Multiple Conformations of Catalytic Serine and Histidine in Acetylxylan Esterase at 0.90 Å*

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Acetylxylan esterase (AXEII; 207 amino acids) from *Penicillium purpurogenum* has substrate specificities toward acetate esters of d-xylopyranose residues in xylan and belongs to a new class of α/β hydrolases. The crystal structure of AXEII has been determined by single isomorphous replacement and anomalous scattering, and refined at 0.90- and 1.10-Å resolutions with data collected at 85 K and 295 K, respectively. The tertiary structure consists of a doubly wound α/β sandwich, having a central six-stranded parallel β-sheet flanked by two parallel α-helices on each side. The catalytic residues Ser90, His187, and Asp175 are located at the C-terminal end of the sheet, an exposed region of the molecule. The serine and histidine side chains in the 295 K structure show the frequently observed conformations in which Ser90 is trans and the hydroxyl group is in the plane of the imidazole ring of His187. However, the structure at 85 K displays an additional conformation in which Ser90 side-chain hydroxyl is away from the plane of the imidazole ring of His187. The His187 side chain forms a hydrogen bond with a sulfate ion and adopts an altered conformation. The only other known hydrolase that has a similar tertiary structure is *Fusarium solani* cutinase. The exposed nature of the catalytic triad suggests that AXEII is a pure esterase, i.e. an α/β hydrolase with specificity for nonlipidic polar substrates.

Plant cell wall hemicelluloses are complex mixtures of heteropolysaccharides. Their main component is xylan, which is composed of a linear chain of β(1→4)glycosidic-linked d-xylopyranoses, having various substitutions at carbon 2 and 3 positions (1). Biodegradation of xylan is a complex process catalyzed by several fungal and bacterial enzymes (2). Although the linear chain is cleaved by endoxylanases and β-xylosidases, acetylxylan esterases (AXE) hydrolyze O-acetyl substitutions of d-xylopyranose moieties. *Penicillium purpurogenum* secretes at least two forms of AXEs, I and II, that demonstrate substrate specificities toward acetate esters of d-xylopyranose and belong to a new class of α/β hydrolases (3).

Purification and characterization of AXEs from other xylanolytic microorganisms have been described previously (4, 5). Although considerable amounts of work have been performed on the enzymology of xylanases and AXEs (6–10), the structure-function characterization of this new class of esterases with regard to their catalytic activities and substrate specificities is yet to be carried out. We reported the crystallization and structure determination using the room temperature data of the first member of the family, *P. purpurogenum* AXEII (11–13). Recently, crystallization and preliminary diffraction studies of the catalytic core of *Trichoderma reesei* AXE have been published (14). Here we present for the first time the complete description of the three-dimensional structure of *P. purpurogenum* AXEII and its active site at 0.90-Å resolution and 85 K, compare it with the 1.10-Å structure (Protein Data Bank Code: 1BS9) determined at 295 K (room temperature), and investigate the structural basis for its acetyl d-xylopyranose specificity.

MATERIALS AND METHODS

Crystallization and Data Collection—The enzyme was crystallized from ammonium sulfate solution in 50 mM citrate buffer at pH 5.3 (11). Diffraction data were collected at the A-1 station of Cornell High Energy Synchrotron Source using a 2000 × 2000 pixel charge-coupled device detector. The crystal (0.5 mm × 0.6 mm × 0.1 mm) was flash-frozen in a stream of liquid nitrogen vapor using a mixture of glycerol and polyethylene glycol as the cryoprotectant. The x-ray beam was tuned to the wavelength 0.920 Å. The entire data collection was carried out in two separate modes, a high (better than 1.6 Å) and a low resolution. A total of 403 frames of data were collected from two crystals, yielding 420,882 observations and 95,343 unique reflections between 99.0- and 0.90-Å resolution, an 86.6% complete set in the resolution range. The intensities were measured and processed with DENZO software package (15). Table I gives a summary of the results from the diffraction experiment.

