Positioning the Acid/Base Catalyst in a Glycosidase: Studies with *Bacillus circulans* Xylanase†

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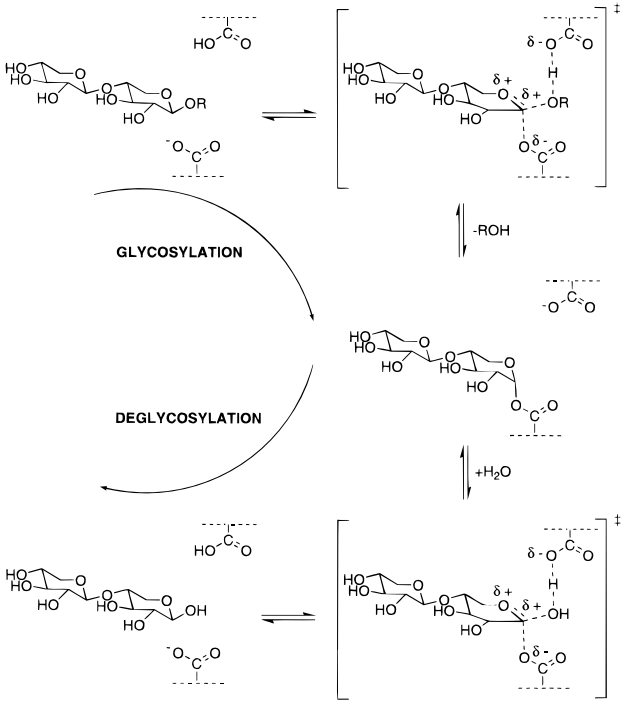
ABSTRACT: The mechanism of action employed by a glycosidase is dictated, in part, by the distance between the two catalytic carboxylic acids. In the retaining endo-β-1,4-xylanase from *Bacillus circulans*, this critical distance (∼5.5 Å) has been altered by mutagenesis of the putative acid/base catalyst Glu172. An increase in the separation (Glu172Asp) resulted in a 400-fold decrease in the k_{cat} value for xylan hydrolysis. By contrast, a decrease in the separation, achieved by the selective carboxymethylation of the Glu172Cys mutant, caused only a 25-fold reduction in the rate of xylan hydrolysis. Altering the length of the acid/base catalyst had a less detrimental effect on the hydrolysis of aryl xylobiosides, with k_{cat}/K_m values being reduced only 3−23-fold relative to the wild-type enzyme. Complete removal of the carboxyl group had a more dramatic effect. The Glu172Cys and Glu172Gln mutants exhibited no measurable activity on xylan or phenyl xylobioside, substrates which require acid catalysis. However, these mutants were capable of hydrolyzing aryl xylobiosides with relatively good leaving groups ($pK_a < 5.5$), which need little protonic assistance. The addition of sodium azide caused significant rate increases for the hydrolysis of 2,5-dinitrophenyl β-xylobioside ($pK_a = 5.15$) by Glu172Cys and Glu172Gln. Thus, the absence of an acid/base catalyst can be partially compensated for by the addition of an anionic nucleophile. These results are consistent with Glu172 functioning as the acid/base catalyst in *B. circulans* xylanase and emphasize the functional importance of the carboxyl group found at this position.

The endo-β-1,4-xylanase from *Bacillus circulans* catalyzes the hydrolysis of xylan, and β-xylobiosides, with net retention of anomeric configuration (Gebler et al., 1992). As with other retaining β-glycosidases, the proposed double displacement mechanism involves a covalent α-glycosyl-enzyme intermediate, as illustrated in Scheme 1 (Koshland, 1953; Sinnott, 1990). The formation and hydrolysis of this covalent intermediate occur via oxocarbenium ion-like transition states, with the assistance of two key active site residues. One residue acts as a nucleophile, attacking the anomeric carbon of the substrate to displace the leaving group and form the covalent intermediate. The other residue plays the dual role of acid/base catalyst, assisting in the formation of this intermediate by proton donation to the departing leaving group. In the subsequent hydrolysis step, this residue functions as a base catalyst, deprotonating the attacking water. These catalytic residues, typically aspartic or glutamic acids, are positioned ∼5.5 Å apart in the active site. In inverting glycosidases, which employ a direct displacement mechanism, these two residues are typically further apart (∼10−11 Å) (Davies & Henrissat, 1995; McCarter & Withers, 1994; Wang et al., 1994; White et al., 1994). This distance between the two essential carboxylic acids therefore appears to be important for controlling the mechanism, though it is unlikely to be the only factor.

Scheme 1: Proposed Mechanism of a Retaining Glycosidase[a]



[a] OR represents the aglycon where R corresponds to an aryl group or one or more xylose residues.

B. circulans xylanase is an excellent model system on which to investigate the effects of altering this critical

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distance (~5.5 Å) between the two essential carboxylic acids. The enzyme is a relatively small, cysteine-free glycosidase (20 kDa) for which an X-ray crystal structure has been solved (Campbell et al., 1993; Wakarchuk et al., 1994). A member of glycosidase family 11 (Henrissat & Barrioch, 1993), *B. circulans* xylanase possesses two conserved active site carboxylic acids, Glu78 and Glu172, which are mechanistically important (Wakarchuk et al., 1994). Glu78 was previously identified as the active site nucleophile by trapping the covalent glycosyl-enzyme intermediate using the mechanism-based inactivator, 2′,4′-dinitrophenyl 2-deoxy-2-fluoro-β-xylobioside (Miao et al., 1994). Detailed kinetic analysis of native xylanase and several Glu78 mutants has since confirmed this assignment (Lawson et al., 1996). The acid/base catalyst must therefore be Glu172. The key catalytic residues of two other family 11 xylanases, *Schizophyllum commune* and *Bacillus pumilis*, have also been identified as carboxylic acids (Bray & Clarke, 1990; Katsube et al., 1990; Ko et al., 1992).

Our strategy for altering the distance between Glu78 and Glu172 involved selectively replacing each glutamic acid with its shortened or lengthened analogue. Substituting a glutamic acid with aspartic acid, its shortened analogue, was easily accomplished using site-directed mutagenesis. To produce the lengthened glutamic acid analogue, the combined techniques of site-directed mutagenesis and chemical modification were employed. A cysteine, introduced at the position of interest, was selectively carboxymethylated with iodoacetic acid, yielding a carboxylic side chain approximately 1.6 Å longer than glutamic acid. Conveniently, wild-type *B. circulans* xylanase contains no cysteines prior to mutagenesis; thus, the problem of multiple cysteine labeling was avoided. Several investigators have introduced unnatural amino acids into other enzymes' active sites by using this combined approach of genetic engineering followed by chemical modification (Amano et al., 1994; Dhalla et al., 1994; Gloss & Kirsch, 1995; Lukac & Collier, 1988; Matsushima et al., 1994).

