Three-dimensional Structure of the Barley \( \beta \)-D-Glucan Glucohydrolase in Complex with a Transition State Mimic*

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Glucophenylimidazol (PheGlcIm), a tetrahydroimidazopyridine-type inhibitor and \( 4 \)H\( \) conformer mimic of a glucoside, binds very tightly to a barley \( \beta \)-D-glucan glucohydrolase, with a \( K_\text{c} \) constant of \( 2 \times 10^{-9}\) M and a change in Gibbs free energy of 51 kJ mol\(^{-1}\). PheGlcIm binds to the barley \( \beta \)-D-glucan glucohydrolase \( \sim 2 \times 10^7 \) times tighter than laminarin, which is the best non-synthetic ground-state substrate found so far for this enzyme, \( 10^9 \) times tighter than D-nitrophenyl \( \beta \)-D-glucopyranoside, and \( 2 \times 10^7 \) times tighter than glucose. The three-dimensional structure of the \( \beta \)-D-glucan glucohydrolase with bound PheGlcIm indicates that the complex resembles a hypothetical transition state during the hydrolytic cycle, that the enzyme derives substrate binding energy from the "aglycone" portion of the ligand, and that it also reveals an anti-protonation trajectory for hydrolysis. Continuous electron densities at the 1.6 \( \sigma \) level form between the three active site residues Asp\(^{95}\), His\(^{207}\), and Asp\(^{285}\), and the C6OH, C7OH, GlcNAc, and C9OH groups of PheGlcIm. These electron densities correspond to the most favorable interactions in the three-dimensional structure of the \( \beta \)-D-glucan glucohydrolase-PheGlcIm complex and indicate atomic distances equal to or less than 2.55 Å. The crystallographic data were corroborated with \( ab \) \( \text{initio} \) molecular orbital calculations. The data indicate that the \( 4 \)E\( \) conformation of the glucose part of PheGlcIm is critical for tight binding and provide the first evidence for probable substrate distortion during catalysis by this enzyme. The enzymic transformation of a substrate into product by glucoside hydrolases proceeds through a series of intermediates and oxocarbenium (cation) ion-like transition states and is mediated via distortion/relaxation cycles of a ground-state low energy \( \text{C}_1 \) chair conformation of the substrate (1–3). Evidence for the existence of oxocarbenium ion-like transition states arises from secondary kinetic isotope effects and inhibition studies with transition state analogues (3–7).

Barley \( \beta \)-D-glucan glucohydrolase is a family GH3 glycoside hydrolase that catalyzes hydrolytic removal of non-reducing glucosyl residues from a broad range of \( \beta \)-D-glucans and \( \beta \)-D-glucooligosaccharides (8). In our previous crystallographic work we used conduritol B epoxide, 2,4-dinitrophenyl 2-deoxy-2-fluoro-\( \beta \)-D-glucopyranoside (2F-DNPGlc) and 4\( ^1 \), 4\( ^{II} \), 4\( ^{IV} \)-S-trithiocellulosexide to identify catalytic amino acid residues and to define three key intermediates in the catalytic sequence (9). The glucose product of the reaction does not immediately diffuse away from the enzyme surface, but instead remains bound at subsite –1. It is likely that this bound glucose is displaced from the active site by the incoming substrate during the first stage of the next hydrolytic event (9, 10). The second stage of the hydrolytic pathway involves C1-O glucosidic bond cleavage, which proceeds through a double displacement. The crystallographic analysis of a S-cellulobioxy-enzyme complex, supported by quantum mechanical modeling, suggested that the S-cellulobioxy-enzyme complex mimics an oxonium intermediate, rather than the enzyme-substrate complex (9). It has also been suggested that formation of a stable oxonium intermediate inhibits distortion of a glycosyl ring (11).

The data obtained in our previous work with the \( \beta \)-D-glucan glucohydrolase indicated that no significant distortion occurred either in the enzyme-product complex or in the covalent glycosyl-enzyme intermediate (9, 10). Similarly, no significant distortions could be detected in sugar conformations at subsites –1 or –1 of the \( S \)-glycosyl-enzyme complexes (9, 10). However, crystallographic analyses of several other endo-acting glycoside hydrolases in complex with sugars indicate distortions of the glycosyl residue that is bound at the –1 subsite. It is also assumed that distortion during oxocarbenium ion-like transition state formation is a necessary part of catalysis (3).

To further investigate intermediates and to test whether ring distortions play a role during catalysis of the barley \( \beta \)-D-glucan glucohydrolase, the three-dimensional structure of the enzyme in complex with glucophenylimidazol (PheGlcIm) has now been solved. PheGlcIm is a representative of the glucimidazoles (GlcIm), which are excellent chemical tools for studying three-dimensional structures that mimic transition states. For
example, PheGlcIm is a nagnat-stype mimic of the reactive intermediate that can be isolated from culture filtrates of *Streptomyces amakusaensis* (12), or prepared synthetically; nagatatin is an inhibitor of *N*-acetyl-β-β-glucosaminidase (13). PheGlcIm has a trigonal anomeric center attached to an exocyclic N atom, which corresponds to the “glycosidic heteroatom,” and an endocyclic N atom of the tetrahydrodiazopripyridine moiety. PheGlcIm possesses a conformation similar to that of gluconiritetrazole, which in solid state or in D2O adopts a 4H4 conformation that is similar to E (14, 15). Tetrahydrodiazopyridine-π-primes do not contain a lone pair electron at the “anomeric” N atom, but possess an anti-oriented, doubly occupied, non-bonding orbital at this heteroatom and are therefore suited as inhibitors of anti-protoglycosidase hydrolases (15–17).

Barley β-β-glucan glucohydrolase has two substrate-binding sites, designated −1 and +1, in its active site (9, 10), and the structure of PheGlcIm suggested that the inhibitor might be a useful mechanistic probe for the investigation of the enzyme-bound reactive intermediate and thus the transition state. It was expected that the tetrahydrodiazoprodiazopyridine moiety and the phenyl ring would be located at the −1 and +1 subsites, respectively. The primary objectives of the present work were to analyze binding interactions of PheGlcIm in the active site of the barley β-β-glucan glucohydrolase by x-ray crystallography, to define the structure of a possible transition state complex, and to reconcile these findings with quantum mechanical modeling.

