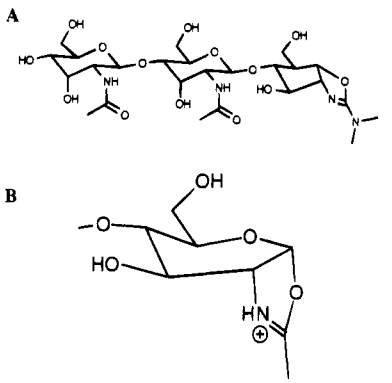

FIGURE 2: (A) Structure of allosamidin. (B) Covalent oxazolinium intermediate.

Table 1: Statistics of Data Collection and Refinement

number of measurements		91378	
number of unique reflections		22218	
R_{merge} (1.85−1.82 Å)		0.039 (0.149)	
completeness (1.85−1.82 Å)		0.732	
Refinement			
resolution range used (Å)		8.00−1.85	
completeness of working set		0.89	
completeness of test set		0.10	
R factor		0.146	
R_{free}		0.200	
number of protein atoms		2087	
number of allosamidin atoms		43	
number of water molecules		189	
RMS Deviations from Ideality			
bond lengths (Å)		0.014	
bond angles (deg)		1.7	
dihedrals (deg)		23	
B values (Å^2)		2.7	
temperature factors	B_{min}	B_{max}	$\langle B \rangle$
all protein atoms	1.00	85.31	12.54
main chain atoms	1.00	30.46	9.60
side chain atoms	1.00	85.31	15.76
allosamidin atoms	1.72	56.54	10.07
water atoms	5.85	74.79	29.22

Interestingly, a strong family 18 chitinase inhibitor, which contains an oxazoline derivative, has been isolated from *Streptomyces* sp. (Sakuda et al., 1987). This inhibitor, allosamidin, has been shown to inhibit family 18 glycosyl hydrolases, but not HEW NOR human lysozyme (Koga et al., 1987). It consists of two β(1,4)-linked *N*-acetylallosamine units and a unique oxazoline derivative, allosamizoline (Figure 2A). Allose differs from glucose only at C3, with the hydroxyl group axial in allose and equatorial in glucose. The allosamizoline moiety is surprisingly similar to the putative reaction intermediate described above.

Structure of the Hevamine−Allosamidin Complex. To investigate whether the active site of hevamine can accommodate an oxazoline-like intermediate, we determined the X-ray structure of hevamine complexed with allosamidin at 1.85 Å resolution. Details on the structure determination are shown in Table 1.

Possible hydrogen bonding contacts between hevamine and allosamidin are shown in Figure 3C. Most contacts of the two *N*-acetylallosamine residues bound at subsites B and C are similar to the contacts observed for *N*-acetylglucosamine residues bound at the same subsites (Terwisscha van Scheltinga et al., 1994). The *N*-acetylallosamine O3 atom at subsite B is at hydrogen bonding distance from the Oδ1 atom of Asn45, whereas the *N*-acetylglucosamine O3 atom hy-