Structure Solution and Refinement—Details of structure determination were previously described (12). Briefly, the crystal structure of AXEII was determined by the single isomorphous replacement and anomalous scattering (SIRAS) method using an iodine derivative. A complete atomic model of the protein was built into this SIRAS map. This starting model was refined separately with both the 1.10-Å and the 0.90-Å data sets collected at 295 K and 85 K, respectively. The refinements were carried out first with XPLOR (16) and then with SHELX97 (17), implemented on a Silicon Graphics Indigo2 workstation. We describe here the procedure carried out with the cryogenic data. The initial XPLOR refinement was conducted using a 2.0σ cut off on structure amplitudes between 99.0- and 0.90-Å resolution, which included 94,055 reflections. The refinement process consisted of 500 cycles of positional re

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1 The abbreviations used are: AXE, acetylxylan esterase; SIRAS, single isomorphous replacement and anomalous scattering.
finement followed by simulated annealing starting at 3000 K. Individual atomic temperature factors were refined isotropically yielding an R factor of 0.333. The refinement was further continued with SHEXL97. Twelve cycles of conjugate gradient least squares minimization using SHEXL97 were carried out leading to a convergence. A test data set of 5% of the total was used for calculating R_{total}. During first six cycles, the model was refined isotropically and 290 fully occupied solvent waters and 3 sulfate ions were included in the model. Multiple conformers of serines 31, 36, 50, 58, 70, 74, 90, 120, 204 and tyrosine 177 were also included in the model. At this stage of the refinement, the R factor was 0.154 with an R_{total} of 0.169. The seventh cycle of minimization included modeling of the disorder of Ser^{160}, Ser^{166}, and Gin^{174} side chains. The following cycles of anisotropic refinement of all non-hydrogen atoms reduced the R factor by 0.04 and R_{total} by 0.035. Modeling of multiple conformations of His^{187} and further addition of solvent led to the convergence of the refinement process at an R factor of 0.107 for all data. All the computer graphics work was performed on a Silicon Graphics Indigo2 workstation running CHAIN (18). A summary of refinement results is provided in Table II.

In contrast to other high resolution structures in which there were small regions of disorder (19, 20), neither the 295 K nor the 85 K structure of AXEII shows any weak or poorly defined electron density usually associated with dynamic disorder. The difference between the low and high temperature structures appears to be in the number of amino acid side chains with multiple conformations. The 85 K structure has more multiply observed side chains. However, this could also result from better modeling of side-chain conformations owing to better data quality and higher resolution. The low temperature structure also shows more ordered solvent molecules, with higher number of tightly bound water oxygens and sulfate ions, including the sulfate at the active site, which is absent in the 295 K structure.

RESULTS

Description of the Secondary and Tertiary Structure—The crystal structure of AXEII and the catalytic triad Ser^{90}-His^{187}-Asp^{178} are shown in Fig. 1a. About 60% or 125 of its 207 amino acids are distributed in ten b-strands (b1 to b10) and six a-helices (a1 to a6). The remaining 82 residues are distributed in five type 1 and 2 b-strands, one y-hairpin turn, and five extended loop regions. Fig. 16 is a schematic showing the topology of the secondary structure of AXEII. The strands, loops, and helices have been numbered based on their sequence of occurrence in the polypeptide. All ten cysteine residues are involved in five intrachain disulfide bridge formation (Cys^{2}-Cys^{79}, Cys^{46}-Cys^{55}, Cys^{101}-Cys^{161}, Cys^{147}-Cys^{179}, and Cys^{171}-Cys^{178}). These disulfide bridges involve at least one cysteine in the loop regions. The tertiary structure consists of a doubly wound a/b sandwich, having a central parallel b-sheet flanked by two parallel a-helices on each side. The catalytic cleft is located at the C-terminal end of the b-sheet near the center, bordered by helical residues 183–193 from one side and the loop 105–113 containing an anti-parallel pair of strands from the other. The geometry and relative orientations of side chains of the catalytic triad are similar to those previously observed in other members of the a/b hydrolase family, such as in cholesteryl esterase (21).

