Crystal Structure of Barley 1,3–1,4-β-Glucanase at 2.0-Å Resolution and Comparison with Bacillus 1,3–1,4-β-Glucanase

(Received for publication, May 16, 1997, and in revised form, November 5, 1997)

Jürgen J. Müller‡‡, Karl K. Thomsen¶, and Udo Heinemann‡‡

From the ‡‡Forschungsgruppe Kristallographie, Max-Delbrück-Centrum für Molekulare Medizin, Robert-Rösse-

Strasse 10, D-12122 Berlin, Germany, the ¶Department of Physiology, Carlsberg Laboratory, Gamle Carlsberg Vej 10,

DK-2500 Copenhagen-Valby, Denmark, and the ‡‡Institut für Kristallographie, Freie Universität Berlin,

Takustrasse 6, D-14195 Berlin, Germany

Both plants and bacteria produce enzymes capable of degrading the mixed-linked β-glucan of the endosperm cell walls of cereal grains. The enzymes share the specificity for β-1,4 glycosyl bonds of O-3-substituted glucose units in linear polysaccharides and a similar cleavage mechanism but are unrelated in sequence and tertiary structure. The three-dimensional structure of the 1,3-β-glucanase isoenzyme EII from barley was determined from monoclinic crystals at a resolution of 2.0 Å. The protein is folded into a β̇α̇β̃ barrel structure as has been shown previously (Varghese, J. N., Garrett, T. P. J., Colman, P. M., Chen, L., Høj, P. B., and Fincher, G. B. (1994) Proc. Natl. Acad. Sci. U.S.A. 91, 2755–2789) by diffraction analysis at lower resolution of tetragonal crystals. It contains one N-glycosylation site which is described in detail with the sugar moieties attached to residue Asn190. The geometry and hydration of the barley 1,3-1,4-β-glucanase is analyzed; a model β-glucan fragment is placed into the binding site by molecular dynamics simulation, and the β-glucan binding grooves of the plant and bacterial enzymes are compared. Their active sites are shown to have a small number of common features in generally dissimilar geometries that serve to explain both the identical substrate specificity and the observed differences in inhibitor binding.

The 1,3–1,4-β-glucanase (1,3–1,4-β-d-glucan 4-glucanohydrolase, EC 3.2.1.73) from barley (Hordeum vulgare, cv. Alexis) degrades mixed linked β-glucans in the cell walls of the starchy endosperm in grains (1). It acts as an endohydrolase on a long linear polysaccharide with about 30% β-1,3 and 70% β-1,4 glycosyl linkages (2). The scissile bond is the β-1,4-linkage of an O-3-substituted glucose in the mixed linked β-glucan. The crystal structure of the tetragonal form of the 1,3–1,4-β-glucanase (isoenzyme EII) is known at 2.2-Å resolution as well as that of the homologous 1,3-β-glucanase from barley (3). EII is glycosylated at Asn190 by a branched oligosaccharide of mixed composition (4). This oligosaccharide may function in increasing the thermostability of the protein (5).

1,3–1,4-β-Glucanases occur also in bacterial strains (6). Both native and engineered enzymes have been structurally characterized (7, 8) including the 1,3–1,4-β-glucanases MAC from Bacillus macerans and LIC from Bacillus licheniformis (9, 10). The crystal structure of H(A16-M), a hybrid β-glucanase with residues 1–16 derived from the Bacillus amyloliquefaciens enzyme (AMY) and residues 17–214 from MAC, was solved at 1.6-Å resolution (11). The crystal structures of MAC, LIC, and H(A16-M) are sufficiently similar overall and around the active sites to suggest that these 1,3–1,4-β-glucanases, as well as AMY and SUB, the enzyme from Bacillus subtilis, share a common mode of substrate binding and hydrolysis.

There is neither sequential nor structural homology between the plant and the bacterial enzymes, and following the universally adopted nomenclature (6), they belong to the families 17 and 16 of glycosyl hydrolases, respectively. Despite this difference they cleave the same substrate at the same cutting site and are inhibited by the same covalently binding inhibitors, 3,4-epoxyalkyl-β-D-cellubiosides (12). However, the barley 1,3–1,4-β-glucanase binds the epoxide preferentially with a propyl linker, whereas SUB prefers a butyl linker (13). Because both endohydrolases follow the same stereochemical pathway in glycosyl bond cleavage with retention of the β-configuration at the anomeric carbon (14, 15), the differences in inhibitor binding are surprising at first sight and warrant a structural explanation. A cleavage mechanism with overall retention of configuration requires the presence in the catalytic site of a general acid separated by about 5.5 Å from a nucleophilic residue (6). These roles have been assigned to Glu288 and Glu232 of EII, respectively (3).

Here we present the structure analysis of monoclinic crystals of the barley 1,3–1,4-β-glucanase (isoenzyme EII) at 2.0-Å resolution. We discuss structural differences between three molecules of EII in two space groups and compare the active center structures of plant and Bacillus 1,3–1,4-β-glucanases. Structural reasons for inhibitor binding preferences are considered, and possible conformations and positions of a hexameric β-glucan fragment are investigated by molecular dynamics calculations.