This paper describes studies in which Glu172, the putative acid/base catalyst, has been subjected to modification. Mutants have been constructed in which Glu172 has been replaced with a cysteine (Glu172Cys),[1] glutamine (Glu172Gln), or an aspartic acid (Glu172Asp). The detailed kinetic evaluation of these mutants, using both xylan and synthetic β-xylobioside substrates, is presented in this paper in addition to the preparation, characterization, and kinetic analysis of the carboxymethylated Glu172Cys mutant (denoted IAA-Glu172Cys).

[1] Abbreviations: AMPSO, 3-[(1,1-dimethyl-2-hydroxyethyl)amino]-2-hydroxypropanesulfonic acid; BSA, bovine serum albumin; CD, circular dichroism; CNBr, cyanogen bromide; 2,5-DNPX_2, 2,5-dinitrophenyl β-xylobioside; 3,4-DNPX_2, 3,4-dinitrophenyl β-xylobioside; DTT, 1,4-dithiothreitol; EDTA, ethylenediaminetetraacetic acid; ESMS, electrospray mass spectrometry; Glu172Asp, *B. circulans* xylanase in which Glu172 has been replaced with an Asp; Glu172Cys, *B. circulans* xylanase in which Glu172 has been replaced with a Cys; Glu172Gln, *B. circulans* xylanase in which Glu172 has been replaced with a Gln; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; HPLC, high-performance liquid chromatography; IAA, iodoacetate; IAA-Glu172Cys, Glu172Cys carboxymethylated at Cys172; MES, 2-(*N*-morpholino)ethanesulfonic acid; MS, mass spectrometry; NMR, nuclear magnetic resonance; ONPX_2, *o*-nitrophenyl β-xylobioside; PhX_2, phenyl β-xylobioside; PNPX_2, *p*-nitrophenyl β-xylobioside.

EXPERIMENTAL PROCEDURES

General. The compounds 2,5-dinitrophenyl β-xylobioside (2,5-DNPX_2), 3,4-dinitrophenyl β-xylobioside (3,4-DNPX_2), *o*-nitrophenyl β-xylobioside (ONPX_2), *p*-nitrophenyl β-xylobioside (PNPX_2), and phenyl β-xylobioside (PhX_2) were synthesized by Dr. Lothar Ziser according to published procedures (Ziser et al., 1995; Ziser & Withers, 1994). Cyanogen bromide (CNBr) (97%) was purchased from Aldrich Chemical Co., Inc. All other chemicals and buffer materials were obtained from Sigma Chemical Co. unless otherwise stated.

All spectrophotometric experiments, with the exception of the thiol titrations and the xylan assays, were done with 1 cm path length micro black quartz cuvettes using a Unicam 8700 UV/Vis spectrophotometer equipped with a circulating water bath. Thiol titrations were performed with plastic cuvettes using a thermostated double-beam UV/Vis spectrophotometer (Pye Unicam PU 8800). The xylan assays were done using a single-beam UV/Vis spectrophotometer equipped with an autocell (Pye Unicam PU 8600). Circular dichroism (CD) spectra of xylanase were obtained with a JASCO J-710 spectropolarimeter using a protein concentration of 0.2 mg/mL. All CD spectra were acquired from 205 to 260 nm and corrected for the buffer background. Electrospray mass spectrometry (ESMS) experiments were done using an HPLC-ESMS setup consisting of a microbore HPLC (Michrom UMA) connected on-line to either a PE-Sciex API III or a API 300 MS as described by Hess et al. (1993). Protein samples (10−20 μg) were injected onto a microbore PLRP column (1 × 50 mm), while a Reliasil C18 column (1 × 150 mm) was used for the cyanogen bromide digests. Following elution with a water/acetonitrile/trifluoroacetic acid solvent system and UV detection, 15% of the eluant was introduced directly into the MS by an ion spray ion source and analyzed.

Molecular Biology, Enzyme Production, and Purification. The following standard laboratory strains of *Escherichia coli* were used for the propagation of recombinant plasmids and the production of recombinant gene products: MV1190: Δ (*lac-proAB*), *thi*, *supE*44, deletion (*sr*1-*recA*)306::Tn10 (tet^r) [F': *traD*36, *proAB*, *lacI*^{q} ΔM15]; RZ1032: HfrKL16 PO/45 [*lysA* (61−62)], *dut*1, *ung*1, *thi*1, *relA*1, *Zbd*-279::Tn10, *supE*44; BHM 71−18: Δ (*lac-proAB*), *thi*, *supE*44, [*mutS*::Tn10 (tet^r)], [F': *proAB*, *lacI*^{q} ΔM15]; HB101: *hsdS*20 (r-m-), *leu*, *supE*44, *ara*14, *galK*2, *lacY*1, *proA*2, *rpsL*20 (Str^r), *xy1*-5, *mt1*-5, *recA*13, *mcrB*.

The mutagenesis, detection of xylanase mutants, and the production and purification of mutant proteins and wild-type xylanase were performed as previously described (Sung et al., 1993; Wakarchuk et al., 1994). The oligonucleotides for mutagenesis of *B. circulans* xylanase were the following: for Glu172Cys, 5′ CTG GTA GCC **GCA** GGT CGC CAT; for Glu172Gln, 5′ CTG GTA GCC **CTG** GGT CGC CAT; and for Glu172Asp, 5′ G GCG ACA **GAC** GGA TAT CAA. The Glu172Asp mutant was constructed with the natural gene, whereas the other two mutants were constructed with the synthetic gene (Sung et al., 1993).

Basic recombinant DNA methods such as plasmid DNA isolation, restriction enzyme digestions, the purification of DNA fragments for cloning, ligations, and transformations, and DNA sequencing were performed as recommended by the enzyme supplier or the manufacturer of the kit used for

the particular procedure. Restriction and DNA modification enzymes were purchased from New England Biolabs Ltd., Mississauga, Ontario. Prep-A-Gene DNA purification matrix was purchased from Bio-Rad Laboratories, Mississauga, Ontario. Sequenase, DNA sequencing kit was purchased from US Biochemicals, Cleveland, OH. Oligonucleotide 3′ end labeling with digoxigenin−ddUTP and the subsequent chemiluminescent detection were performed using a kit and some additional reagents from Boehringer Mannheim Canada, Laval, Quebec.