**EXPERIMENTAL PROCEDURES**

**Materials**—The glucose diagnostic kit, 4-nitrophenyl β-β-glucopranoside (4NPGlc), gluconolactone, castanospermine, and bovine serum albumin (BSA) were purchased from Sigma Chemical Co. (St. Louis, MO). Microcon microconcentrators were from Amicon (Beverly, MA), and Sep-Pak® Plus cartridges were from Waters (Milford, MA). GlcIm, GlcIm with the phenyl substituent (PheGlcIm), and GlcIm with the phenethyl substituent (PheThGlcIm) (Table I) were synthesized as described previously (18, 19).

**Enzyme Isolation and Purity**—Barley β-β-glucan glucohydrolase isoenzyme ExoI was purified from a homogenate of 8-day-old seedlings described previously (18, 19). Inactivation of β-β-Glucan Glucohydrolase by Castanospermine and Glucosidicidozole—Inactivation of β-β-glucan glucohydrolase by castanospermine and glucosidicidozole. PheGlcIm, PheGlcIm, and monitored at 37 °C by incubating 1–2 nmol of the purified enzyme in 100 mM sodium acetate buffer, pH 5.25, containing 0.2% (w/v) 4NPGlc, 160 μg/ml BSA, and 0–64 μg castanospermine or 0–100 nm glucosidicidozole. Each inhibitor was tested at six concentrations, in a duplicate. The residual enzyme activity was monitored spectrophotometrically at 410 nm (8). The first and second order rate inhibition constants were determined by a proportional weighted fit, using a non-linear regression analysis (20) and the GraFit program (Erithacus Software Ltd. (21)).

**Dependence of kcat, Km, and K1,2 on pH**—The effects of pH on catalytic efficiency and inhibitor binding were determined by incubating 0.5–1 nmol of the purified enzyme at 37 °C in 50 mM citric acid/100 mM sodium dihydrophosphate (Mellilvaine) buffers (pH 4.0–7.7) containing 160 μg/ml BSA and appropriate substrate (4NPGlc) concentrations. The kcat, Km, and K1,2 versus pH profile was calculated from time-course curves at low 4NPGlc concentration (0.17 mm and 1/9 Km). Division of the pseudo-first-order rate constants by the enzyme concentration produced the final K1,2 values (22, 23). The pH of each reaction mixture was checked during the time course to make certain that no pH changes occurred during the assays. The Ks values at each pH were estimated using concentrations of inhibitor that were 0.25–3 times the Ks and concentrations of 4NPGlc substrate that were 0.5–1 times the Ks constant. Dixon plots were used to determine the final Ks values at each pH. Substrate hydrolysis during both types of measurements never exceeded 0.5% of the initial substrate concentration. Enzyme values in assays were within the range of 6–9%. The kinetic constants were calculated using non-linear regression analyses (20) and the GraFit program (21).

**X-ray Crystallography of the β-β-Glucan Glucohydrolase-PheGlcIm Complex**—Crystals of β-β-glucan glucohydrolase isoenzyme ExoI (24) were grown from a solution of 140–150 μM were transferred into a solution of 100 mM Hepes-NaOH buffer, pH 7.0, containing 1.2% (w/v) polyethylene glycol 400, 0.8 mM ammonium sulfate (solution A) and 10 mM PheGlcIm. After 2 h at 4 ± 2 °C, the crystal was transferred into solution A with 11% (w/v) glycerol as a cryo-protectant. The crystal was subsequently mounted on a goniometer and flash-frozen to 100 K in a stream of nitrogen gas (Oxford Instruments, Oxford, England). Data were collected to 2.62-Å resolution using a rotating copper anode x-ray generator (MacScience Co. Ltd., Yokohama, Japan) operating at 40 kV and 50 mA, and a Rigaku Raxis IV detector (Rigaku/MSC, The Woodlands, TX) fitted with capillary optics (AXCO, Melbourne, Australia). A total of 80 data frames were collected using 1° oscillations and 1-h exposure times with a charge II detector within a 2.0° 2θ range, using a 0.1°/2θ step size. The diffraction data were integrated, scaled, and reduced using the HKL program (25). Autoindexing determined that the primitive tetragonal crystals belonged to the space group P4_2_2_2. Model refinement was performed with CNS (26). Atomic B-factor values for the protein, water molecules, and carbohydrates were reset to an overall thermal parameter of 30 Å². The residues within a 10-Å radius of the active site, excluding the bound glucose, were set to zero occupancy. The initial model used to locate the position of the protein in the unit cell was constructed with a restrained rigid body refinement technique from 15 to 3.0 Å. After a final round of the rigid body refinement, the crystallographic Rwork was 22.29%, Rfree was 27.40%, and the overall B value was 23.4 Å². Geometrical refinement of the overall B factor refinement was subsequently calculated using the β-β-glucan glucohydrolase model wit a bulk solvent correction applied and a maximum likelihood method implemented. The electron density map was calculated from the observed structure factors and phases using the β-β-glucan glucohydrolase isoenzyme ExoI structure as the starting model (PDB accession code IIEQ (9)) and MAPMAN package (27). PheGlcIm was located in the active site pocket by examining a difference Fourier electron density map at a level greater than 4 σ. This showed peaks for the two rings of the PheGlcIm that were manually built into the electron density, using the graphics program “O” (28) and refined. The PROCHECK program (29) was used to assess the geometrical quality of the model, and the structure was found not to deviate from the ideal geometry. The final refinement statistics are summarized in Table II.

**Quantum Mechanical Calculations**—Ab initio molecular orbital calculations were performed with the GAMESS-US program (30). A model of the active site was constructed from the side-chain groups of residues in contact with the inhibitor. Small molecule representations of the amino acid side chains were used; acetate for Asp95, Asp256, and Glu91, guanidinium for Arg256, methylammonium for Lys206, imidazole for His89, and indole for Trp54. All atoms of the inhibitor were included. The systems included 66 non-hydrogen atoms, and 49–51 hydrogen atoms, depending on the protonation state of the inhibitor and acetate group. Crystallographic data processing and molecular modeling of the inhibitor were performed using the CCP4 package (31). The initial model contained a water molecule at 21G level was applied, in which non-hydrogen atoms were restrained to their position found in the x-ray crystallographic analysis. Atoms were allowed to move 0.5 Å before restraints were applied; a harmonic restraint with force constant of 0.5 J Å⁻² (mdyn Å⁻¹) was used.