EXPERIMENTAL PROCEDURES

Diffraction Data and Structure Analysis by Molecular Replacement—The purification, crystallization, and x-ray diffraction data of barley N-acetyl-β-glucosamine; PDB, Brookhaven Protein Data Bank; 1ghr, Ighs, 2ayh, 1byh, PDB entries for the tetragonal crystal form of EII, 1,3-glucanase isoenzyme GII from barley, H(A16-M), and H(A16-M) with 3,4-epoxybutyl β-D-cellubioside inhibitor, respectively; NCS, non-crystallographic symmetry.
1,3–1,4-β-glucanase, isoenzyme EII, have been described (16). The diffraction data set was collected from one crystal at room temperature with a 180-mm MarResearch imaging plate system on an Enraf Nonius FR571 rotating anode x-ray generator (45 kV, 90 mA). The images were evaluated with MOSFLM, AGRVATA, and ROTAVATA from the CCP4 suite (20). Relevant crystallographic parameters in the monoclinic space group P2₁ are summarized in Table I. Space group and unit cell dimensions are consistent with the presence of two protein molecules per asymmetric unit. The 40,727 unique reflections correspond to 99.3% of the observations expected for 13.65 to 2-Å resolution, and the completeness in the outermost resolution shell from 2.09 to 2.0 Å is 96.6%. The crystal and x-ray diffraction data for EII in the tetragonal space group P4₃2₁2 were collected (77.56°, 109.5°) with TIP3 waters provided by the XPLOR package (24). After this procedure, molecular Mol1 of EII was oriented with its inertia equivalent ellipsoid axes parallel to the P1 cell axes. The overlapping water molecules were removed so that Mol1 finally was embedded in 5,069 non-overlapping water molecules. The water box is large enough to cover the substrate by a water shell of at least 13-Å thickness. The charged residues of the protein are made net neutral to mimic the effects of solvation and counterions. The nonbonded interaction cutoff was specified to 8 Å. The molecular dynamics calculation, done by XPLOR, preceded by 120 steps of standard repel nonbonded energy minimization and 80 steps of Powell Lennard-Jones minimization in vacuum, started with the fixed enzyme-substrate complex to relax the water molecules around the macromolecule. The solvent molecules were relaxed further during 120 steps of energy minimization followed by molecular dynamics for 5 ps at 100 K. At 200 K, 20 ps at 300 K with 0.2-Å reassignment using the CHARMM force field. The energy was then minimized for the solute (100 steps Powell) followed by several steps of molecular dynamics for the solute (5 ps at 100 K, 5 ps at 200 K, 5 ps at 300 K). Finally, molecular dynamics were run for 80 ps at 300 K with temperature coupling for the whole system, followed by 80 steps of Powell Lennard-Jones minimization. Over the entire simulation the Mol1 molecule remained fixed, but those side chains in the binding cleft containing atoms in a 4-Å surface shell, the hexaglucan, and the waters were allowed to move. Alternative protocols without water relaxation at 100, 200, and 300 K and without temperature coupling resulted in similar sugar conformations and positions.

RESULTS AND DISCUSSION

Refinement Results—After combining working and test data sets, final positional and B-value refinement yielded an R-value of 17.0%. The R-value was 16.5% for all observations with a low resolution cutoff at 6 Å as used in the previous structure analysis (3). Two residues of N-acetyl-D-glucosamine (NAG) per molecule of the barley 1,3–1,4-β-glucanase, we used the hexameric β-glucan fragment proposed earlier (11) as start model in simulating substrate binding to EII. The glucose moieties are numbered from 1 to 6 beginning at the non-reducing end. They bind to enzyme subsites p4, p3, p2, p1, p1’, and p2’, and cleavage occurs at the catalytic site between p1 and p1’. Manual primary positioning of the hexamer in the binding cleft using O (21) took into account restrictions deduced from chemical probing with barley 1,3–1,4-β-glucanase (22) and inhibitor binding to the structurally very similar 1,3-β-glucanase of barley (1g8; Ref. 23).

To start the molecular dynamics simulation, a FORTRAN program written for this purpose but not specific for the glucanase molecule first filled a P1 cell with dimensions 66.379 x 60.241 x 52.643 Å with TIP3 waters provided by the XPLOR package (24). After this procedure, the small molecules were added following the standard slow-cooling protocol (100 steps Powell) followed by several steps of molecular dynamics for the solute (5 ps at 100 K, 5 ps at 200 K, 5 ps at 300 K). Finally, molecular dynamics were run for 80 ps at 300 K with temperature coupling for the whole system, followed by 80 steps of Powell Lennard-Jones minimization. Over the entire simulation the Mol1 molecule remained fixed, but those side chains in the binding cleft containing atoms in a 4-Å surface shell, the hexaglucan, and the waters were allowed to move. Alternative protocols without water relaxation at 100, 200, and 300 K and without temperature coupling resulted in similar sugar conformations and positions.