The N terminus of the polypeptide chain is anchored at the second residue by a disulfide bond and then flows into a long strand, b1. The C-terminal end of b1 is adjacent to the catalytic serine at the active site. The polypeptide chain then describes a loop structure, loop 1 (l1) between residues 13 and 20, before entering the helix a1. After completing the terminal strand b2 of the central b-sheet via a right-handed b/b-crossover, the polypeptide chain forms a long loop, a2, between residues 41 and 55, the conformation of which is stabilized by an intra-loop disulfide between Cys^{46} and Cys^{55}. The helix, a2, the longest secondary element in AXEII, comprises residues 56 through 79. The C-terminal Cys^{101} of a2 is involved in the disulfide bridge with Cys^{79}, which anchors the N terminus. b3, having residues 83–89, is the central strand at the C-terminal end of which resides the catalytic Ser^{90} residue. The backbone conformation of Ser^{90} belongs to the very restrictive (+,-) quadrant of the Ramachandran plot, but otherwise is normal for a catalytic serine in esterases and lipases (22). b3 turns sharply into a3, a three-turn helix, which is the only helix in the interior of the molecule, surrounded completely by protein atoms. The C-terminal Cys^{101} of the helix is disulfide-bridged to Cys^{161} from loop l3. This is followed by a 17-residue loop l3 between 101 and 117, containing a short anti-parallel segment formed by b4 and b5. The loop ends in a two-turn helix a4 oriented nearly perpendicularly to the sheet, unlike other helices. The next segment of the polypeptide chain between residues 126 and 171 contributes three strands b6, b7, and b8, and b10 with terminal residues 127–131, 142–145, and 167–171, respectively, to the central b-sheet, as well as to a short anti-parallel segment, b7 and b9, consisting of residues 135–138 and 148–151, respectively, away from the sheet. The intervening loop l4, the longest loop in the structure between residues 152 and 166, is held in place by a disulfide bridge between Cys^{161} and Cys^{101} from the C-terminal end of a4. All of the secondary structure crossovers

<table>
<thead>
<tr>
<th>Resolution range (Å)</th>
<th>Total observations</th>
<th>Unique reflections</th>
<th>Percent possible measured overall (%)</th>
<th>Percent measured in last shell (0.94–0.90Å)</th>
<th>Intensity to S.D. ratio overall</th>
<th>Intensity to S.D. ratio in last shell</th>
<th>R_{merge} (last shell)</th>
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</thead>
<tbody>
<tr>
<td>99.00–0.90</td>
<td>420,882</td>
<td>95,343</td>
<td>86.6</td>
<td>40.4</td>
<td>29.2</td>
<td>5.5</td>
<td>0.058 (0.185)</td>
</tr>
</tbody>
</table>

Table II: Refinement statistics

| Protein atoms in the model | 1442 |
| No. of protein residues with multiple conformations | 13 |
| Solvent sulfates | 4 |
| Solvent waters | 300 |
| Solvent glycerols | 4 |
| Resolution range (Å) | 99.0–0.90 |
| Unique data used (F > 0) | 95,283 |
| Overall completeness (%) | 86.6 |
| Weighted R^2 | 0.276 |
| Crystallographic R | |
| All data | 0.107 |
| F > 4oF | 0.103 |
| R_{merge} | |
| All data | 0.132 |
| F > 4oF | 0.128 |
| Goodness of fit | 2.46 |
| Restrainted goodness of fit | 2.28 |
| r.m.s. deviations | |
| Bond distance (Å) | 0.016 |
| Bond angle (°) | 1.982 |
| Dihedral angle (°) | 25.230 |
| Planarity (°) | 1.717 |
| Ramachandran plot statistics | 91.1 |
| residues excluding glycines and prolines in the most favored region | 91.1 |

**TABLE I** Data collection statistics

**TABLE II** Refinement statistics
are right-handed, whereas the $\beta_8$-loop-$\beta_{10}$ crossover is left-handed. This segment of the polypeptide consisting of peripheral strands and loops are held together by two additional disulfide bonds, between Cys$^{147}$ and Cys$^{179}$, and between Cys$^{171}$ and Cys$^{178}$, which also anchor the next loop, $\lambda_5$ consisting of residues 172–183. Asp$^{176}$ is contributed by this loop to the catalytic triad. Held by two disulfide bonds, the polypeptide chain makes a right-handed turn into a short helix $\alpha_5$ (residues 184–189), which contributes the last of the catalytic side chains, His$^{187}$. This helix is separated from the C-terminal helix $\alpha_6$ (residues 195–207) by five intervening residues.