Several protonation states of the models were considered. The acetate representing Glu91 was either ionized (corresponding to a charge of −1), or neutral. The protonation states of the inhibitor PheGlcIm were represented either by the ionized state, which corresponded to a charge of +1 and a proton on the ring N1 atom, or by a neutral form. Four configurations were considered as follows: 1, Glu91 ionized; 2, Glu91 ionized and PheImGlc neutral; and 3 and 4, Glu91 and PheGlcIm neutral, with the formal HOCO angle of 0° (see Table III).

**Improved relative energies of configurations 1, 3, and 4 were calculated at the B3LYP8/6–31G(d) level (Gaussian Inc., Pittsburgh, PA (31)), using the GAUSSIAN 98 program (Revision A.7, Gaussian Inc. (32)). The electrostatic component of the protein environment was included by computing partial atomic charges for all residues, except the inhibitor, excluding those that were specified above. Partial atomic charges for amino acids were taken from the Solvent Interaction Potential (SIP) dataset (33). Ionizable residues Asp, Glu, Arg, and Lys beyond 8 Å had integer charges positioned at the Cz, Ca, Ce, and Ne atoms, respectively. The effects of solvent on the relative energies of 1, 3, and 4 were determined through calculations of solvation energies. The electrostatic component of the solvation energies was calculated using the DelPhi program (Delphi 97.0 Molecular Simulations Inc., San Diego, CA (34)). Hydrogen atoms were added to fill in unsatisfied valences using the
InsightII package (InsightII 97.0, Molecular Simulations Inc., 1997). The positions of all hydrogen atoms were subjected to energy minimization, while keeping protein, inhibitor, and water atoms fixed at the positions determined by x-ray crystallography, by using the Discover program and conventional valence force field parameters (Discover 97.0, Molecular Simulations Inc., 1997). Electrostatic potential-derived charges (35) were calculated for the inhibitor at the HF/6-31+/G(d) level and used to determine SIP atomic radii. Charges and radii for protein atoms were taken from the SIP data set of amino acids (36). All water molecules were removed for the calculation of solvation energies. The ionic strength was set to zero, and dielectric constant values of 1 and 78.54 were assumed for the solute and solvent, respectively. A grid of 197^3 points was used, yielding a grid resolution of 0.5 Å. This maintained a solvent boundary of at least 10.0 Å around the protein.

Coordinates—The coordinates of β-D-glucan glucohydrolase with bound PheGlcIm have been deposited with the Protein Data Bank (37), under the code 1LQ2.

### RESULTS

#### Inhibition of β-D-Glucan Glucohydrolase

Table I summarizes first order rate constants of inhibition $k_i$, dissociation constants for the enzyme-inhibitor complex $K_i$, and second-order rate constants of inhibition $k_i K_i^{-1}$, for a range of “glucose-derived” inhibitors. Although 2F-DNPGlc, conduritol B epoxide, castanospermine, and gluconolactone all inhibit within the micromolar range, the $K_i$ constants for glucoimidazoles are in the low nanomolar ranges (3, 17, 39). Thus, PheGlcIm binds to β-D-glucan glucohydrolase $\sim 2 \times 10^5$ times tighter than laminarin, which is the best non-synthetic inhibitor. The coordinates of β-D-glucan glucohydrolase with bound PheGlcIm have been deposited with the Protein Data Bank (37), under the code 1LQ2.

| Inhibitor * | $K_i$ (M) | $k_i$ (min$^{-1}$) | $k_i K_i^{-1}$ (min$^{-1}$ mM$^{-1}$) | $\Delta G$ * (kJ mol$^{-1}$) | Chemical structure |
|-------------|-----------|------------------|-------------------------------------|--------------------------|------------------|
| Glucose b   | $8.8 \times 10^{-3}$ | ND d             | -                                   | -12.2                    |                  |
| 2F-DNPGlc c | $25.5 \times 10^{-6}$ | 0.1              | 3.9                                 | -27.3                    |                  |
| Conduritol B | $42.6 \times 10^{-6}$ | 0.3              | 7.0                                 | -25.9                    |                  |
| Epoxide c   |           |                  |                                     |                          |
| Castanospermine |       |                  |                                     |                          |
| Glucono-lactone b | $2.1 \times 10^{-5}$ | ND d             | -                                   | -33.7                    |                  |
| GlcIm       | $60.2 \times 10^{-9}$ | 0.2              | $3.3 \cdot 10^3$                    | -41.9                    |                  |
| PheGlcIm (Phenyl GlcIm) | $1.7 \times 10^{-9}$ | 0.04             | $23.5 \cdot 10^3$                   | -50.9                    |                  |
| PhethGlcIm (Phenethyl GlcIm) | $1.8 \times 10^{-9}$ | ND d             | -                                   | -50.7                    |                  |

* Using 4NPGlc as a substrate with $K_m$ of 1.4 mM (40).

* Data for glucose are taken from Ref. 8.

* Data for 2F-DNPGlc and conduritol B epoxide are taken from Ref. 9.

* ND, not determined.

* Calculated according to $\Delta G = -RT \ln [K_i]$ (41).