### Table I

|                | EII | 1 ghr |
|----------------|-----|-------|
| Space group    | P2₁ | P4₂,2₂ |
| a (Å)          | 85.58 | 87.4 |
| b (Å)          | 82.99 | 87.4 |
| c (Å)          | 77.56 | 109.5 |
| β (°)          | 104.36 | 90.0 |
| V (Å³)         | 309.161 | 836.444 |
| \( V₀ (Å³ Da⁻¹) \) | 2.41 | 3.26 |

Molecules per asymmetric unit 2 1

Residues 306 306

Number of reflections 40,727 16,399

Number of reflections per degree of freedom 1.96 NA

Resolution (Å) 13.6–2.0 6–2.2

Completeness (%) 99.3 82.0

R₁ (%), R₁ (no NCS) 14.4/8.1/59.1 14.0/7.9/66.8

Rmerge (%) 25.2 26.3%

Sym (%) 8.1 5.5

merge (%) 8.1 10.1

Number of waters 418 47

### Table II

|                | Mol1 | Mol2 |
|----------------|------|------|
| Deviation from target geometry | 0.007 | 1.314 |
| rms bond lengths (Å) | 24.06 | 1.184 |
| rms Δ bond angles (°) | <0.2 | <0.2 |
| rms Δ torsion angles (°) | 0.260/0.54/0.38 | 0.270/0.54/0.39 |

| Solvent structure | NCS waters (number/%) | 130/63.2 |
|--------------------|-----------------------|-----------|
|                     | B, NCS waters (Å²) average/max | 28.8/58.2 |
|                     | B, other waters (Å²) average/max | 32.8/61.0 |
|                     | Δ, NCS waters (Å²) rms/average | 10.8/7.9/4.35 |
|                     | SD/max | 0.57/0.46/0.34|

* a NCS waters are related by non crystallographic symmetry, other waters are not.
EII were located at the glycosylation site, Asn190. The side chains of Asp183 and Ile304 in Mol1 and Gln41 in Mol2 were refined in two alternative positions with 2/3 and 1/3 occupancy, and two acetate molecules were identified in non-related positions. In the Ramachandran diagram, more than 91% of the non-glycine and non-proline residues are within the most favored regions, and all other residues are within additional allowed regions. Two cis-peptides preceding Pro137 and Ala276, identical to those in 1ghr, are present in both Mol1 and Mol2. The average correlation of the calculated Fc map with the 2Fo − Fc electron density map is better than 0.9 for both molecules according to an analysis with O (21). The correlation coefficient drops below 0.7 for the highly flexible side chains of Arg100, Arg197, and Arg261, which have B values of about 50 Å² and are not sterically restricted by hydrogen bonds or van der Waals contacts in both molecules.

Stereoechemical parameters of the main and side chains are strongly restrained by standard weights and are better than or inside of the bandwidth defined in PROCHECK (25) for structures with comparable resolution. The final atomic coordinate set representing the monoclinic form of EII was scrutinized with WHATCHECK (26), PROCHECK, and several programs of the CCP4 suite. The results are summarized in Tables I and II. The experimental data and the refined atomic coordinates of both molecules in the asymmetric unit were submitted to the Brookhaven Protein Data Bank (27) entry code 1aq0.

**Overall Structure and Crystal Packing—** The overall structure and the active site of EII have been described by Varghese et al. (3) and are very similar in the monoclinic crystal form. The overall shape of the 1,3–1,4-β-glucanase can be approximated by its inertia equivalent ellipsoid with half-axes of 29.1, 25.2, and 16.4 Å. The global folding pattern belongs to the β8 barrel type, although β-strand number 8 of the barrel is truncated to just two residues (Fig. 1). The active site is located in an open cleft at the bottom of the barrel defined by the C-terminal ends of the parallel intra-barrel β-strands. It is about 36 Å long and 8 to 9 Å deep, allowing the binding of oligosaccharide substrates.