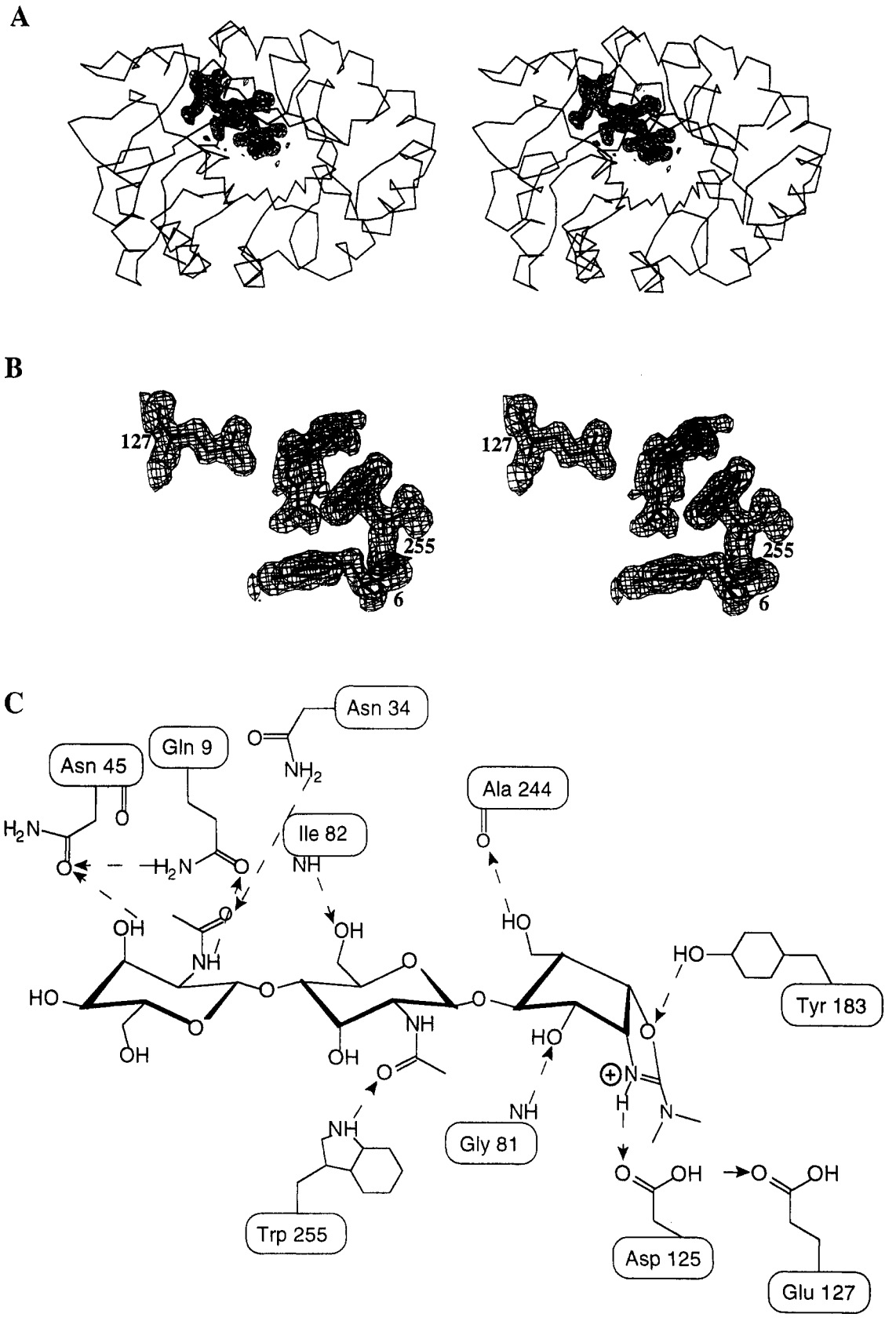

FIGURE 3: (A) Stereofigure of electron density of allosamidin, with the Cα-trace of hevamine. Allosamidin binds to hevamine at subsites B, C, and D. (B) Hydrophobic interactions between the allosamizoline group and the side chains of Tyr6 and Trp255. The proton donor Glu127 is also indicated. (C) Direct hydrogen bonds between allosamidin and hevamine.

drogen bonds to the Nδ2 atom. The side chain of Asn45 has in both cases the conformation with a hydrogen bond between its Oδ1 atom and the main chain nitrogen atom of Ala47. The C3 hydroxyl group at subsite C is not within hydrogen bonding distance from hevamine neither in the allosamidin or the chitotriose structure.

The allosamizoline moiety of allosamidin is bound at subsite D, where the reaction intermediate is formed. Its

hydrophobic face stacks on the aromatic side chain of Trp255, while the two methyl groups make van der Waals interaction with the side chain of Tyr6 (Figure 3B). Furthermore, the allosamizoline group appears to be firmly fixed by hydrogen bonds. The pseudosugar ring can make hydrogen bonding contacts with main chain atoms of Gly81 and Ala244. The oxygen atom of the oxazoline group is within hydrogen bonding distance from the Oη atom of Tyr183; the nitrogen atom is close to the Oδ1 atom of Asp125. Trp255, Tyr6, and Tyr183 are strictly conserved in plant and fungal family 18 chitinase sequences. Also Asp125 is conserved, except in *Arabidopsis thalliana*, where it is an asparagine (Samac et al., 1990). The interactions of the allosamizoline group with these residues observed in hevamine can probably be made in the other chitinases; the asparagine residue in the *A. thalliana* sequence can interact in a similar way with allosamidin as Asp125 (Figure 3C). Glu127 is close to the oxazoline group; its Oϵ2 atom is 4.3 Å away from the C1 atom. This residue was proposed to act as a proton donor during catalysis (Terwisscha van Scheltinga et al., 1994) and seems to be in a perfect position to do so (Figure 3B).

The nitrogen atom of the oxazoline ring is bound at the same position where the heavy atom compound $(CH_3)_3Pb^+$ binds, indicating the preference for a positive charge at this position. An induced positive charge at the nitrogen atom of the acetamido group would cause the carbonyl oxygen to be more negatively charged, making it more effective in stabilising the positive charge at C1. Thus it seems that an induced negative charge on the carbonyl oxygen of the *N*-acetyl group is an excellent substitute for the nucleophilic carboxylate. Alternatively, a covalent bond between the carbonyl oxygen and the C1 atom might be formed to stabilize the reaction intermediate.

There are other enzymes which hydrolyze β(1,4)-*N*-acetylglucosaminic linkages where a C2 anchimeric assistance could provide a reasonable alternative to the apparent lack of a catalytic base. Two well known examples are goose lysozyme (Weaver et al., 1995) and the soluble lytic transglycosylase (Thunnissen et al., 1994, 1995). Anchimeric assistance of the C2 acetamido group cannot be excluded even in the case of the thoroughly studied HEW lysozyme where Asp52 has yet long been put forward as "stabilizing" the intermediate oxocarbonium ion and has been suggested before (Lowe et al., 1967). Arguments in favor of some C2 assistance in the HEW lysozyme mechanism include the observation that removal of the C2 acetamido group of the substrate leads to a greater reduction in k_{cat} (Lowe & Sheppard, 1968) than that resulting from the D52N mutation (Malcolm et al., 1989).

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