Comparison with Fusarium solani Cutinase—The only other known structure that is similar to AXEII is that of cutinase, an esterase that hydrolyzes cutin, a polyester component of the waxy layer of a plant’s cuticle. The structure of cutinase has been determined at 1.0-Å resolution (20). Fig. 2 shows a superposition of backbones of AXEII and cutinase. Table III is an alignment of the AXEII amino acid sequence (7) with that of cutinase based on the secondary structures. In comparison with AXEII, cutinase has 29 additional residues at the N terminus of which the first 16 belong to a signal peptide. The next 13 are present in the crystal structure, where residues are numbered from 17 to 213 (197 amino acids; Protein Data Bank code: 1CEX). Although the overall similarity between the two tertiary structures is striking (the root mean squared deviation for 170 Ca atoms is 1.2 Å), long insertions and deletions, substitutions, and altered disulfide bond structure account for a large number of local conformational changes, especially in loops around the active site. AXEII has five disulfide bonds, whereas cutinase has only four of these cysteines bonded into two disulfides. All three of these additional disulfides (Cys$^{46}$-Cys$^{55}$, Cys$^{101}$-Cys$^{161}$, and Cys$^{147}$-Cys$^{179}$) are involved in anchoring loops surrounding the active site that are associated with large conformational differences between two polypeptides. In contrast, loop $\lambda_1$ (residues 13–21) between $\alpha_1$ and $\beta_1$, adjacent to the active site but away from the substrate-binding cleft, is conformationally quite similar to the one in cutinase. The conformations of $\lambda_2$ (residues 41–55) in these two structures are such that they project in opposite directions: in cutinase more toward the interior, in the direction of the active site, whereas in AXEII it is pulled outward away from the active site by the disulfide bridge between Cys$^{46}$ and Cys$^{52}$. A conformation of this loop like the one in cutinase would be in direct steric conflict with $\lambda_3$ absent in cutinase. Also, the region 41–43 of the loop has a deletion of three residues in comparison with cutinase, which shortens the path to the disulfide bridge.
The loop \( \lambda_3 \) consists of an insertion of residues 101–115 that contain a short anti-parallel segment (\( \beta_4-\beta_5 \)). This segment of polypeptide is absent in cutinase. Its strategic positioning at the rear wall of the active site opening suggests its distinctive role in recognition and processing of acetate side groups of a chain of D-xylopyranose as opposed to cutin molecules by cutinase. The next major difference between the two structures is in loop regions, including \( \lambda_4 \), which spans between residues 148 and 166 for cutinase, corresponding to residues 132 and 166, respectively, in AXEII, in spatial positions. This 16-residue insertion in this segment is distributed mainly between two short anti-parallel strands \( \beta_7 \) (135–138) and \( \beta_9 \) (148–151), and the sixth strand of the central \( \beta \)-sheet, \( \beta_8 \). Not one of these features of the region is present in cutinase. The presence of Cys\(^{101} \)-Cys\(^{161} \) disulfide in AXEII also drastically alters the course of the backbone between residues 154 and 166, although these residues in the two proteins are analogous to each other in spatial positions and sequence alignment. The loop \( \lambda_5 \) (residues 172–183 in AXEII and 172–184 in cutinase) contributes Asp\(^{175} \) to the catalytic triad and has one conserved disulfide (Cys\(^{171} \)-Cys\(^{178} \)); however, the additional disulfide in AXEII (Cys\(^{147} \)-Cys\(^{179} \)) alters the course of the main chain by pulling it away from the catalytic cleft. In addition to one residue insertion between 179 and 184, the backbone of cutinase in this segment adopts a different path such that the distance between C\( \alpha \) positions of 181 in AXEII and the equivalent 182 in cutinase is 10.4\( \AA \). This altered conformation of the polypeptide chain in cutinase partially shields catalytic Asp\(^{175} \) and His\(^{187} \) from exposure to the solvent, in contrast to exposed catalytic His\(^{187} \) in AXEII. Besides, a cutinase-like path for this segment of \( \lambda_5 \) in AXEII would put residues 178 and 179 in steric conflict with the backbone of 150–152 of \( \lambda_4 \). Residue 184 marks the beginning of a two-turn helix \( \alpha_5 \), which contains the catalytic histidine (His\(^{187} \) in AXEII and cutinase, respectively) and is quite similar in both structures. The C-terminal helix \( \alpha_6 \) in AXEII is similar to that in cutinase, except for the fact that cutinase has four additional C-terminal residues in the helix.

The secondary structure elements of the two proteins are otherwise quite similar, except for occasional insertion or deletion of few residues and their spatial displacement. The \( \beta \)-sheet core of the two structures superimposes almost perfectly, except for the terminal strand \( \beta_2 \) and the missing \( \beta_8 \) in cutinase. The C\( \alpha \) positions and side-chain atoms of the catalytic residues superimpose with an average deviation of 0.3 \( \AA \) from each other.