Thiol Titrations. To a solution of 0.2 mM 5,5′-dithiobis(2-nitrobenzoic acid), 6 M guanidine hydrochloride, 20 mM HEPES, and 1 mM EDTA (pH 7.4) warmed to 25 °C was added enzyme (2.5−5 μM). The absorbance at 412 nm, due to the released 2-nitro-5-thiobenzoate, was monitored over a 30 min period. The concentration of free thiol groups was calculated from the net ΔA_{412}, corrected for a buffer blank, using the extinction coefficient ϵ = 14 900 $M^{-1}\cdot cm^{-1}$ (determined from a cysteine standard curve generated under the same conditions). Division of this obtained value by the protein concentration yielded the number of free thiol groups.

Iodoacetate Labeling of the Glu172Cys Mutant under Denaturing Conditions. Enzyme (1 mg/mL; 0.05 mM) in 7.1 M urea, 11% glycerol, 400 mM glycylglycine, 40 mM HEPES, and 1 mM EDTA (pH 7.5) was incubated at 60 °C for 20 min. The reaction mixture was then cooled to 40 °C, IAA added to give a final reagent concentration of 1.1 mM (22-fold molar excess of IAA over cysteine residues), and the reaction mixture incubated in the dark for 24 h. To stop the reaction and decrease the enzyme concentration to 0.05 mg/mL (to minimize aggregation upon refolding), the reaction mixture was diluted 20 times with the above 7.1 M urea solution. The labeled enzyme was then refolded by dialyzing the diluted enzyme mixture against 20 mM HEPES, 6% glycerol buffer (pH 7.0, room temperature, 48 h). The dialyzed sample was filtered (0.2 μm) and concentrated using a 10 kDa nominal cut-off centrifugal concentrator (Amicon Corp., Danvers, MA). The protein concentration was determined from the molar extinction coefficient for xylanase: ϵ = 81 790 $L\cdot mol^{-1}$, $A^{0.1\%}_{280}$ = 4.08 (Wakarchuk et al., 1994).

Steady-State Kinetic Studies Using Synthetic β-Xylobioside Substrates. The rates of enzymatic hydrolysis for all the synthetic substrates, with the exception of PhX_2, were determined using a continuous assay. To an appropriate concentration of substrate in 0.1% BSA, 20 mM MES, and 50 mM NaCl buffer (pH 6.0), warmed to 40 °C, was added an aliquot of enzyme. Substrate hydrolysis was monitored by measuring the rate of phenolate release at the appropriate wavelength: 2,5-$DNPX_2$, 440 nm, $\Delta\epsilon$ = 3.57 $mM^{-1}\cdot cm^{-1}$; 3,4-$DNPX_2$, 400 nm, $\Delta\epsilon$ = 11.71 $mM^{-1}\cdot cm^{-1}$; $ONPX_2$, 400 nm, $\Delta\epsilon$ = 1.07 $mM^{-1}\cdot cm^{-1}$; $PNPX_2$, 400 nm, $\Delta\epsilon$ = 1.66 $mM^{-1}\cdot cm^{-1}$.

Xylanase-catalyzed hydrolysis rates for the substrate PhX_2 were determined using a stopped assay. Different concentrations of PhX_2 in 0.1% BSA, 20 mM MES, and 50 mM NaCl buffer (pH 6.0, 190 μL) were warmed to 40 °C, and the reaction was initiated by the addition of a 10 μL aliquot of enzyme. After an appropriate time, 0.6 mL of 0.2 M Na_3PO_4/H_2O (pH 12.15) was added to stop the reaction. The absorbance of the released phenolate at 288 nm was determined immediately and corrected for the spontaneous hydrolysis of PhX_2 and the background absorbance of the enzyme ($\Delta\epsilon$ = 2.17 $mM^{-1}\cdot cm^{-1}$ for phenol at pH 12.15).

Steady-State Kinetic Studies Using Xylan. The substrate used in these studies, soluble xylan, was prepared as follows. A 5% suspension of birchwood xylan (Sigma Chemical Co.) was magnetically stirred at room temperature for 3 h. Upon removal of insoluble material via centrifugation, the supernatant was freeze-dried and stored in a desiccator.

Xylanase-catalyzed hydrolysis rates for the soluble birchwood xylan were determined using a reducing sugar assay. Various concentrations of xylan in 0.1% BSA, 20 mM MES, and 50 mM NaCl buffer (pH 6.0) were warmed to 40 °C, and the reaction was initiated by the addition of enzyme. At an appropriate time, a 50 μL aliquot was removed and diluted with 50 mM NaOH (1 mL) to stop the reaction. The concentration of reducing sugars produced was determined spectrophotometrically with hydroxybenzoic acid hydrazide reagent (Lever, 1972), using xylose as a standard. Protein concentrations and reaction times were chosen so that each enzyme hydrolyzed xylan to the same extent, thereby making a relative comparison of kinetic parameters possible.

Hydrolysis rates for both the synthetic substrates and xylan were determined at 6−8 different substrate concentrations ranging from 0.01 to 10 times the estimated K_m value, where possible. From the experimental rate versus substrate concentration data, values of K_m and k_{cat} were calculated directly using the program GraFit (Leatherbarrow, 1992), while the k_{cat}/K_m values were determined from the slope of the Lineweaver−Burk plot.

Pre-Steady-State Kinetic Study of Native Xylanase with 2,5-$DNPX_2$. A pre-steady-state kinetic study of native xylanase with 2,5-$DNPX_2$ was conducted, using stopped-flow techniques, to determine the rate-determining step for this substrate. A substrate solution (1 or 3.8 mM 2,5-$DNPX_2$) and an enzyme solution (1 mg/mL; 0.05 mM) in 20 mM MES, 50 mM NaCl (pH 6.0) were cooled to 5 °C. The reaction was started by driving together 50 μL aliquots of the above two solutions and the release of 2,5-dinitrophenolate ($\Delta\epsilon$ = 3.57 $mM^{-1}\cdot cm^{-1}$) monitored at 440 nm over a 2 s time period.

Effects of Exogenous Nucleophiles on Reaction Rates and Products. The reaction rates for the synthetic substrates, $ONPX_2$ and 2,5-$DNPX_2$, with native xylanase and the Glu172 mutants were determined in the presence of sodium azide as follows. A solution containing various concentrations of sodium azide (0−500 mM), 20 mM $ONPX_2$ (or 2 mM 2,5-$DNPX_2$), 0.1% BSA, 20 mM MES, and 50 mM NaCl (pH 6.0) was warmed to 40 °C. The reaction was initiated by the addition of enzyme and the release of product monitored at 440 or 400 nm for 2,5-$DNPX_2$ and $ONPX_2$, respectively. The possible cleavage of the substrates by azide (500 mM) alone was also monitored and proved to be negligible. Analysis of the reaction products was performed by thin-layer chromatography on 60 F_{254} silica gel aluminum plates (Merck) run in 7:2:1 (v/v/v) ethyl acetate/methanol/water and developed with 10% H_2SO_4 in methanol. The reaction products were also analyzed by ^1H NMR spectroscopy and ESMS after removal of the enzyme from the reaction mixture by centrifugal ultrafiltration, lyophilization of the filtrate, and repeated dissolution/lyophilization into D_2O.