**TABLE III**

Statistics measuring the degree of similarity of molecules Mol1, Mol2, and 1ghr

|                | Mol1/Mol2 | Mol1/1ghr | Mol2/1ghr |
|----------------|-----------|-----------|-----------|
| Complete molecules |           |           |           |
| Atom displacement after least-squares fit (Å) | 0.40/0.16/3.81 | 0.28/0.24/1.10 | 0.47/0.30/3.96 |
| Ca atoms, rms/average/max | 0.41/0.17/4.58 | 0.31/0.25/2.33 | 0.48/0.31/4.29 |
| Main chain, rms/average/max | 1.13/0.46/8.52 | 0.86/0.53/4.41 | 1.05/0.59/8.48 |
| Side chains, rms/average/max | 0.88/0.33/8.56 | 0.62/0.37/4.47 | 0.79/0.43/8.45 |
| All atoms, rms/average/max | 0.40/0.16/3.81 | 0.28/0.24/1.10 | 0.47/0.30/3.96 |
| Thermal parameters |           |           |           |
| <Δ B>, main chain (Å²) | 0.47 | -9.115 | -9.58 |
| <Δ B>, side chains (Å²) | 0.18 | -6.88 | -7.06 |
| <Δ B>, all atoms (Å²) | 0.34 | -8.09 | -8.43 |
| rms Δ B, Ca atoms (Å²) | 3.74 | 6.39 | 6.89 |
| rms Δ B, extended main chain (Å²) | 3.87 | 6.81 | 6.89 |
| rms Δ B, all atoms (Å²) | 4.50 | 8.07 | 8.12 |
| Torsion angles |           |           |           |
| rms Δ ϕ (°) | 9.61 | 11.94 | 14.31 |
| Residues with | 3.28 | 6.84 | 7.56 |
| Δ ϕ | 4.58 | 21.24 | 22.22 |
| rms Δ ϕ (°) | 16.75 | 8.60 | 18.82 |
| Residues with | 4.16 | 5.98 | 7.95 |
| Δ ϕ | 3.27 | 16.34 | 20.92 |
| Residues in the active site cleft |           |           |           |
| Atom displacement after least-squares fit of whole structure (Å) | 0.14/0.09/0.56 | 0.23/0.21/0.52 | 0.27/0.24/0.52 |
| Ca atoms, rms/average/max | 0.15/0.10/0.60 | 0.26/0.23/0.79 | 0.29/0.26/0.80 |
| Main chain, rms/average/max | 0.43/0.19/2.84 | 0.85/0.54/3.16 | 0.78/0.49/3.28 |
| Side chains, rms/average/max | 0.35/0.15/2.88 | 0.63/0.39/3.21 | 0.59/0.38/3.33 |
The two molecules of EII in the asymmetric unit make numerous intermolecular contacts with distances between 2.5 and 4 Å. 13 amino acids of Mol1 are in contact with 14 amino acids of Mol2 through non-crystallographic symmetry. 39 and 43 amino acids of Mol1 and Mol2, respectively, engage in additional crystallographic contacts. These contacts result from a tighter packing of the molecules in the monoclinic cell ($V_m = 2.41 \text{ Å}^3$ Da$^{-1}$) in comparison with tetragonal 1ghr ($V_m = 3.26 \text{ Å}^3$ Da$^{-1}$), where 44 residues make intermolecular contacts. The contacts do not include any residue of the catalytic site and of the potential substrate-binding site (see below) of Mol1 and Mol2, as well as of 1ghr, and therefore do not exert a major influence on the protein structure within the binding cleft. This is suggested by the analysis of distances (LSQKAB, Ref. 28) between 45 equivalent residues within the 4-Å surface of the substrate binding cleft. A comparison of Mol1, Mol2, and 1ghr shows the residues in the cleft to be structurally conserved to a higher degree than those in the rest of the molecules (Table III). The differences between the atom positions of the residues in the catalytic and substrate-binding site (Glu288, Glu232, Tyr33, Glu93, Asn92, Val134, Phe135, Asn168, Tyr170, Leu173, Phe275, Glu280, Lys283, and Trp291) are below the average coordinate error for Mol1 and Mol2 determined to 0.2 Å from the Luzzati (29) analysis.

**Structural Heterogeneity, Subdomains, and Hydration**—The most prominent difference between the main chains of the three molecules is found for amino acid residues 190–200 of Mol2 compared with the other two (Fig. 2). In this region, the structure of Mol2 is influenced by the NCS interactions of Gly199 and Ala200 in a $\beta$-turn of Mol1 with Val196 in Mol2, and by numerous crystallographic contacts to Mol2 itself. The shift of the Ca backbone preceding the small $\beta$-sheet by a maximum of 4.8 Å at Thr194 indicates a certain flexibility of this region. Statistics measuring the degree of similarity of molecules (30) show clearly that Mol1 is more similar to 1ghr than is Mol2 (Table III). Therefore, all considerations of general aspects of the structures will refer to Mol1.

By using the program PUU (31) two structural domains were detected with a boundary crossing the $\beta_8$ barrel perpendicular to the binding cleft. Residues Ile1 to Ser126 and Tyr272 to Phe296 constitute domain I; residues Val127 to Thr272 belong to the glycosylated domain II (right side in Fig. 3). Only two strands link both domains at Ser126/Val127 and Thr272/Tyr273. This assignment of structural domains differs from that proposed earlier (3) and is supported by a least-squares superposition of domains I of Mol1 and 1ghr yielding a rotation by about 1° between their domains II. The rotation axis traverses the center of mass of domain II and is inclined by about 50° against the cleft axis marked by a modeled hexoglucan substrate (see below) in Fig. 3. Interestingly, the two $\beta$-strands crossing the inter-domain boundary are not regularly structured. Strand $\beta_8$ is truncated to just two residues in Mol1 and Mol2 and is next to the cis-peptide preceding Ala276. The long strand $\beta_5$ is inter-
ruptured by a $3_{10}$ turn at Gln129 to Ile131 where it crosses from domain I into domain II also reflecting some structural irregularity. Functional implications of this domain structure will be discussed below.

Water molecules in 130 pairs related by NCS were identified allowing for a maximal separation of 1.5 Å. These water-binding sites present in both Mol1 and Mol2 are expected to have biological relevance. 37 out of these 130 sites have identical positions within 1.5 Å with any of the 48 water sites present in 1ghr indicating that their positions are independent of crystal contacts and preparation conditions. The water distribution around EII is quite asymmetric, since the molecular surface near the C and N termini shows significantly higher hydration than other parts of the protein molecule. This finding is true also for the waters detected in the molecule 1ghr. Interestingly, only 12 waters out of the 130 are found within the substrate-binding cleft of EII. The lack of complete cleft hydration may be due to the presence of exposed hydrophobic residues (see below).