### Table III

<table>
<thead>
<tr>
<th>Element</th>
<th>( \beta )</th>
<th>( \alpha )</th>
<th>Loop</th>
<th>( \beta )</th>
<th>Loop</th>
<th>( \alpha )</th>
<th>Loop</th>
<th>( \beta )</th>
<th>Loop</th>
<th>( \alpha )</th>
<th>Loop</th>
<th>( \beta )</th>
</tr>
</thead>
</table>

### FIG. 3

A view of the catalytic triad in A and B states. Carbon atoms are shown in gray, nitrogen in blue, oxygen in red, and sulfur in yellow. Gray bonds depict the A and green the B states. Bonds that belong to both states are also shown in gray. The electron density shown is from a final \( (2F_{\text{obs}} - F_{\text{calc}})^2 \) map, contoured at 1.8\( \sigma \). The view is oblique to the imidazole ring of His\(^{187} \).

### FIG. 4

Movement of Tyr\(^{177} \) from A (gray) to B (green) states. The electron density is from a final \( (2F_{\text{obs}} - F_{\text{calc}})^2 \) map, contoured at 1.0\( \sigma \).
The Active Site and Multiple Conformations of Catalytic Residues—The active site gorge (Fig. 1a) is bordered by the following segments of the tertiary structure: $\lambda_1$ (residues 13–20) and $\lambda_2$ (residues 41–55) to the left, in the view in Fig. 1a; $\beta_3$-to-$\alpha_3$ turn at the center of the gorge, where the three side chains of the catalytic residues are located; and, helix $\alpha_5$, $\lambda_5$ (residues 175–183), $\beta_6$-to-$\alpha_7$ turn (residues 132–134), and $\lambda_4$ (residues 152–161) to the right. The opening of the active site is 11 Å across, 7 Å thick from the surface to the catalytic triad, and has a depth of 20 Å to the back wall delineated by residues of $\lambda_3$ (101–117). Residues lining the gorge are Glu189, Tyr187, Ser90, Thr13, Tyr57, Gln91, Phe152, Tyr177, and Gln188. Carbon atoms are shown in gray, nitrogen in blue, oxygen in red, and sulfur in yellow. In this orientation Ser90 Oy is roughly normal to the acetate plane at a distance of 2.6 Å to the carbon atom.

Fig. 5. Hydrogen bonding interactions at the active site of AXEII in (a) B states and (b) A state. Side chains of residues Ser$^{90}$, His$^{187}$, Asp$^{175}$, Thr$^{13}$, Tyr$^{57}$, Gln$^{91}$, Phe$^{152}$, Tyr$^{177}$, and Gln$^{188}$. Carbon atoms are shown in gray, nitrogen in blue, oxygen in red, and sulfur in yellow. Bonds in the B state are shown in green.

Fig. 6. Docking of 2-acetyl xylopyranose in the active site of AXEII. Side chains shown are Ser$^{90}$, His$^{187}$, Asp$^{175}$, Glu$^{12}$, Thr$^{13}$, Tyr$^{57}$, Gln$^{91}$, Phe$^{152}$, Tyr$^{177}$, and Gln$^{188}$. Carbon atoms are shown in gray, nitrogen in blue, oxygen in red. Disulfide bonds are shown in yellow. I n this orientation Ser$^{90}$ Oy is roughly normal to the acetate plane at a distance of 2.6 Å to the carbon atom.
His\textsuperscript{187} side chain in the A state. Tyr\textsuperscript{177} moves about 2 Å away to accommodate His\textsuperscript{187} in the B state (Fig. 4). The transition to the B state at 85 K could have been initiated by binding of a sulfate ion to the His\textsuperscript{187} Ne\textsubscript{2} atom through a hydrogen bond formation (His\textsuperscript{187} Ne\textsubscript{2}...O\textsubscript{2S O\textsubscript{4}} 210: 2.75 Å) (Fig. 5a). O\textsubscript{2} of SO\textsubscript{4} 210 is also hydrogen-bonded (2.75 Å) to water oxygen 473, which, in turn, forms a hydrogen bond with Tyr\textsuperscript{57} side-chain hydroxyl (2.89 Å). The sulfate ion makes four additional hydrogen bond-forming contacts with protein atoms as shown in Fig. 5a: O\textsubscript{3} to Thr\textsuperscript{13} O\textsubscript{g} (3.03 Å), to Thr\textsuperscript{13} backbone NH (3.03 Å), and to Gln\textsuperscript{91} backbone NH (2.98 Å), and O\textsubscript{4} to Thr\textsuperscript{13} O\textsubscript{y} (2.75 Å). In addition, the O\textsubscript{1} oxygen of sulfate is coordinated to three water oxygens 465, 488, and 489 through strong hydrogen bonds (average distance; 2.80 Å) in a tetrahedral manner, all of which are linked to protein atoms by hydrogen bonds (to Gln\textsuperscript{188} Ne\textsubscript{2}, Thr\textsuperscript{13} backbone carbonyl, and Ser\textsuperscript{90} O\textsubscript{y}; average distance ~2.84 Å). In the B state, binding of the sulfate ion eliminates three water oxygens that are present only in the A state, namely, 588, 599, and 600. O\textsubscript{3} of the sulfate ion replaces water oxygen 598. However, binding of the sulfate ion does not affect water oxygens 465, 473, 488, and 489, all of which are strongly coordinated to protein atoms. These water molecules are also present in the 295 K structure. There is an estimated net gain of two hydrogen bonds in the transition from the A state to the B state.