The reaction rates for 2,5-$DNPX_2$ with native xylanase were also determined in the presence of neutral nucleophiles. A solution of 3 mM 2,5-$DNPX_2$, 0.1% BSA, 20 mM MES,

and 50 mM NaCl (pH 6.0) was warmed to 40 °C. The reaction was initiated by the addition of enzyme (or buffer) and various concentrations of the exogenous nucleophiles (0−1 M MeOH, 0−500 mM DTT, or 0−1 M β-mercaptoethanol). The reaction was monitored at 440 nm in both the presence and absence of enzyme. Cleavage of the substrate by the exogenous nucleophiles alone proved to be significant, thus requiring the enzyme-catalyzed rates to be corrected for this background reaction.

pH Dependence of k_{cat}/K_m. The k_{cat}/K_m values for the hydrolysis of $ONPX_2$ at each pH were determined from progress curves at low substrate concentrations as follows. A solution of $ONPX_2$ (0.5 mM, 0.01−0.2 × K_m, for the IAA-Glu172Cys, Glu172Asp, and Glu172Cys mutants; and 0.3 mM, 0.02 × K_m, for native xylanase), 0.1% BSA, and the appropriate buffer was warmed to 25 °C. The reaction was initiated by the addition of a 10 μL aliquot of enzyme (or buffer) and the release of *o*-nitrophenolate monitored at 400 nm until greater than 75% substrate depletion was observed. The pH of the reaction mixture was then determined and an aliquot assayed for activity, at pH 6.0, to check enzyme stability. The progress curves were analyzed using a first-order rate equation to yield the pseudo-first-order rate constants. Division of these obtained rate constants by the enzyme concentration yielded the k_{cat}/K_m values. The buffers used were as follows: 20 mM succinic acid, 50 mM NaCl (pH 2.5−5); 20 mM MES, 50 mM NaCl (pH 5−7); 20 mM HEPES, 50 mM NaCl (pH 7−8.5); 20 mM AMPSO, 50 mM NaCl (pH 8.5−9.0). The pK_a values were assigned by analyzing the k_{cat}/K_m versus pH plots using GraFit.

ESMS Analysis of Digested IAA-Glu172Cys and Native Xylanase. The IAA-Glu172Cys mutant and native xylanase were degraded with CNBr as follows. To the enzymes (1 mg/mL; 0.05 mM) diluted in 0.1 M HCl was added CNBr (dissolved in acetonitrile) to give a final reagent concentration of 19 mM (190-fold molar excess over methionine residues). The reaction mixture was incubated in the dark, under N_2(g), at room temperature for 25 h. After the reaction was quenched by quick freezing (using liquid N_2), the excess CNBr and reaction by-products were removed by freeze-drying (repeated twice). The freeze-dried, cleaved enzymes, dissolved in 6 M guanidine hydrochloride, 20 mM HEPES, and 1 mM EDTA (pH 7.4), were then subjected to ESMS analysis.

The peptide mixtures were first separated by reverse-phase HPLC using the ESMS as a detector, as described previously. A daughter ion MS/MS experiment of peptide 170−185, in digested IAA-Glu172Cys, was then conducted by selectively introducing the doubly charged *m/z* 853 ion through Q1 into the collision cell (Q2) and observing the daughter ions in Q3. The CAD (collision-assisted dissociation) setting was 3, and the scan range for Q3 was set from 50 to 1720 Da at 0.5 Da step sizes so that it would capture the entire mass range of the possible daughter ions as well as the parent ion.[2]

RESULTS

Preliminary Characterization of the Glu172Cys, Glu172Asp, and Glu172Gln Mutants. The replacement of a glutamic acid by a cysteine and an aspartic acid was confirmed by electrospray mass spectrometry (ESMS). The expected molecular mass reductions of 26 and 14 Da, relative to native xylanase, were observed for Glu172Cys and Glu172Asp, respectively. Thiol titrations provided additional evidence of the introduction of a unique cysteine in the Glu172Cys mutant. The Cys mutant contained 1.05 free cysteines, while native xylanase contained none. These mutations had little effect on the enzyme's conformation as indicated by the similar CD spectra shown in Figure 1.

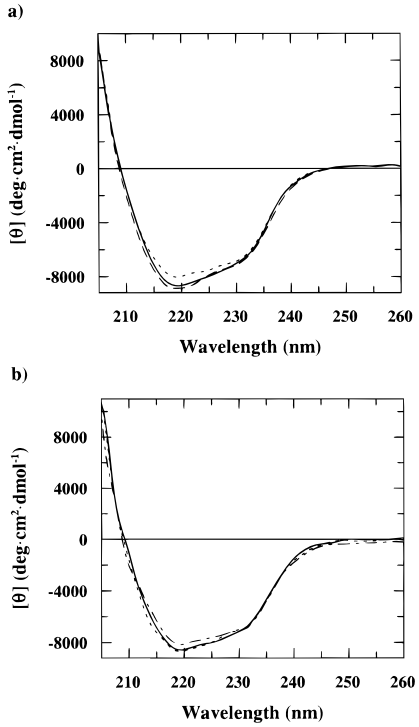

FIGURE 1: CD spectra of native xylanase and the Glu172 mutants. (a) A comparison of the CD spectra of native xylanase (—), Glu172Asp (− −), and the Glu172Gln (- - -) mutant. (b) The CD spectra of Glu172Cys (- - -) and refolded IAA-Glu172Cys (− - −) versus native xylanase (—).

Iodoacetate Labeling of the Glu172Cys Mutant under Denaturing Conditions. Previous attempts to selectively carboxymethylate the nucleophile mutant, Glu78Cys, under nondenaturing conditions were unsuccessful, suggesting that Cys78 is relatively inaccessible to iodoacetate (Lawson et al., 1996). Similar problems were anticipated with the Glu172Cys mutant since analysis of the modeled X-ray structures revealed that both Cys78 and Cys172 have small exposed surface areas (the calculated exposed surface area of the sulfhydryl group of Cys78 and Cys172 is 2.6 and 0.2 Å^2, respectively[3]). Consequently, the iodoacetate labeling of Glu172Cys was attempted only under denaturing conditions using the procedure developed for the nucleophile cysteine mutant, as described under Experimental Procedures. ESMS analysis of the refolded labeled mutant (IAA-Glu172Cys) showed essentially complete conversion of Glu172Cys to a monolabeled species having a molecular mass of 20 426 (±6) Da (molecular mass of untreated Glu172Cys was 20 368 ± 5 Da). Evidence that Cys172 is the amino acid being alkylated was obtained by both ESMS analysis and thiol titrations. Native xylanase exposed to the same labeling conditions, and possessing no cysteine at

[2] The ESMS analysis of the CNBr digests was performed on a PE-Sciex API 300 MS.