#### Table I

| Kinetic parameters of barley β-D-glucan glucohydrolase inhibition |
|---------------------------------------------------------------|
| Inhibitor | $K_i$ (M) | $k_i$ (min$^{-1}$) | $k_i K_i^{-1}$ (min$^{-1}$ mM$^{-1}$) | $\Delta G$ * (kJ mol$^{-1}$) | Chemical structure |
|-----------------|-----------|------------------|-------------------------------------|--------------------------|------------------|
| Glucose b       | $8.8 \times 10^{-3}$ | ND d             | -                                   | -12.2                    |                  |
| 2F-DNPGlc c     | $25.5 \times 10^{-6}$ | 0.1              | 3.9                                 | -27.3                    |                  |
| Conduritol B Epoxide c | $42.6 \times 10^{-6}$ | 0.3              | 7.0                                 | -25.9                    |                  |
| Castanospermine |           |                  |                                     |                          |
| Glucono-lactone b | $2.1 \times 10^{-5}$ | ND d             | -                                   | -33.7                    |                  |
| GlcIm           | $60.2 \times 10^{-9}$ | 0.2              | $3.3 \cdot 10^3$                    | -41.9                    |                  |
| PheGlcIm (Phenyl GlcIm) | $1.7 \times 10^{-9}$ | 0.04             | $23.5 \cdot 10^3$                   | -50.9                    |                  |
| PhethGlcIm (Phenethyl GlcIm) | $1.8 \times 10^{-9}$ | ND d             | -                                   | -50.7                    |                  |

* Using 4NPGlc as a substrate with $K_m$ of 1.4 mM (40).

* Data for glucose are taken from Ref. 8.

* Data for 2F-DNPGlc and conduritol B epoxide are taken from Ref. 9.

* ND, not determined.

* Calculated according to $\Delta G = -RT \ln [K_i]$ (41).
aglycones, is or phenethyl substituents on their GlcIm core, mimicking strength of the inhibition with glucoimidazoles containing phenylhydrolytic reaction and a competitive inhibitor (8). The technique and the three-dimensional structure of the solved to 2.62°. The three-dimensional structure was obtained in the latter case with a 6.7, respectively. The dependence of the catalytic nucleophile and the catalytic acid/base are 4.6 and M was observed at pH 5.3. The apparent pKa values of the catalytic amino acids of Glucohydrolase-glucose complex as a search model. The final dependence of Ki values of the catalytic amino acids of the barley D-glucan glucohydrolase during the inhibition with PheGlcIm were calculated over a range of pH values. Fig. 1

![Image](https://via.placeholder.com/150)

FIG. 1. The pH dependence of kcat Km −1 and Km −1 of the barley β-D-glucan glucohydrolase during the inhibition with PheGlcIm. Non-linear fitting of the line through the individual kcat/Km data points (C) yielded apparent pK values of 4.6 and 6.7 for the catalytic nucleophile and the catalytic acid/base, respectively. Non-linear fitting of the Km −1 values (○) versus pH indicates that the lowest K values were monitored within the pH range of 5.0–5.5.

ground-state substrate found so far for this enzyme (40), 106 times tighter than the synthetic substrate 4NPGlc (40), and 2×107 tighter than glucose, which is the product of the hydrolytic reaction and a competitive inhibitor (8). The strength of the inhibition with glucoimidazoles containing phenyl or phenethyl substituents on their GlcIm core, mimicking aglycones, is ~30 times lower than the K value for GlcIm (Table I). The inhibitor with the lowest K value was PheGlcIm, that is, the inhibitor with the phenyl substituent (Table I), and this compound was therefore chosen for structural studies.

To investigate any correlation between binding of the inhibitor and apparent pK values of the catalytic amino acids of β-D-glucan glucohydrolase, kcat Km −1, and Km −1 values for PheGlcIm were calculated over a range of pH values. Fig. 1 shows the pH dependence of kcat Km −1 values for β-D-glucan glucohydrolase and indicates that the apparent pK values for the catalytic nucleophile and the catalytic acid/base are 4.6 and 6.7, respectively. The dependence of Km −1 of PheGlcIm versus pH shows that the lowest K value for PheGlcIm of 1.7×10−9 M was observed at pH 5.3. The apparent pK constant of PheGlcIm is 4.99 (19), and this indicates that the loss of binding at pH values lower than ~5.0 reflects the formation of positive charge on the N1 of the imidazole moiety of PheGlcIm. The basic side of the Km −1 versus pH profile shows gradual loss of binding of the inhibitor at pH values above 5.3.

Crystallography of the β-D-Glucan Glucohydrolase-PheGlcIm Complex—The crystallographic data were collected to 2.62-Å resolution at 100 K from at least three different crystals using various soaking strategies to obtain the best resolution. PheGlcIm was added directly to the crystal in mother liquor in a solid form, or a solution of PheGlcIm mixed with the mother liquor to reach 2 and 10 mM concentrations. The best 2.62-Å diffraction data set was obtained in the latter case with a mosaic spread of 0.4°. The three-dimensional structure was solved to 2.62-Å resolution using a rigid body refinement technique and the three-dimensional structure of the β-D-glucan glucohydrolase-glucose complex as a search model. The final crystallographic Rwork and Rfree factors for β-D-glucan glucohydrolase-PheGlcIm complex were 20.96% and 27.29%, respectively (Table II). The structure of the β-D-glucan glucohydrolase consists of 602 amino acid residues, 241 bound water molecules, and 1 PheGlcIm molecule. Furthermore, we detected a heavily occupied glycosylation site at N600, which was disordered in previous crystals (1EX1 (42)), contained no detectable sugars (1IEQ, 1IEV, and 1IEX), or contained either one (1IEW (9)) or two (1J8V (10)) N-acetyl-D-glucosamine moieties at this site. The oligosaccharide attached to N600 has the following minimal structure (Structure I).

**Table II** Data collection and refinement statistics of barley β-D-glucan glucohydrolase-PheGlcIm complex

| Parameter | Value |
|-----------|-------|
| Resolution (Å) | 50.0–2.62 |
| Unit cell dimensions: a = b, c (Å) | 100.69, 182.68 |
| Total observations | 70,377 |
| Unique observations | 44,625 |
| Multiplicity | 2.58 |
| Rmerge (%) | 13.0; 58.3 |
| Data completeness (%) | 73.6; 45.4 |
| Rwork (%) | 20.96 |
| Rfree (%) | 27.29 |
| r.m.s. bonds (Å); angles (°) | 0.01; 1.7 |
| Luzzati coordinate error (Å) | 0.30 |

* Rmerge = 100 Σ[|Ii(1)−⟨I⟩|2]/Σ[I1]2 summed over all independent reflections.
* For the highest resolution shell (2.69–2.62 Å).
* Represents approximately 5% of the data.