An oxyanion binding site is comprised of two backbone amide groups of Thr\textsuperscript{13} and Gln\textsuperscript{91}, as well as Ser\textsuperscript{90} O\textsubscript{y} and Thr\textsuperscript{13} O\textsubscript{y} hydroxysls in the A state, as shown in Fig. 5b. The Thr\textsuperscript{13} side chain is stabilized by a hydrogen bond from Gln\textsuperscript{91} Ne\textsubscript{2} (2.90 Å). The water oxygen 598 is thus bound at this oxyanion binding site in a roughly tetrahedral geometry. In the B state, SO\textsubscript{4} 210 occupies the oxyanion hole by replacing water 598; the position of O\textsubscript{3} of the sulfate nearly coincides with the oxygen atom of water 598. Furthermore, in the B state, O\textsubscript{2} of SO\textsubscript{4} 210 occupies what used to be the Ser\textsuperscript{90} O\textsubscript{y} position relative to the His\textsuperscript{187} imidazole ring in the A state, thus maintaining an orientation to the imidazole ring similar to that of O\textsubscript{y} in the A state.

**Other Residues in Alternate Conformations**—In addition to Ser\textsuperscript{90}, Tyr\textsuperscript{177}, and His\textsuperscript{187}, 10 serine (residues 31, 36, 50, 58, 70, 74, 120, 160, 196, and 204) and Gln\textsuperscript{154} side chains possess two alternate conformations. These were identified from positive and negative difference electron density maps. The occupancies of the major conformations range between 55 and 75%. All of the alternate conformers have hydrogen bond-forming partners, either protein or solvent atoms, in both conformations. Side chains of the rest of the amino acid residues have well-characterized electron densities that uniquely define their conformations. Unlike other high resolution structures, no dynamically disordered region of the polypeptide chain is identifiable. Only the residues at two termini have significantly higher than average temperature factors; their average equivalent isotropic temperature factors are 25 and 45 Å\textsuperscript{2} for main-chain and side-chain atoms, respectively. The average equivalent isotropic temperature factor for the rest of the protein C, N, and O atoms is about 12 Å\textsuperscript{2}.

**Packing Interactions in the Crystal**—One of the closest pack-
ing interactions between secondary structure elements in the crystal occurs between α1 of one molecule and α2 of another. However, very little direct hydrophobic packing surfaces are involved in these close contacts. Instead, several solvent molecule-mediated interactions dominate this molecular interface. Notable among these include a sulfate ion (213)-mediated contacts between Thr24 O of α1 and Asn73 N of Ser70 O of α2. Several water molecules line this interface. The closest approach between the helices is at Ala62 Cβ of α2, which is at a van der Waal’s contact distance (3.84 Å) from Ser31 Cα of α1. In the second conformation, Ser31 Oγ also makes a water-mediated (449) contact to OH of Tyr110 of β5 of the same neighboring molecule. The backbone amide of N-terminal Gly35 of α1 donates the proton to the backbone carbonyl oxygen of Gly109 from β5, thereby forming an intermolecular hydrogen bond. A second intermolecular hydrogen bond is formed in this region between the backbone carbonyl of Ser36 from β2 and Thr111 Oγ. In addition, the C-terminal end of α2 is involved in two intermolecular hydrogen bonds: Ser77 backbone carbonyl to Asn180 side chain and Gln78 side chain to Gly48 backbone carbonyl. Gly78 Nε2 also accepts a proton from an alternate conformation of Ser30 Oγ across the molecular interface. Asn180, Ser30, and Gly48 are all from extended loop regions of the molecule. Gly139 Cα from the disulfide stabilized loop α2 packs (3.83 Å) against Ala149 Cα, from the adjacent molecule. Other short contacts in this loop-interaction between two molecules include an approach (3.07 Å) of Asn174 backbone carbonyl oxygen to Gly139 Cα and packing of Pro17 side chain against Thr140 Cα.