The Glycosylation Site—Barley 1,3–1,4-$\beta$-glucanase, isoenzyme EII, has only one site for potential N-glycosylation, the sequence Asn190-Ala191-Ser192. The attached carbohydrate moieties were analyzed by Harthill and Thomsen (4). They identified five different branched N-glycans comprised of different sugars contributing with different relative amounts between 15 and 30%. All have a common core sequence starting with two $\beta$-1,4-linked NAG molecules (Fig. 4). The sugars in the two NCS-related protein molecules are differently well defined. The average temperature factors are 30.0 and 60.7 Å$^2$ for NAG$^1_1$ and NAG$^2_1$ of Mol1, and 49.1 and 63.6 Å$^2$ for the corresponding residues in Mol2. Whereas NAG$^1_1$ and NAG$^2_1$ are well defined, a third sugar residue, $\alpha$-L-fucose, attached to O-3 of NAG$^1_2$ is seen at very low electron density (0.3 e Å$^{-3}$) but was not modeled. Both glycosylation sites are sterically accessible to different degrees.

Both disaccharides are in the energetically preferred $4C_1$ chair conformation, and the torsions of the $\beta$-1,4 glycosyl bonds $\Phi$(H-1–C-1–O-4–C-4) and $\Psi$(C-1–O-4–C-4–H-4) are 49.8° and 15.2° in NAG$^1_1$/NAG$^2_1$, and 44.9° and 3.9° in NAG$^1_2$/NAG$^2_2$. These dihedrals correspond to $\Phi$ and $\Psi$ angles also found in saccharides bound to lysozyme (PDB entry 1lzr) or influenza virus neuraminidase (PDB entry 1nnc).

The Active Site: Structure and Hydration—The substrate binding cleft of barley 1,3–1,4-$\beta$-glucanase was geometrically defined using SURFNET (32), and residue accessibilities were characterized by NACCESS (33). The largest cleft found by SURFNET (1 Å ± sphere radius < 4 Å; cutoff distance between atoms and mask region 4 Å) has a volume of 2031 Å$^3$ (Fig. 5, top) and comprises the catalytic site and the potential substrate binding site as described recently (3, 23). The global dimensions are 7.5 Å in width, 8 to 9 Å in depth around the catalytic center, and 36 Å in length. All accessible atoms within a distance ≤4 Å from the cleft surface were selected, and their polarity was estimated by NACCESS. The resulting patterns are given in Table IV. The "left" side of the cleft (in the orientation of Fig. 5) is mainly decorated with apolar residues, whereas the "right" side, especially in the vicinity of the catalytic site, is covered with polar residues. The residues Tyr$^{170}$, Glu$^{232}$, Tyr$^{173}$, Glu$^{288}$, and Trp$^{291}$ in or near the catalytic center are accessible, also Leu$^{173}$, Phe$^{275}$, Asn$^{92}$, Glu$^{93}$, and Asn$^{168}$.
which possibly interact with the substrate (3) as partially proven by chemical probing (22).

Water molecules are mainly located at the polar right side of the cleft floor (Fig. 5) where several residues form hydrogen bonds to five water molecules. Only the three water molecules at the lowest end of the cleft are also present in the 1ghr model (3). Three water molecules form H bonds to the Ala\textsuperscript{172} backbone and the Tyr\textsuperscript{172} and Glu\textsuperscript{280} side chains at the left side. Thus, the mostly polar right half of the cleft is covered by a water layer.

A comparison with the water structure in the active site of the Bacillus 1,3–1,4-\(\beta\)-glucanase will follow below.

Molecular Dynamics Simulation of Substrate Binding—A model of a hexaglucan substrate bound to the active site of EII was constructed to assist our understanding of the enzymology of the barley 1,3–1,4-\(\beta\)-glucanase. The \(\beta\)-glucan before and after the simulated docking procedure is shown in Fig. 6 (bottom). In the start configuration, C-1 of glucose residue Glc\textsubscript{4} points to the nucleophile Glu\textsuperscript{232} of EII; Glc\textsubscript{4} is situated halfway between Glu\textsuperscript{232} and the general acid Glu\textsuperscript{288}; Glc\textsubscript{3} O-6 forms hydrogen bonds to Glu\textsuperscript{280} and Lys\textsuperscript{283} (as the inhibitor in 1ghs); Glc\textsubscript{3} O-2 makes an H bond to Asn\textsuperscript{92} (as in 1ghs), and the face of Glc\textsubscript{3} is in hydrophobic contact with Tyr\textsuperscript{33} (as in 1ghs). The water molecules localized in the cleft were removed completely, because no fixed waters could be detected between inhibitor and 1,3–1,4-\(\beta\)-glucanase in the crystal structure of an enzyme-inhibitor complex (34).

The molecular dynamics simulation of substrate binding was equilibrated after 70 ps. For further discussion we use the middle structure of the inhibitor as determined by LSQMAN (35). Sugar residues Glc\textsubscript{2}, Glc\textsubscript{3}, Glc\textsubscript{4}, and Glc\textsubscript{5} are situated within the binding cleft, their root mean square distances from the middle structure are 0.38, 0.31, 0.72, and 0.66 Å, respectively. The first residue, Glc\textsubscript{1}, is outside of the binding cleft as defined by SURFNET (Fig. 5). The root mean square distances of Glc\textsubscript{1} and Glc\textsubscript{6} are about 0.66 and 0.68 Å, respectively. Because the position of the substrate is strongly influenced by