Manβ1→4GlcNAcβ1→4GlcNAcβ1→N-Asn600

![Image](https://via.placeholder.com/150)

**Structure 1**

Three-dimensional Structure of β-D-Glucan Glucohydrolase-PheGlcIm Complex—The interactions of PheGlcIm with β-D-glucan glucohydrolase are schematically shown in Fig. 2. At the resolution achieved from the data at 2.62 Å, there are three continuous electron densities at the 1.6 σ level formed between three active site residues Asp95, His2285, and Asp2855, and the C6OH, C7OH, C8OH, and C9OH groups of PheGlcIm (Fig. 3). The C9OH group of PheGlcIm (corresponding to the C2OH in the glucosyl residue at substrate −1 (9)) interacts with the catalytic nucleophile O62265 with a very short interaction of 2.48 Å, whereas the N1 atom of PheGlcIm interacts with the acid/base catalyst making hydrogen bond interactions to Oe1491 and Oe2491 of 3.07 and 2.95 Å, respectively (Fig. 2). The latter interactions are significantly longer than those observed previously in the Bacillus agaradherens endo-1,4-β-D-glucanase-cellulobiose-derived imidazole (16) and Cellulomonas fimii endo-1,4-β-D-xylanase-xylo-imidazole (17) complexes, where the distances of the inhibitor N1 atom to the catalytic acid/base are 2.58 Å and 2.49 Å, respectively. Residue Asp35 interacts with the C4OH and C6OH groups of the glucose moiety in the β-D-glucan glucohydrolase-glucose complex, and His2285, which interacts with the C3OH of the glucose in the β-D-glucan glucohydrolase-glucose complex, form very short interactions of 2.45 Å and 2.55 Å, respectively. Naturally, the resolution of 2.62 Å at which the structure was determined precludes placing any significance on these short distances.

There are five other residues involved in the PheGlcIm binding, namely Lys206, Arg158, Trp286, Trp434, and Glu220, and three water molecules that interact with amino acid residues bound to PheGlcIm (Figs. 2 and 3). One water molecule is associated with the catalytic nucleophile Asp285, and two water molecules are associated with the catalytic acid/base Glu491 and Glu220. The two latter water molecules were similarly positioned in the β-D-glucan glucohydrolase-2-deoxy-2-fluoro-α-D-glucopyranosyl complex and were coordinated through hy-
hydrogen bonding interactions with Glu491 and Glu220 (9). These water molecules were implicated in the hydrophilic “channel” through which they are precisely directed as candidate nucleophiles for a second displacement reaction of the hydrolytic mechanism.

The sugar component of PheGlcIm is in the $^4E$ (envelope) or the $^7E$ conformation with atomic numbering shown in Fig. 2. In this structure the C8-C9-C10-N1-C5 atoms are co-planar. The plane of the phenyl moiety of PheGlcIm is tilted by $45^\circ$ to the planes of the side chains of Trp286 and Trp434 (Fig. 3). The tilt of the phenyl substituent of PheGlcIm provides more favorable localized hydrophobic interactions with the phenyl components of indole moieties of Trp286 and Trp434 than if the phenyl rings were parallel to the planes of the indole moieties. These hydrophobic interactions restrict the conformational mobility of the phenyl ring of PheGlcIm, producing very clear electron density at the 4 $\sigma$ level for the phenyl substituent of PheGlcIm (Fig. 3).

The superpositions of the $\beta$-d-glucan glycohydrolase-PheGlcIm complex onto the three-dimensional structures with bound glucose, cyclohexitol, and $S$-cellobioside moieties (9) are shown in Fig. 4. The comparison with $\beta$-d-glucan glycohydrolase-glucose, which represents an enzyme-product complex, indicates that O$^1$ and O$^2$ of Asp285 and Asp95, and His206 make pronounced movements toward the “glucose” component of PheGlcIm. On the other hand, Oe$^1$ rotates slightly away from the N1 atom of the imidazole moiety. Despite these movements, the glucose moieties in both structures are almost perfectly superimposable (Fig. 4B). The glucose and the cyclohexitol moiety (Fig. 4C) do not superpose well, because the C1 atom of the cyclohexitol moiety migrates $1.2$ Å toward O$^1$ (9). The superposition of $\beta$-d-glucan glycohydrolase-PheGlcIm and the $\beta$-d-glucan glycohydrolase-$S$-cellobioside complexes (Fig. 4D) shows that Oe$^2$ is placed $0.2$ Å closer to the $S$ atom of the $S$-cellobioside moiety than Oe$^2$ is to the N1 atom in the...
In contrast, $O^{295}, O^{101}$ and $O^{207}$ of Asp$^{285}$ and His$^{207}$ all make significant movements toward the glucose component of PheGlcIm. These comparisons indicate that there is mobility of amino acid residues involved in binding of PheGlcIm, which might be critical for formation of transition states during catalysis.

Quantum Mechanical Calculations—Ab initio molecular orbital calculations comparing distances between the carboxyl oxygen atoms of acetate that represents Glu$^{491}$ and the imidazole ring nitrogen of the inhibitor show that in none of the four 