[3] The surface area of a fully exposed sulfhydryl group is >40 Å^2.

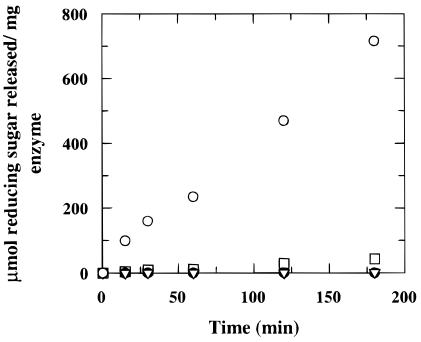

FIGURE 2: Hydrolysis of soluble birchwood xylan by Glu172Asp (□), Glu172Cys (●), and refolded IAA-treated Glu172Cys [1.1 mM (○) or 0 mM (▽) IAA]. For each enzyme, the concentration of xylan used was 10 mg/mL.

position 172, showed no sign of modification by ESMS. Thiol titrations revealed that IAA treatment results in the blocking of Cys172, with the IAA-Glu172Cys mutant having only 0.07 free cysteine. Iodoacetate labeling did not impede correct refolding, as indicated by Figure 1b, for the CD spectra of IAA-Glu172Cys and native xylanase are essentially identical.

Identification of the Iodoacetate-Labeled Peptide by ESMS. Native xylanase, possessing two methionines at positions 158 and 169, yields three peptides (peptides 1−158, 159−169, and 170−185) when degraded with CNBr. Similar treatment of the IAA-Glu172Cys mutant should therefore produce the identical three peptides, the only difference being that the IAA-labeled peptide should have a greater molecular weight than the corresponding peptide in native xylanase. The HPLC traces of CNBr-degraded native xylanase and IAA-Glu172Cys were identical, as expected, with each chromatogram containing three peaks corresponding to the three predicted peptides. The peptide corresponding to the first HPLC peak had molecular masses of 1673 and 1705 Da in the native and IAA-Glu172Cys digests, respectively. These masses are identical to those predicted for peptide 170−185 in native and IAA-Glu172Cys, with the observed 32 Da increase being as expected for the substitution of Glu172 with a carboxymethylated cysteine. Subsequent sequencing of this unique IAA-Glu172Cys peptide (*m/z* 853, $z = +2$), via an MS/MS experiment, confirmed that the IAA-labeled peptide is indeed peptide 170−185 having the sequence AT**X**GYQSSGSSNVTVW (**X** = carboxymethylated cysteine) (data not shown).

Kinetic Evaluation of Native Xylanase and the Glu172 Mutants Using Xylan. Replacement of Glu172 with a cysteine had a dramatic effect on activity with the mutant exhibiting no observable activity on the natural substrate, xylan, as had been noted in preliminary studies (Wakarchuk et al., 1994). Substantial reactivation of the Glu172Cys mutant was observed upon IAA treatment, as illustrated in Figure 2. The kinetic parameters k_{cat}(app), K_m(app), and k_{cat}/K_m(app), determined for xylan hydrolysis by native xylanase, IAA-Glu172Cys, and the Glu172Asp mutant, are summarized in Table 1. For the IAA-Glu172Cys mutant, the highest substrate concentration tested was below the listed K_m value. The k_{cat}/K_m value was, therefore, more accurately determined from the slope of the Lineweaver−Burk plot.

Kinetic Evaluation of Native Xylanase and the Glu172 Mutants Using Synthetic β-Xylobioside Substrates. The kinetic parameters k_{cat}, K_m, and k_{cat}/K_m were determined for five aryl β-xylobioside substrates and are summarized in Table 2. Values of k_{cat} and k_{cat}/K_m for IAA-Glu172Cys are based upon the assumption of 100% labeling, consistent with the ESMS and thiol titration results. Presented in Figure 3 are the Brønsted relationships for native xylanase, Glu172Asp, and IAA-Glu172Cys. The calculated slopes (β_{1g}) and correlation coefficients (ρ) for Figure 3a, the log (k_{cat}/K_m) versus pK_a plot, are as follows: $\beta_{1g} = -0.7$, $\rho = 0.97$ for native xylanase; $\beta_{1g} = -0.8$, $\rho = 0.99$ for Glu172Asp; $\beta_{1g} = -0.7$, $\rho = 0.99$ for IAA-Glu172Cys. The corresponding values for Figure 3b, the log k_{cat} versus pK_a plot, are $\beta_{1g} = -0.5$, $\rho = 0.88$ for native xylanase; $\beta_{1g} = -0.7$, $\rho = 0.98$ for Glu172Asp; and $\beta_{1g} = -0.4$, $\rho = 0.96$ for IAA-Glu172Cys. No pre-steady-state phenolate burst was observed for native xylanase with either 0.5 or 1.9 mM 2,5-DNPX_2 using stopped-flow techniques (data not shown). This result indicates that the glycosyl-enzyme intermediate is not accumulating, thereby suggesting that glycosylation is the rate-determining step. In light of this finding, a straight line has been drawn through the native xylanase data in Figure 3b. The Brønsted plots for Glu172Cys (data not shown) were very scattered and yielded the following values: $\beta_{1g} = -0.9$, $\rho = 0.74$ for the log (k_{cat}/K_m) versus pK_a plot; $\beta_{1g} = -0.4$, $\rho = 0.59$ for the log k_{cat} versus pK_a plot.

Table 1: Kinetic Parameters for Soluble Xylan with Native Xylanase and Glu172 Mutants

enzyme	K_m(app)[a] (mg/mL)	k_{cat}(app) [a] (min^{-1})	k_{cat}/K_m(app)[a] (min^{-1}•mg^{-1}•mL)
native	1.4	6700	4800
IAA-Glu172Cys	29	270	6.5[b]
Glu172Asp[c]	4.4	17	3.9

[a] The (app) designations are used since the actual value observed depends upon the source and batch of xylan used, as well as the extent of reaction followed. These parameters were kept consistent in all studies described here. [b] The k_{cat}/K_m value was determined from the slope of the Lineweaver−Burk plot. [c] Data taken from Wakarchuk et al. (1994).