![Fig. 5. Stereographic drawings of the substrate binding cleft of barley 1,3-1,4-\(\beta\)-glucanase and of the hybrid Bacillus 1,3-1,4-\(\beta\)-glucanase H(A16-M). Top, barley 1,3-1,4-\(\beta\)-glucanase, active site atoms of Glu\textsuperscript{232}, Tyr\textsuperscript{33}, and Glu\textsuperscript{280} are labeled. Bottom, H(A16-M). The active site residues Glu\textsuperscript{105}, Tyr\textsuperscript{123}, and Glu\textsuperscript{109} are labeled. Water molecules binding to the corresponding cleft are shown as blue spheres. The maps defining the clefts were estimated by SURFNET (32). Drawn with BOBSCRIPT (42) and RASTER3D (43).](https://www.jbc.org/)

### TABLE IV

| Active site residues in barley and Bacillus 1,3-1,4-\(\beta\)-glucanases |
|---------------------------------|---------------------------------|---------------------------------|
| **EII**                         | **H(A16-M)**                    |
| **Left side**                   | **Right side**                  | **Left side**                   | **Right side**                  |
| Ala\textsuperscript{174} a      | Phe\textsuperscript{135} a       | Asp\textsuperscript{191} a      | Glu\textsuperscript{131} p w    |
| Pro\textsuperscript{171} a      | Leu\textsuperscript{132} p       | Trp\textsuperscript{192} a      | Asp\textsuperscript{191} p w    |
| Tyr\textsuperscript{172} p w    | Ala\textsuperscript{130} a w     | Val\textsuperscript{29} a       | Glu\textsuperscript{109} pw w   |
| Leu\textsuperscript{173} a      | Glu\textsuperscript{139} p       | Trp\textsuperscript{184} a p w  | Asp\textsuperscript{107} p      |
| Tyr\textsuperscript{170} a ile\textsuperscript{169} a | Asn\textsuperscript{182} a p w  | Gly\textsuperscript{28} a p w   | Glu\textsuperscript{109} p      |
| Glu\textsuperscript{288} a      | Ser\textsuperscript{128} a p w   | Phe\textsuperscript{20} a       | Tyr\textsuperscript{125} a p     |
| Trp\textsuperscript{291} a      | Asn\textsuperscript{168} a       | Gly\textsuperscript{28} a p     | Tyr\textsuperscript{125} a p     |
| Phe\textsuperscript{273} a      | Glu\textsuperscript{5} p w      | Gly\textsuperscript{28} a       | Glu\textsuperscript{109} p      |
| Lys\textsuperscript{283} a      | Glu\textsuperscript{282} p w    | Asn\textsuperscript{92} p w w w | Trp\textsuperscript{192} a p     |
| Gly\textsuperscript{280} p w    | Asp\textsuperscript{92} p w     | Ser\textsuperscript{72} a p w   | Met\textsuperscript{160} a p     |
| Gly\textsuperscript{6} a        | Asp\textsuperscript{94} a w     | Gly\textsuperscript{53} p w     | Tyr\textsuperscript{124} a p     |
| Ser\textsuperscript{8} p        | Arg\textsuperscript{11} p       | Trp\textsuperscript{34} a       | Pro\textsuperscript{97} a       |
| Met\textsuperscript{7} a        | Pro\textsuperscript{55} a       | Trp\textsuperscript{34} a       | Lys\textsuperscript{178} a p     |
| Tyr\textsuperscript{33} a       | Glu\textsuperscript{12} a p     | Asp\textsuperscript{92} p w     | Arg\textsuperscript{60} p w     |
| Leu\textsuperscript{17} a       | Ala\textsuperscript{34} a       | Glu\textsuperscript{63} p w     | Ala\textsuperscript{38} a p w   |
| Tyr\textsuperscript{33} a       | Glu\textsuperscript{12} a p     | Glu\textsuperscript{63} p w     | His\textsuperscript{69} a p     |

Note: The catalytic site glutamic acids and the tyrosine defining subsite p2 are emphasized by boldface.
FIG. 6. Structural comparison of barley (left) and Bacillus 1,3-1,4-\(\beta\)-glucanases (right). Top, the EII folding pattern belongs to the \(\beta\alpha\beta\) barrel type; H(A16-M) adopts the jellyroll fold. The key residues for the catalytic event are marked in both enzymes. Middle, molecular surface drawings of the 1,3-1,4-\(\beta\)-glucanases. The hexameric \(\beta\)-glucan models are oriented such that the reducing ends point upwards and shown together with the catalytic site residues Glu\(^{232}\) and Glu\(^{288}\) (EII, left) and Glu\(^{105}\) and Glu\(^{109}\) (H(A16-M), right). Tyrosine residues Tyr\(^{33}\) (EII) and Tyr\(^{94}\) Barley 1,3-1,4-\(\beta\)-Glucanase Crystal Structure.
Tyr$^{33}$ and the contacts are built in manually, most of the interactions found in the inhibitor complex of the 1,3-β-glucanase (1ghs; Ref. 3) are present in this model, too. The global course of the hexamer is visible in Fig. 3. The smallest root mean square Δ value for Glc3 coincides with the longest retention of any glucose residue in one position during the simulation. This is in agreement with the results of the subsite mapping of barley 1,3-β-glucanase isoenzyme GII (36), where the binding affinity at the second subsite toward the non-reducing end relative to the cutting point is at the maximum. In the Bacillus as well as in the barley 1,3,4-β-glucanases the first and sixth residues of the β-glucan model substrates are not tightly bound to the ends of the clefts.