\[ \begin{align*} 
\text{FIG. 4.} & \quad \text{Stereo representation of the active site of the barley } \beta-\text{d-glucan glucohydrolase with bound PheGlcIm (A), and PheGlcIm with superposed glucose (B), cyclohexitol moiety (C), and S-celllobioside moiety (D). A MOLSCRIPT (44) diagram of PheGlcIm is colored in cyan, and the superposed glucose, cyclohexitol, and S-celllobioside moieties are shown in yellow. Transparent magenta and green colors represent the molecular surfaces (45) of domains 1 and 2, respectively. Black, red, blue, and yellow spheres represent carbon, oxygen, nitrogen, and sulfur atoms, respectively. The structures were superposed over the } C_{\alpha} \text{ atoms of Asp}^{95}, \text{Phe}^{144}, \text{Arg}^{158}, \text{Lys}^{206}, \text{His}^{207}, \text{Glu}^{220}, \text{Tyr}^{253}, \text{Asp}^{285}, \text{Trp}^{286}, \text{Glu}^{287}, \text{Asp}^{291}, \text{Met}^{316}, \text{Trp}^{434}, \text{and Glu}^{491}, \text{with root mean square deviations in the } C_{\alpha} \text{ chain of 0.203 } \text{Å for PheGlcIm and glucose, 0.252 } \text{Å for PheGlcIm and cyclohexitol moiety, and 0.249 } \text{Å for PheGlcIm and S-celllobiosyl moiety. The subsites } -1 \text{ and } +1 \text{ are indicated. To improve clarity of the diagrams, the inverted inside-out views of the active site with the residues Arg}^{158}, \text{Asp}^{285}, \text{Trp}^{286}, \text{Trp}^{434}, \text{and Glu}^{491} \text{ are presented. The entrance to the active site is located toward the upper left-hand corner.} 
\end{align*} \]
 configurations was a good correlation with the experimental values achieved (Table III). In configurations 1 and 3 the N1-O1 distance is shorter than the N1-O2 distance, in disagreement with the crystallographic data, whereas in configuration 3 the N1-O2 distance is longer and the N1-O1 distance is shorter than the values found in the experiment. In configuration 2 both distances are much longer than those found experimentally, even though this configuration represents the theoretical ionization states of Glu491 and the phenyltetrahydroydrazopyridine moiety in the crystalline environment. We have also considered configurations in which only one of either Glu491 or PheGlcIm were charged (data not shown). These calculations did not provide a better agreement with the experimental values than those observed in configurations 1–4.

Configurations 1–4 include the three most likely, albeit different logical considerations of the ionization states of PheGlcIm and Glu491 (15, 16). Configurations 3 and 4 represent the same ionization states of PheGlcIm and Glu491, but differ in the position of a proton (Table III) that is present either on Oe1 Glu491 or on Oe2 Glu491, respectively; Oe1 Glu491 and Oe2 Glu491 have the same formal HOCO angles of 0°. At the highest level of theory studied here, B3LYP energies incorporating solvation effects, we predicted that configuration 3 lies 12.9 kJ mol⁻¹ lower than configuration 1, whereas configuration 4 is predicted to lie 9.7 kJ mol⁻¹ higher than 1. In summary, these calculations suggest that the energies of both ionization states are very similar. The state in which both PheGlcIm and Glu491 are neutral is slightly lower in energy than the state in which both components are charged. The Cremer-Pople ring pucker parameters of the configurations 1–4 show that the glucosyl moieties in PheGlcIm in each of the model structures are in an E-type conformation. Notably, no differences in the Cremer-Pople ring pucker parameters of the N1 protonation state of PheGlcIm are found, compared with the neutral state of PheGlcIm, and suggest that higher resolution x-ray data are unlikely to distinguish between them.

**DISCUSSION**

We have previously shown for the barley β-D-glucan glucohydrolase with bound S-cellobiose or S-laminaribiose moieties (9, 10) that pyranose ring distortion from the 4C1 conformation is not necessary for occupation of the –1 subsite and have never observed distortion of intermediates or substrate analogues in the active site. Similarly, Sulzenbacher et al. (46) with an endoglucanase-cellobiose complex and Fort et al. (47) with an endoglucanase-mixed-linkage cellooligosaccharide complex showed that there is no substrate distortion at –1 subsite, although in the latter case the mixed-linkage oligosaccharide evades binding at the –1 subsite. However, the fact that the sugar is locked tightly at the –1 subsite of the β-D-glucan glucohydrolase through extensive hydrogen bonding is consistent with the theory that distortion into the transition state geometry occurs at this subsite, along the reaction coordinate (3, 7).

In contrast, distortions at the –1 subsite have been reported in other enzyme systems, such as a 1Sβ skew boat for endoglucanase-thiooligosaccharide (48) and endoglucanase-2-deoxy-2-fluoro-α-cellobiose complexes (2), a 2Sβ conformation for a cellobiohydrolase-thiooligosaccharide complex (49), 4-sofa-like conformations for chitibiose-N-acetylgalcosamine and chitin-chitobiose complexes (50, 51), a 1,4-B conformation for a chitobiose-chitotriacose complex (52), and a 2,3B conformation for the 2-deoxy-2-fluoro-α-xylobiol moiety in complex with endo-1,4-β-D-xylanase (53).

The mechanistic aspect of substrate distortion during development of ion-like transition states has been addressed previously using transition-state-like mimics or transition-state-like analogues (3, 6). Notenboom et al. (17) suggested that there are five principal factors contributing to the development of transition state characteristics, including charge distribution, a trigonal anemeric center, a half chair conformation (or close to), an appropriate relative configuration of OH groups, and an ability of the glycosidic oxygen to be directionally protonated.

In the light of these suggestions and the observed distortion of substrates in many other glycoside hydrolases, we further investigated the possibility that substrate or intermediate ring distortion occurs during hydrolysis by the barley β-D-glucan glucohydrolase, using transition state mimics. A comparison of the Kᵢ values for barley β-D-glucan glucohydrolase inhibitors revealed large differences (Table I). Castanospermine, which has previously been described as a transition state analogue (54), inhibited the barley enzyme in the micromolar range. Withers et al. (55) showed that castanospermine, being structurally unrelated to the transition state, is a “fortuitous binder” rather than a transition state analogue; no correlation was observed between pH dependences of inhibitor binding of castanospermine and catalytic efficiencies of the *Agrobacterium* sp. β-D-glucosidase. The most potent inhibitor of barley β-D-glucan glucohydrolase was PheGlcIm, with the Kᵢ constant of 1.7 × 10⁻⁵ M. This value places PheGlcIm among the strongest inhibitors found so far for glycoside hydrolases (3, 19). The low Kᵢ value of PheGlcIm reflects the strong binding, expected from a transition state analogue. Although the Kᵢ constant for PheGlcIm is in the low nanomolar ranges, the potential to design even more powerful mimics of the transition states exists. It has been suggested that these analogues should bind 10¹⁰⁻¹⁰¹⁵ times tighter (6, 14, 56) or even 10⁻¹⁹⁻¹⁰⁻²³ times tighter (3, 57) than the ground-state substrates.