Effects of Exogenous Nucleophiles on Reaction Rates and Products. The enzymatic cleavage of two synthetic substrates, ONPX_2 and 2,5-DNPX_2, by native xylanase and the Glu172 mutants was performed in the presence of different concentrations of sodium azide (0−500 mM), the results being shown in Figure 4. Addition of sodium azide resulted in enhanced reaction rates (up to 8-fold) for both substrates with the Glu172Gln and Glu172Cys mutants. No such increases were seen with native xylanase, Glu172Asp, or the IAA-Glu172Cys mutant. Thin-layer chromatographic analysis of the Glu172Gln and Glu172Cys reaction mixtures containing sodium azide revealed the formation of a new product ($R_f = 0.39$), distinct from xylobiose ($R_f = 0.08$) or substrate ($R_f = 0.42$ and 0.51 for ONPX_2 and 2,5-DNPX_2, respectively). The ^{1}H NMR spectrum and ESMS data obtained for this new product ($R_f = 0.39$) were consistent with the product being β-xylobiosyl azide: ^{1}H NMR (400 MHz, D_2O) δ: 4.46 (1 H, d, $J_{2,1} = 7.8$ Hz, H-1), 4.16 (1 H, dd), 3.97 (1 H, dd), 3.29 (4 H, m)[4] (MacLeod et al., 1994); ESMS calcd for $C_{10}H_{17}N_3O_8$ (M + Na^+), 330.3; found (M + Na^+), 330.1. Xylobiose and the corresponding phenol were the only reaction products of native xylanase reaction mixtures containing sodium azide.

[4] The remaining five sugar protons were buried under the buffer peaks.

Table 2: Kinetic Parameters for Aryl β-Xylobiosides with Native Xylanase and Glu172 Mutants

substrate	kinetic parameter	native	Glu172Gln	Glu172Cys	IAA-Glu172Cys	Glu172Asp
2,5-DNPX$_2$ (pK_a = 5.15)[a]	k_{cat} (s^{-1})	76[c]	0.61	1.5	3.2	4.1
	K_m (mM)	2.2[c]	0.45	0.29	2.0	2.1
	k_{cat}/K_m ($s^{-1}\cdot mM^{-1}$)[b]	35[c]	1.3	4.2	1.8	2.1
3,4-DNPX$_2$ (pK_a = 5.36)[a]	k_{cat} (s^{-1})	8.3[c]	—[d]	0.18	1.4	1.0
	K_m (mM)	3.4[c]	—[d]	1.2	3.6	1.1
	k_{cat}/K_m ($s^{-1}\cdot mM^{-1}$)[b]	2.7[c]	—[d]	0.17	0.46	0.90
ONPX$_2$ (pK_a = 7.22)[a]	k_{cat} (s^{-1})	9.6	0.62	0.40	1.0	0.25
	K_m (mM)	14.2	8.3	2.5	36	7.2
	k_{cat}/K_m ($s^{-1}\cdot mM^{-1}$)[b]	0.66	0.075	0.16	0.028	0.032
PNPX$_2$ (pK_a = 7.18)[a]	k_{cat} (s^{-1})	24	—[d]	0.018	0.41	0.16
	K_m (mM)	49	—[d]	8.6	13.4	6.4
	k_{cat}/K_m ($s^{-1}\cdot mM^{-1}$)[b]	0.43	—[d]	0.002	0.032	0.026
PhX$_2$ (pK_a = 9.99)[a]	k_{cat} (s^{-1})	0.051	—[d]	—[e]	0.017	0.002
	K_m (mM)	8.7	—[d]	—[e]	28.2	5.8
	k_{cat}/K_m ($s^{-1}\cdot mM^{-1}$)[b]	0.005	—[d]	—[e]	0.0006	0.0003

[a] The pK_a value given is for the aglycon. [b] All the k_{cat}/K_m values were determined from the slope of the Lineweaver−Burk plot. [c] Data taken from Ziser et al. (1995). [d] Not determined. [e] Enzyme-catalyzed hydrolysis was not detected.

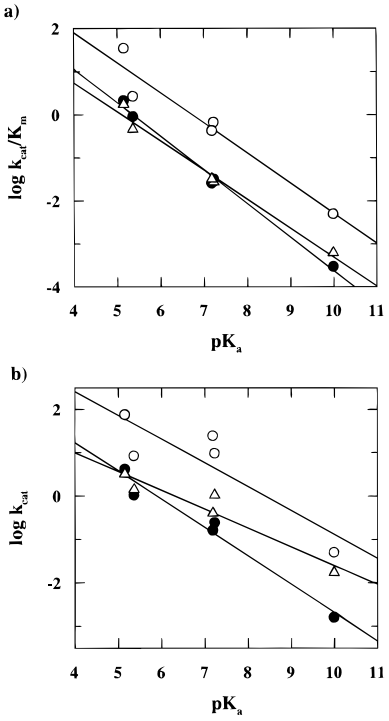

FIGURE 3: Brønsted relationships for native xylanase (○), Glu172Asp (●), and IAA-Glu172Cys (△). The data for the (a) log (k_{cat}/K_m) vs pK_a plot and (b) log k_{cat} vs pK_a plot were taken from Table 2.

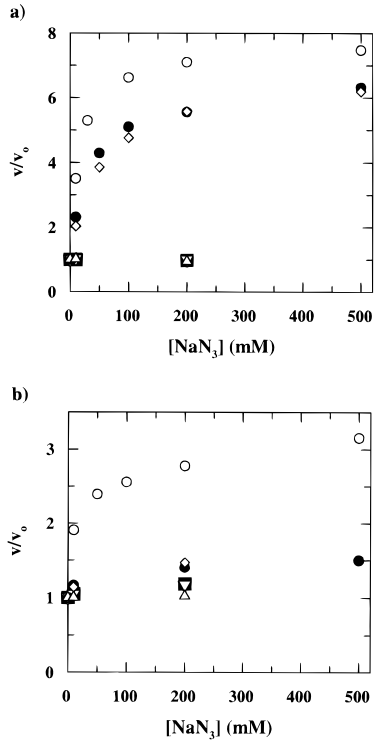

FIGURE 4: Effects of azide on xylanase-catalyzed hydrolysis rates for (a) 2,5-DNPX$_2$ and (b) ONPX$_2$. The enzymes examined were native xylanase (▽), Glu172Asp (■), Glu172Gln (○), Glu172Cys (●), and refolded IAA-treated Glu172Cys [1.1 mM (△) or 0 mM (◇) IAA].