All glucose units of the modeled substrate are in the preferred 4C$_1$ chair conformation and the torsion angles are in the low energy regions between 28 and 54° for Ψ and −52 to +14° for Ψ in β,1-3 glycosyl bonds (37), and around Ψ = 40°, Ψ = −20° for β,1-4-bonds (38). Only the torsion angle Ψ = 72.5° at the scissile β,1-4-bond is outside the minimum, indicating strain which may be relieved by bond cleavage. A deviation from the low energy conformation of similar magnitude but with opposite sign was observed in the modeled β-glucan substrate of the Bacillus 1,3,4,1-β-glucanase (11).

In rationalizing substrate binding and conversion, the domain structure and possible structural rearrangements must be taken into account. The general acid, Glu$^{288}$, and the substrate fix point Tyr$^{193}$ are located at the same domain I, whereas the nucleophile Glu$^{232}$ at the bottom of the cleft belongs to the glycosylated domain II. The substrate residues Glc1 to Glc4 are attached to domain I, and the scissile bond O-4 of the modeled hexamer is positioned at the inter-domain boundary, and residues Glc5 and Glc6 are attached to domain II (Fig. 3). A slight rotation of the domains relative to each other may support a rearrangement of the catalytic residues and/or the substrate position, thus providing a fine tuning of the catalytic event. A domain rearrangement of this kind is observed between Mol1 and Mol2 of EII (see above).

Comparison of Active Site Structures and Modeled Substrate Binding by Barley and Bacillus 1,3,4,1-β-Glucanases—Active site directed inactivation of 1,3,4,1-β-glucanases from B. subtilis and barley (13) by covalent modification with epoxyalkyl cellobiosides reveals subtle differences in their active site geometries. Both enzymes with unrelated primary, secondary, and tertiary structures (Fig. 6) have identical substrate specificity, and in both cases the clefs are somewhat branched out at the molecular surface (Fig. 5). All residues within the active site (Asp$^{107}$, Glu$^{105}$, Glu$^{109}$, Trp$^{192}$, Glu$^{119}$, and Trp$^{192}$) or interacting with the cellobiose inhibitor (Tyr$^{24}$, Asn$^{26}$, Glu$^{65}$, and His$^{79}$) as described (34) and additional potential substrate binding partners (Arg$^{65}$, Tyr$^{28}$, Ser$^{90}$, Glu$^{131}$, and Asn$^{121}$) have accessible surface fractions. These residues are summarized in Table IV for comparison with EII. No obvious similarity between both patterns is found. Whereas the branched cleft in EII has a nearly isometric cross-section in the substrate binding region, the cleft of H(1A16-M) is narrow and deep. The hydration pattern of the H(1A16-M) groove is quite different from the water structure in the EII cleft (Fig. 5).

The six glucose residues cover the whole length of the cleft in H(1A16-M), but eight can be accommodated in EII (36). In any case, only the innermost four appear tightly bound within the reaction center. In Fig. 6 (middle), the surfaces, modeled substrates, catalytic residues, and the hydrophobic binding centers at subsite p2 (Tyr$^{292}$ of EII and Tyr$^{292}$ of H(1A16-M)) are shown. The basic differences between both families of 1,3,4,1-β-glucanases are the relative positioning of the general acid, the nucleophile, and these tyrosine residues. Given the same orientation of the substrate with the reducing end of Glc6 facing upward in Fig. 6 (middle), the general acid, Glu$^{288}$, of EII is at the left side of the hexameric β-glucan, but the corresponding Glu$^{109}$ is at the cleft bottom of H(1A16-M). To follow the same stereospecific pathway a global rotation is necessary between both substrates by about 90°.

In conclusion, the active sites of the plant and Bacillus 1,3,4,1-β-glucanases are surprisingly different in view of their close functional similarity. The enzymes show nicely how the same catalytic activity can evolve on completely different protein folds and in dissimilar local geometries.

(H(1A16-M)) stacking against Glc3 of the substrate at subsite p2 are also shown. Surface colors indicate electrostatic potential (blue, positive, and red, negative) as calculated with DELPHI (41). Note the different rotational setting of the substrate chain in the active site cleft and the different orientation of the protein side chains. Bottom, superposition of β-glucan model substrates for barley and hybrid Bacillus 1,3,4,1-β-glucanases. The molecules are superimposed with the glucose moiety Glc3 interacting with a tyrosine in subsite p2. The substrate strand fitting into the active site of H(1A16-M) (Ref. 11, green) defines the start conformation used in molecular dynamics modeling of the substrate bound to EII (red).
Acknowledgments—Kim Henrick is thanked for helpful discussions regarding the carbohydrate moieties and Yves Muller for critically reading the manuscript.