To investigate whether the PheGlcIm satisfies the definition of a transition state mimic (3, 22, 57), catalytic efficiency and inhibitor binding constants were monitored across a range of

**Table III**

Calculated geometries of barley β-D-glucan glucohydrolase-PheGlcIm models evaluated by ab initio molecular orbital calculations with GAMESS-US

| Model   | RotamerGlu491 | R(N1-O1) | R(N1-O2) |
|---------|---------------|----------|----------|
| Model 1 | NA            | 3.07     | 2.95     |
| Model 2 | NA            | 2.67     | 2.20     |
| Model 3 | NA            | 8.67     | 3.59     |
| Model 4 | NA            | 2.60     | 3.23     |

*Charge on N1 of PheGlcIm.

¹Charge on Glu491 acetate (cf. *Experimental Procedures*).

²Distance between Oe1 Glu491 and N1 of PheGlcIm.

³Distance between Oe2 Glu491 and N1 of PheGlcIm.

⁴NA, not applicable.

⁵The formal HOCO angle is 0°.
pH values (Fig. 1). Barley \( \beta \)-\( d \)-glucan glucohydrolase is a retaining enzyme (8) and has a pH optimum between pH 5.2 and 5.6 (Fig. 1) (40). The two ionizable amino acid residues Asp 285 and Glu 491 act as the catalytic nucleophile and catalytic acid/base, respectively (9). A correlation between binding of an inhibitor and apparent pK$_a$ constants of the catalytic machinery is one of the criteria proposed for transition state mimics (3, 22, 57). Fig. 1 shows a close similarity in catalytic efficiency and inhibitor binding over the pH range 4.0–7.5 and would therefore suggest that the inhibitor is acting as a transition state mimic. In addition, the inhibitor binding versus pH curve indicates that the inhibitor is active in its neutral form (Fig. 1).

Here we report the first three-dimensional structure of family GH3 glycoside hydrolase in complex with glucoimidazole-type of inhibitor, which may be regarded as a transition state mimic or a transition state analogue. An important finding arising from the three-dimensional structure of the \( \beta \)-\( d \)-glucan glucohydrolase-PheImGlc complex is that the glucose component of PheGlcIm is in the \( \text{4E} \) conformation. Similarly, a xylobiose-derived imidazole in complex with an endo-1,4-\( \beta \)-\( d \)-glucanase from Bacillus agaradherans (8A3H) has a 4-sofa conformation (16), which is similar to \( \text{4E} \). The superposition in the active sites of all three ligands shows that their coincidence is almost perfect (Fig. 5).

Values of dihedral angles of the imidazole ring of the \( B. \) agaradherans endo-1,4-\( \beta \)-\( d \)-glucanase-cellobiose-derived imidazole (16), the \( C. \) fimi endo-1,4-\( \beta \)-\( d \)-xylanase-xylobiose-derived imidazole complexes (17) and the \( \beta \)-\( d \)-glucan glucohydrolase-PheGlcIm complex (this work) are in conformity; the C5-N4-C10-C9 dihedral angles span the range 0.8–0.1°, the N4-C10-C10-C9 dihedral angles are within 0.3–5.9°, and the C7-C5-N4-C10 dihedral angles span the range 24.3–30.0°. Remarkably, the relative positions of catalytic nucleophiles in the three active sites are very similar (Fig. 5). However, the relative dispositions of catalytic acid/bases in the enzyme-ligand complexes are different. Whereas in the \( B. \) agaradherans endo-1,4-\( \beta \)-\( d \)-glucanase (16) the catalytic acid/base Glu 139 is positioned in the plane of the imidazole ring (Fig. 5A), the catalytic acid/base Glu 127 in the \( C. \) fimi endo-1,4-\( \beta \)-\( d \)-xylanase complex (17) is slightly above the plane of the imidazole (Fig. 5B). On the contrary, the catalytic acid/base Glu 491 in the barley \( \beta \)-\( d \)-glucan glucohydrolase-PheGlcIm complex is markedly above the imidazole plane.
This comparison implies that a distortion of the sub-
strate must take place during the hydrolytic event and during
this distortion the glycosidic oxygen might move “upwards,”
that is, closer to the position of the catalytic acid/base Glu491.
This possibility is further illustrated by superpositions of β-
D-glucan glucohydrolase-PheGlcIm and the β-D-glucan glucohy-
drolase-S-cellobioside complexes, where the exocyclic N1 atom
in PheGlcIm is below the “glycosidic” S-atom, and thus below a
predicted pseudo-axial orientation expected for the glycosidic
oxygen (Fig. 6). This observation agrees with those made by
Notenboom et al. (17), however, the possibility remains that 4E
represents an artifactual conformation, and the actual transi-
tion-state-like conformation during the hydrolytic cycle might
be 4H3.

Notenboom et al. (17) suggested that the C. fimi endo-1,4-β-
D-xylanase reaction proceeds through a classic 4C1 \( \rightarrow \) 2S3 \( \rightarrow \) 4H3 \( \rightarrow \) 4C1 itinerary, and Sidhu et al. (53) proposed a 4C1 \( \rightarrow \) 2H3 \( \rightarrow \) 2S6 \( \rightarrow \) 2B \( \rightarrow \) 4C1 itinerary for the Bacillus circulans
endo-1,4-β-D-glucanase. In our present work and in previous
studies (9, 10), various stable intermediates during the hydro-
lytic cycle of a barley β-D-glucan glucohydrolase have been
defined. The following itinerary can now be suggested for the
barley β-D-glucan glucohydrolase: 4C1 \( \rightarrow \) (? \( \rightarrow \) 4E (4H3) \( \rightarrow \) 4C1 \( \rightarrow \) 4C1, where the individual events are a free substrate, an
enzyme-substrate complex of unknown conformation, the tran-
sition state, the covalent glycosyl-enzyme intermediate, and
the glucose product, respectively. At this stage we are not able
to conclude whether the β-D-glucan glucohydrolase-PheGlcIm
complex represents an early or a late transition state. Accord-
ing to the current understanding of the mechanism of action of
retaining hydrolases (3, 4, 7), it is expected that an early
transition state or the one that precedes the first transition
state, would be closer to a distorted conformation. On the other
hand, the late transition state or the one that follows the
second transition state might be closer to an oxocarbenium
ion-like intermediate. It then follows that these inhibitors
could mimic both types of transition states, and those that
mimic the distorted substrate or oxocarbenium ion-like inter-
mediate, should bind strongly to the enzyme.