Native xylanase-catalyzed cleavage of 2,5-DNPX$_2$ was also conducted in the presence of the neutral nucleophiles MeOH, DTT, and β-mercaptoethanol. No rate increases were observed; instead, the nucleophiles exhibited a slight inhibitory effect at higher concentrations (data not shown).

pH Dependence of k_{cat}/K_m. The k_{cat}/K_m values for the hydrolysis of ONPX$_2$ by native xylanase and the Glu172 mutants were determined over a range of pH values. The pH studies were performed at 25 °C since the Glu172 mutants are relatively unstable at 40 °C for extended periods of time. Even at this reduced temperature, activity loss was observed for Glu172Asp at the more acidic pH values; thus, only k_{cat}/K_m values for pH 4.5−9 were determined. The resulting k_{cat}/K_m versus pH plots, presented in Figure 5, yielded the following pK_a values: pK_{a1} = 4.6 ± 0.04, pK_{a2} = 6.8 ± 0.03 for native xylanase; pK_{a1} = 4.2 ± 0.13, pK_{a2} = 8.0 ± 0.06 for Glu172Asp; pK_{a1} = 4.0 ± 0.23 for Glu172Cys; pK_{a1} = 3.2 ± 0.08, pK_{a2} = 6.7 ± 0.07 for IAA-Glu172Cys.

DISCUSSION

The importance of the acid/base catalyst, Glu172, is clearly shown by the fact that replacement of this residue by a group with no significant capacity as a proton donor/acceptor (Gln or Cys) results in an enzyme which cannot cleave xylan, a substrate which requires acid catalysis (Wakarchuk et al., 1994). However, these mutants were able to hydrolyze aryl xylobiosides with relatively good leaving groups which need little acid catalysis, as might be expected if Glu172 functions as an acid catalyst. Thus, k_{cat}/K_m values, which reflect the first irreversible step, glycosylation, were decreased only 8−30-fold for substrates of aglycon pK_a < 5.5. Greater

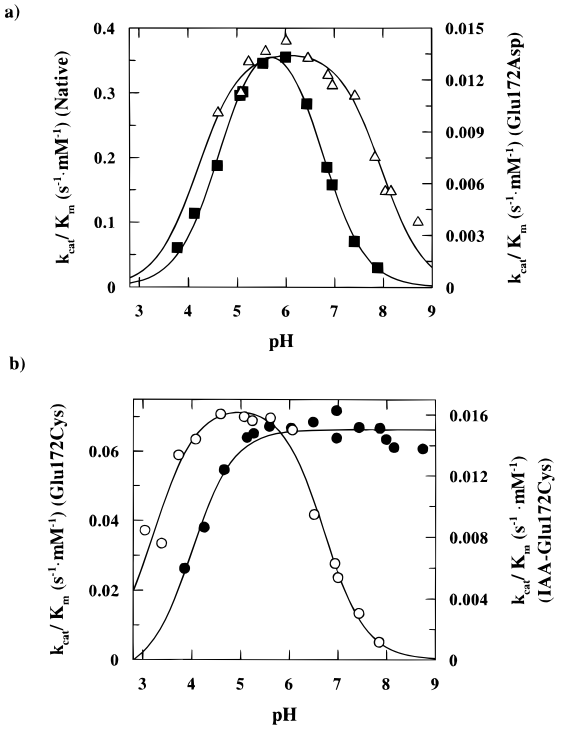

FIGURE 5: pH dependence of k_{cat}/K_m for (a) native xylanase (■) versus Glu172Asp (△) and (b) Glu172Cys (●) versus IAA-Glu172Cys (○). The lines shown for native, Glu172Asp, and IAA-Glu172Cys represent fits to data for enzymes with two ionizable groups. For Glu172Cys, the line shown represents a fit to data for an enzyme with a single ionizable group.

differences were observed for substrates of higher aglycon pK_a, with no hydrolysis being detected for phenyl xylobioside (pK_a = 9.99).

When a carboxyl group is present, even if improperly positioned, xylan hydrolysis rates are much closer to those for the wild-type enzyme, the k_{cat} values for IAA-Glu172Cys and Glu172Asp being reduced only 25- and 400-fold, respectively, much less than that caused by complete removal of the carboxyl side chain. The side chain of IAA-Glu172Cys appears to be better positioned than that of Glu172Asp, which must be displaced by at least 1 Å. This presumably reflects the greater flexibility of the longer side chain, though clearly a price is paid for this in decreased binding as shown by the elevated K_m value. Interestingly, these differences between IAA-Glu172Cys and Glu172Asp essentially disappear when the enzyme is assayed with the aryl xylobiosides, presumably because of their decreased need for protonic assistance. Rates measured for these two enzymes were generally greater than for the Gln and Cys mutants, again showing that a mispositioned acid catalyst is better than none at all.

The absence of an acid/base catalyst can, in part, be compensated for by the addition of the anionic nucleophile, azide, to the reaction buffer. As shown in Figure 4a, large increases in k_{cat} for 2,5-DNPX_2 were observed with Glu172Gln and Glu172Cys as the azide concentration was increased. Further, analysis of reaction mixtures revealed a new product, its ^{1}H NMR spectrum and ESMS data being consistent with those of β-xylobiosyl azide. No such rate increases are seen with either wild-type enzyme or Glu172Asp upon addition of azide, nor is any sugar product other than xylobiose formed. These results are consistent with earlier findings for the *C. fimi* exoglycanase (MacLeod et al., 1994), for *Agrobacterium faecalis* β-glucosidase (Wang et al., 1995), and for *E. coli* β-galactosidase (Huber & Chivers, 1993). For these enzymes, the rate enhancements were shown to be due to azide reacting with the glycosyl-enzyme intermediate more rapidly than does water, thus resulting in increased steady-state rates when deglycosylation is the rate-determining step. Similar, but smaller rate increases were seen for $ONPX_2$ hydrolysis by Glu172Gln, but not for Glu172Cys, suggesting that deglycosylation remains rate-limiting for the Gln mutant with this substrate, but that glycosylation has become rate-limiting with the Cys mutant. This is consistent with the observed k_{cat} values for these two substrates which are identical for Glu172Gln (0.61 s^{-1}), but which differ by a factor of 3.7 for Glu172Cys and by much more when $PNPX_2$ is used as a substrate.

The absence of any azide-induced rate increase with wild-type enzyme, Glu172Asp, and IAA-Glu172Cys, along with the identification of only xylobiose as the reaction product and not xylobiosyl azide, is presumably a consequence of charge screening by the anionic carboxyl group at position 172, inhibiting the access of azide. The fact that azide effects are seen for Glu172Cys, with the substrate 2,5-DNPX_2, strongly suggests that the cysteine side chain is not deprotonated under the assay conditions (pH 6). Further, the absence of azide effects with IAA-Glu172Cys provides additional evidence for the successful carboxymethylation of the cysteine residue.