REFERENCES
1. Fincher, G. B., Lock, P. A., Morgan, M. M., Lingelbach, K., Wettenhall, R. E. H., Mercer, J. F. B., Brandt, A., and Thomsen K. K. (1986) Proc. Natl. Acad. Sci. U. S. A. 83, 2081–2085
2. Bamforth, C. W. (1982) Brew. Dig. 57, 22–35
3. Varghese, J. N., Garrett, T. P. J., Colman, P. M., Chen, L., Høj, P. B., and Fincher, G. B. (1994) Proc. Natl. Acad. Sci. U. S. A. 91, 2785–2789
4. Harthill, J. E., and Thomsen, K. K. (1995) Plant Physiol. Biochem. 33, 9–18
5. Doan, D. N. P., and Fincher, G. B. (1992) FEBS Lett. 309, 265–271
6. Davies, G., and Henrissat, B. (1995) Structure 3, 853–859
7. Heinemann, U., Ay, J., Gaiser, O., Müller, J. J., and Ponnuswamy, M. N. (1996) Biol. Chem. Hoppe-Seyler 377, 447–454
8. Heinemann, U., and Hahn, M. (1996) Trends Biochem. Sci. 20, 349–350
9. Hahn, M., Olsen, O., Politz, O., Borriss, R., and Heinemann, U. (1995) J. Biol. Chem. 270, 3081–3088
10. Høj, P. B., Rodriguez, E. B., Stick, R. V., and Stone, B. A. (1989) J. Biol. Chem. 264, 4939–4947
11. Høj, P. B., and Fincher, G. B. (1995) FEBS Lett. 374, 221–224
12. Hahn, M., Keitel, T., and Heinemann, U. (1995) Eur. J. Biochem. 232, 849–858
13. Høj, P. B., Rodríguez, E. B., Stick, R. V., and Stone, B. A. (1991) J. Biol. Chem. 266, 11628–11631
14. Chen, L., Sadek, M., Stone, B. A., Brownlee, R. T. C., Fincher, G. B., and Høj, P. B. (1992) Biochim. Biophys. Acta 1253, 112–116
15. Malet, C. J.-B., Bernabe, M., Brosa, C., and Planas, A. (1993) Biochem. J. 296, 753–758
16. Keitel, T., Thomsen, K. K., and Heinemann, U. (1993) J. Mol. Biol. 232, 1003–1004
17. Brünger, A. T. (1990) Acta Crystallogr. Sect. A 46, 46–57
18. Brünger, A. T. (1992) Nature 355, 472–474
19. Brünger, A. T., Kraka, A., and Erickson, J. W. (1990) Acta Crystallogr. Sect. A 46, 585–592
20. Collaborative Computational Project, No. 4 (1994) Acta Crystallogr. Sect. D 50, 760–763
21. Jones, T. A., Zou, J.-Y., Cowan, S. M., and Kjeldgaard, M. (1991) Acta Crystallogr. Sect. A 47, 110–119
22. Chen, L., Fincher, G. B., and Hej, P. B. (1993) J. Biol. Chem. 268, 13318–13326
23. Chen, L., Garrett, T. P. J., Fincher, G. B., and Hej, P. B. (1995) J. Biol. Chem. 270, 8093–8101
24. Brünger, A. T. (1992) X-PLOR Manual, Version 3.1, Yale University Press, New Haven
25. Laskowski, R. A., MacArthur, M. W., Moss, D. S., and Thornton, J. M. (1993) J. Appl. Crystallogr. 26, 283–293
26. Friend, G. (1990) J. Mol. Graph. 8, 52–56
27. Bernstein, F. C., Koetzle, T. F., Williams, G. J. B., Meyer, E. E., Jr., Brice, M. D., Rodgers, J. R., Kennard, O., Shimanouchi, T., and Tasumi, M. (1977) J. Mol. Biol. 112, 535–542
28. Kabsch, W. (1976) Acta Crystallogr. Sect. A 32, 922–923
29. Luzzati, V. (1952) Acta Crystallogr. 5, 802–810
30. Kleywegt, G. J. (1996) Acta Crystallogr. Sect. D 52, 842–857
31. Holm, L., and Sander, C. (1994) Proteins Struct. Funct. Genet. 19, 256–268
32. Laskowski, R. A. (1995) J. Mol. Graph. 13, 323–330
33. Hubbard, S. J., and Thornton, J. M. (1993) NACCESS, version 2.1, Computer Program, Department of Chemistry and Molecular Biology, University College London
34. Keitel, T., Simon, O., Borriss, R., and Heinemann, U. (1993) Proc. Natl. Acad. Sci. U. S. A. 90, 5287–5291
35. Kleywegt, G. J., and Jones T. A. (1997) Methods Enzymol., in press
36. Noguchi, K., Okuyama, K., Kitamura, S., and Takeo, K. (1992) Carbohydrate Res. 237, 33–43
37. Sathyarayana, B. K., and Rao, V. S. R. (1971) Biopolymers 10, 1605–1615
38. Nicholls, A., Sharp, K. A., and Honig, B. (1991) Proteins Struct. Funct. Genet. 11, 281–296
39. Kraka, A., and Erickson, J. W. (1990) Acta Crystallogr. Sect. A 46, 585–592
40. Esnouf, R. M. (1997) J. Mol. Graph. Mod. 15, 132–134
41. Merrill, E. A., and Murphy, M. E. P. (1994) Acta Crystallogr. D 50, 869–873
Crystal Structure of Barley 1,3–1,4-β-Glucanase at 2.0-Å Resolution and Comparison with Bacillus 1,3–1,4-β-Glucanase
Jürgen J. Müller, Karl K. Thomsen and Udo Heinemann

J. Biol. Chem. 1998, 273:3438-3446.
doi: 10.1074/jbc.273.6.3438

Access the most updated version of this article at http://www.jbc.org/content/273/6/3438

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC’s e-mail alerts

This article cites 40 references, 9 of which can be accessed free at http://www.jbc.org/content/273/6/3438.full.html#ref-list-1