Intermolecular hydrogen bond formation between two alternate conformations of serine Oγ atoms is also observed at the packing contact between the N terminus of helix α6 and the loop α4 region (residues 159–161) of a neighboring molecule. In one conformation, Ser186 side chain forms a hydrogen bond to an alternate conformation of Ser160 Oγ and in its second conformation, the hydrogen bond is formed between the side chain and the backbone amide of Ser160. Again, several water molecules tightly hydrogen bonded to protein atoms are found at this interface. The other two direct intermolecular contacts between these two regions involve a hydrogen bond between Lys203 side chain of α6 and Gly157 backbone carbonyl, and the packing of Leu199 Cα2 against Gly158 Cα (4.16 Å). Although the C terminus approaches the anti-parallel loop α3, there is no direct contact between the two. Intermolecular contacts in this region involve interactions between Lys83 side chain and backbone carbonyl of Asn107, and between Ala81 backbone carbonyl and Asn107 side chain.

A packing interaction that involves nonpolar contacts occurs in the region where the short helix α4 approaches α4 after β9. Met124 Sε from α4 has a van der Waal’s contact (3.75 Å) with Phe152 Cβ of the adjacent molecule. In the same region, the carbonyl oxygen of Pro80 has a short contact (3.25 Å) to Ca of Phe152. Ser120 side chain from α4, in its more occupied conformation, approaches the loop α5 that contributes Asp125 to the catalytic triad, by donating the proton to the Pro120 backbone carbonyl oxygen. In addition, side chains of residues Asn183 and Ala185, near the N terminus of α5 packs against side chains of Thr14 and Ala15 from α1 and Ser15 from α2, respectively, of a symmetry-related molecule. The crystal packing of AXEII is, thus, dominated by polar interactions; there are only a few interactions among hydrophobic side chains.

**DISCUSSION**

The Active Site and Possible Binding Modes of the Substrate—Coexistence of two different conformational states, perhaps representing two oxidation states of catalytic Ser and His residues in the same crystal, is a major finding that was only possible due to extraordinarily high resolution of the study. In the state A, which is the resting state of the enzyme resembling the geometry of the side chains in the 295 K structure (12), it is likely that Ser30 OγH is the proton donor in the Ser30 OγH . . . Ne2 His187 hydrogen bond. It was shown for cutinase that Ne2 is deprotonated (19). In the presence of a negatively charged sulfate ion, which is a strong nucleophile and makes a direct contact (2.75 Å) to His187 Ne2, it is likely that Ne2 retains the proton due to increase in pKₐ. (Efforts to locate this proton from the difference electron density maps were unsuccessful, perhaps due to complications from modeling the side chain in two alternate conformations.) The B state, therefore, could be a mimic of the binding of the tetrahedral intermediate to His in which the imidazole ring has an overall positive charge. Although the Ser side chain in the B state assumes a catalytically “inactive” position, the proximity and orientation of the Ser Oγ proton to the Ne2 of His187 could contribute to the stability of the B state. The movement of residues His187 and Tyr177, demonstrated by multiple conformations of their backbone and side-chain atoms, is perhaps reflective of the motion within the catalytic cavity during intermediate steps of catalysis.

In an attempt to comprehend substrate-protein interactions, a 2-acetyl xylopyranose molecule, a substrate for AXEII (3), was manually docked at the active site in one of at least two possible orientations of the pyranose ring (Fig. 6). In this position, Ser Oγ is oriented roughly normal to the acetate plane at a distance of 2.6 Å to the carbon atom. In both orientations, however, the pyranose ring stacks against the Tyr177 side chain. Interestingly, the location of the pyranose ring nearly coincides with the only glycerol molecule found in the active site. In this binding mode, the acetate group is at a van der Waal’s contact distance from the backbone and side-chain atoms lining the pocket, namely Thr137 NH and His187 CO and side-chain atoms of His137, Gln12, Tyr89, and Gln188. The acetate group thus fits nicely in this pocket, which seems to be tailored to accommodate such a moiety. This observation is consistent with the biochemical data that AXE II has high specificity for the acetate ester and only weakly hydrolyzes esters of longer fatty acids (3). Alternatively, a small substrate like 2-acetyl xylopyranose may flip around and bind in a direction opposite to the one shown in Fig. 6. The acetyl group would then face the longer end of the active site, thus making room for longer fatty acid side chains to bind and be hydrolyzed. However, in this mode the xylopyranose moiety binds to the interior of the molecule, precluding the possibility of a polymeric substrate such as an acetylated xylan from binding at the active site.