Heightman and Vasella (15) suggested that orientation of
the lone pair of electrons of the glycosidic oxygen with respect
to an acid/base catalyst and relative to the C1–O5 bond of the
sugar moiety, defines the protonation trajectory of glycoside
hydrolases, which can be either anti or syn. Whereas the anti-
protonation class of hydrolases includes retaining enzymes, which have been classified within the GH-A clan (families GH1, GH2, GH5, GH10, and GH51) and the GH-K clan (families GH18 and GH20; available at afmb.cnrs-mrs.fr/CAZY), the syn-protonators represent both retaining and inverting enzymes, which are classified in glycoside hydrolase families GH7, GH11, GH12, GH16, GH22, GH23, and GH45 (3). The barley \( \beta \)-d-glucan glycohydrolase, as a representative of family GH3 glycoside hydrolases, is a typical retaining enzyme (\( e = \omega \); nomenclature of Sinnott (4)) enzyme (8). The structure of the \( \beta \)-d-glucan glycohydrolase-PheGlcIm complex clearly reveals an anti-protonation trajectory for this enzyme (Fig. 5). The family GH5 \( B. \) agaradherans endo-1,4-\( \beta \)-d-glucanase (16) and the family GH10 \( C. \) fimis endo-1,4-\( \beta \)-xylanase (17) are also classified as anti-protonators, with a lateral mode of protonation. The barley \( \beta \)-d-glucan glycohydrolase could similarly be classified as a lateral anti-protonator, although in this case the acid/base catalyst in the active site is positioned more perpendicularly to the glycosidic oxygen (Figs. 5 and 6). However, it is important to point out that the relative positioning of the catalytic acid/base in the active site may be dictated by the overall geometry of the active site. Despite the fact that the three enzymes mentioned above are not closely related (belonging to glycoside hydrolases families GH3, GH5 and GH10; afmb.cnrs-mrs.fr/CAZY), it is remarkable that such an exceptionally good agreement in the positioning of the enzyme catalyts in their active sites has been reached (Fig. 5).

Further to the comparison of the three-dimensional disposition of the catalytic amino acid residues in the \( B. \) agaradherans endo-1,4-\( \beta \)-d-glucanase-cellobiose-derived imidazole complex (16), in the \( C. \) fimis endo-1,4-\( \beta \)-xylanase-xyllose-derived imidazole complex (17) and in the \( \beta \)-d-glucan glycohydrolase-PheGlcIm complex (Fig. 2), we have investigated differences in the disposition of residues that bind the C6OH group and the C5 carbon of the imidazole-sugar inhibitors at the \( -1 \) subsite. As expected for enzymes from different but related families, there is limited conservation of amino acid residues that bind the C6OH group (or the C5 carbon for xyllose-derived imidazole) (data not shown). Nevertheless, amino acid residues that bind the C6OH groups are present in both the \( B. \) agaradherans endo-1,4-\( \beta \)-d-glucanase (family GH5) and in the barley \( \beta \)-d-glucan glycohydrolase (family GH3), which indicates functional conservation of the C6OH binding. In the \( B. \) agaradherans endo-1,4-\( \beta \)-d-glucanase, Ca backbone oxalos of Ala\(^{234} \) and Thr\(^{235} \) bind to the C6OH group of the cellulose-derived imidazole at a \(-1 \) subsite (16), whereas Asp\(^{65} \) alone is sufficient to achieve similar binding of PheGlcIm in the barley \( \beta \)-d-glucan glycohydrolase (9, 10). In contrast, the \( C. \) fimis endo-1,4-\( \beta \)-xylanase (family GH10) has a bulky aromatic residue Trp\(^{281} \) to achieve similar binding of PheGlcIm in the barley \( \beta \)-d-glucan glycohydrolase.

Quantum mechanical calculations were performed to predict the ionization state of the acid/base catalyst Glu\(^{18} \) and the ionization state of the inhibitor PheGlcIm, so as to ascertain whether the shape of PheImGlc or its ionization state were more important for inhibition of the enzyme. These calculations suggest that PheGlcIm is neutral when bound to the enzyme. However, the difference in energy between ionized and neutral states is not large. If there is no proton transfer from the catalytic acid/base to the PheImGlc molecule, as these calculations suggest, it seems likely that the shape (glucose and PheImGlc are in \( c_{1} \) and \( c_{4} \) conformations, respectively) must play a critical role when the inhibitor interacts with the active site of the \( \beta \)-d-glucan glycohydrolase.

\( \beta \)-d-Glucan glycohydrolases in embryophytes have been implicated in wall loosening during cell elongation, in wall remodeling, in defense reactions against fungal pathogens, in the release of glucose from wall polysaccharides as an energy source in dark-grown seedlings, and in the general turnover of glucans from \( \beta \)-d-glucans and \( \beta \)-d-oligosaccharides (9, 10, 59). The broad specificity of the enzyme with respect to linkage position in its \( \beta \)-d-glucan and \( \beta \)-d-oligosaccharide substrates suggests that it could undertake several of these functions. In this context details of the conformational pathway of the substrates during enzymatic catalysis of the \( \beta \)-d-glucan glycohydrolase can be used as a basis for the design of novel transition state analogues, which could be used to investigate the function of the enzymes in \( \textit{planta} \). Furthermore, transition state analogues might find applications as agents controlling plant growth (60), and as probes for the investigation of catalytic mechanisms (3, 6). As an adjunct to such investigations, powerful computational techniques such as quantum mechanical modeling, molecular docking, de novo design, quantitative structure-activity relationships and combinatorial library design, based on structural information, have potential to rationally predict new families of inhibitors (61–63), for application in agricultural and related technologies.
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