Other residues that the substrate may have direct contact with are Tyr57, Gln91, and Phe152 (Fig. 6). In the active site gorge, which is about 20 Å long to the back wall containing the anti-parallel loop α3 (see the description of the active site in the result section), an estimated four to five xylose residues of a xylan chain could be accommodated. If a xylan chain binds at the active site, then xylose residues may additionally make contacts with residues Ser58 Asp153 Glu158, and Asn107.

It is possible that the loop α3 containing the anti-parallel strand β4–β5 plays a special role in recognition and binding of a xylan chain. This loop, absent in cutinase, is nearly 10 Å away from the substrate atoms when a single molecule of xylose binds at the active site (Figs. 7 and 8). However, it may serve as a gate to the active site for binding of a xylan chain. Interestingly, the loop is loosely anchored to the rest of the molecule by hydrogen bonds near the turn, Gln108 Ne2... O61 Asp153 (2.92 Å), Asn107 Nε2... OC Phe102 (2.99 Å), and Asp105 O61... Ox1 Glu94 (2.54 Å). There are several acidic side chains in this region that are linked to each other by hydrogen bonds, as shown in Fig. 7. These residues are, therefore, protonated at the
crystallization pH of 5.3. Deprotonation of these residues, coupled with conformational changes, could trigger breakage of the hydrogen bonds and release of the loop. This structural flexibility could be critical to its role as the "gatekeeper" of the active site gorge and recognition of a xylan chain.

Accessibility of the Active Site—As described under "Results" and in the discussion above, the active site gorge of AXEII is more exposed than the only other known structure of the same family, cutinase. With a water molecule as the probe, the accessibility surface for the catalytic Ser and His is more for AXEII (12.8 Å²) than for cutinase (7.9 Å²). In contrast, catalytic residues are relatively buried in cutinase, as has been illustrated in Fig. 8. Not only the Ser³⁰ and His¹⁸⁷ side chains are more exposed, but also the gorge stretches wider in AXEII than in cutinase. A few side chains, such as Tyr¹⁷⁷, Tyr⁵⁷, and Phe¹⁵² cover the gorge, as shown in Fig. 8, reducing its accessibility. Furthermore, the gatekeeper loop limits the access to the gorge for the xylan chain. However, as has been pointed out, some of these residues and the loop belong to flexible regions of the molecule, and it is likely that they undergo movement to accommodate a xylan chain. The openness of the active site is suggestive that AXEII is a "pure" esterase as opposed to a lipase that functions as an esterase on esters of long-chain fatty acids or substrates containing lipid-like side groups. AXE II, in contrast, uses polar molecules as substrates, as an AXE should.

Molecular Packing—Despite the overall compact nature of the AXEII molecule, 82 of 207 residues are located in loops and turns. Although each of five disulfide bridges involves at least one Cys from loops and turns, three have both Cys residues belonging to extended loop regions of the molecule, namely α2, α3, λ4, and λ5. The internal rigidity of the AXEII molecule is a direct consequence of well-packed secondary structure elements and disulfide-stabilized loops and turns. Some of the loop regions are highly polar and form parts of the outer surface. The central theme in packing of AXEII molecules in the unit cell is: few hydrophobic and many polar interactions, the presence of interfacial solvent molecules and solvent-mediated protein-protein interactions, and strong loop-loop contacts in several extended loop regions. These are all characteristics of a tightly folded monomeric, soluble, globular protein. A high long range order of the crystal could, however, be a consequence of the molecule's overall shape and its highly polar surface property that is conducive to close packing. The overall shape of the molecule resembles a cylinder of an average diameter of 27 (±3) Å and a height of 38 (±3) Å. These cylindrical molecules are packed in the unit cell with the cylinder axes along the z axis, which, interestingly, is also roughly the direction along which major helices and the central β-sheet are aligned.

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REFERENCES