No information regarding the identity of the rate-determining step for the wild-type enzyme or the Glu172Asp and IAA-Glu172Cys mutants can be gleaned from the azide data. For this, it is necessary to consider the Brønsted relationships shown in Figure 3. Good correlations of log (k_{cat}/K_m) with aglycone leaving group ability are seen for these three enzymes with a range of aryl xylobioside substrates, consistent with the notion that k_{cat}/K_m reflects the first irreversible step, glycosylation, since this step involves sugar−aryl bond cleavage. Very similar β_{1g} values are seen for these three enzymes, indicating that the placement of the acid catalyst does not seriously affect the degree of proton donation at the transition state. Even though the data obtained for k_{cat} are more scattered, some correlation is nonetheless observed in all cases. The glycosylation step appears, therefore, to be not only the first irreversible step, but also the overall rate-determining step for these three enzymes. The hydrolysis of 2,5-DNPX_2 by wild-type enzyme did not yield a pre-steady-state phenolate burst, nor were rate enhancements observed upon the addition of neutral nucleophiles. These results are consistent with glycosylation being the rate-determining step for wild type-enzyme with 2,5-DNPX_2.

Modifying the carboxyl side chain at position 172 had interesting effects on the pH dependence of the reaction, as seen in Figure 5. Ionizations in the plots of k_{cat}/K_m versus pH are due to groups in the free enzyme (Fersht, 1985). The pK_a values of 4.6 and 6.8 seen for wild-type xylanase have been shown by ^{13}C NMR studies to be due to Glu78 and Glu172, respectively (McIntosh et al., 1996). Upon substitution of Glu172 by Asp, very little change is observed in pK_{a1}, that of Glu78, as might be expected since this replacement results in only a small change in local charge distribution. However, an increase of just over 1 unit in pK_{a2}, that of Glu/Asp 172, is observed. Since electrostatic effects should diminish upon pulling this carboxyl group further away from the anionic Glu78, this change is opposite to that expected.

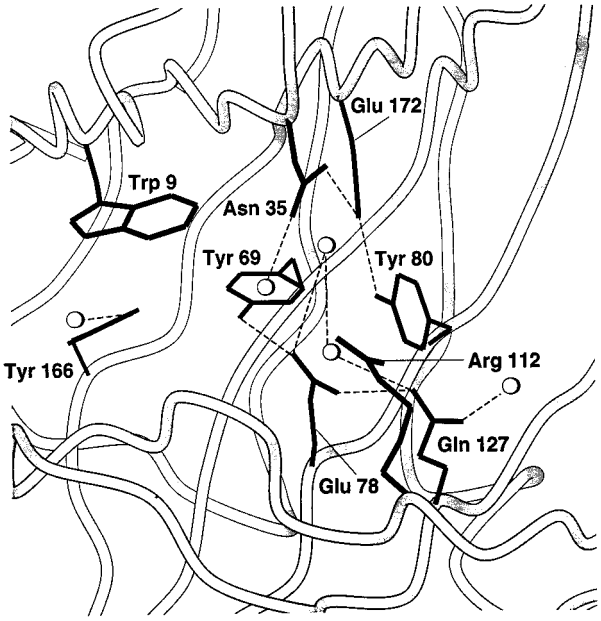

FIGURE 6: Active site of *Bacillus circulans* xylanase, showing the key residues and hydrogen bonding interactions. The nucleophile (Glu78) and acid/base catalyst (Glu172) are located on opposite sides of the active site cleft. Hydrogen bonds are shown as dashed lines while the spheres represent water molecules.

Rather, it suggests either the loss of some hydrogen bonding interactions which ordinarily lower the pK_a of Glu172, presumably by stabilizing the anion, or a closer positioning of Asp172 to some other anionic or hydrophobic group. The X-ray crystal structure of native xylanase (refer to Figure 6) reveals the formation of hydrogen bonds between Glu172 and two active site residues, Asn35 and Tyr80 (Campbell et al., 1993; Wakarchuk et al., 1994). Shortening Glu172 to an aspartic acid likely weakens or completely destroys these hydrogen bonds, thereby causing an elevation in pK_{a2}. Further, placement of the carboxyl group in a less polar environment would have similar consequences. Similar increases in the pK_a of Glu172 have been observed by ^{13}C NMR upon the mutation of residues involved in hydrogen bonding to Glu172 and Glu78 (McIntosh and Joshi, unpublished results).

Lengthening of the acid/base catalyst (IAA-Glu172Cys) has a reciprocal effect on the active site pK_a values, the pK_a of Glu78 (pK_{a1}) being depressed just over 1 pH unit while that of Glu/IAA-Cys172 stays approximately constant. Since the intrinsic pK_a value of carboxymethylated cysteine is expected to be about 1 pH unit lower than that of glutamic acid, based upon the pK_a values for the analogous carboxylic acids $CH_3CH_2CO_2H$ (pK_a = 4.88) and $CH_3SCH_2CO_2H$ (pK_a = 3.72) (Brown et al., 1955), in the absence of other effects a *decrease* of pK_{a2} would have been expected. Thus, the absence of any change suggests the presence of a countering effect which has increased pK_{a2} by approximately 1 unit, with the net result of no change. The simplest explanation is that lengthening of the side chain allows the two essential carboxyl groups to hydrogen bond to each other, most likely via an intervening water molecule. This would lower pK_{a1}, since the monoanion will be relatively stabilized, but raise pK_{a2}. Molecular modelling of IAA-Glu172Cys reveals that, in its fully extended conformation, the side chain carboxyl group of IAA-Cys172 comes within 3.8 Å of Glu78, a distance too great for direct hydrogen bonding. A candidate water molecule for the postulated hydrogen bonding network is that seen hydrogen bonded to Glu78 in the structure shown in Figure 6. Extension of the side chain at position 172 would allow the acid/base carboxyl group to also hydrogen bond to this water molecule, completing the hydrogen bonding network. An alternative explanation for the pK_a results is that lengthening the acid/base catalyst alters the conformation of the active site, placing Glu78 in an environment more capable of stabilizing a carboxylate anion, while positioning IAA-Cys172 in a less favorable environment.

Replacement of Glu172 with a cysteine results in the abolition of the basic limb of the pH profile, as expected, along with a slight decrease in the pK_a of Glu78. This is completely consistent with our observation that Cys172 provides no acid catalysis, as evidenced by the similarity of k_{cat} values for Glu172Cys and Glu172Gln, a mutant incapable of providing acid catalysis. It also agrees with the conclusion that Cys172 remains protonated in the glycosyl-enzyme intermediate, as revealed by the azide effects observed with the substrate 2,5-DNPX_2.

CONCLUSION

In *B. circulans* xylanase, a group at position 172 capable of effective proton transfer is essential for hydrolysis of substrates with leaving groups which require acid catalysis, such as phenyl xylobioside and the natural substrate xylan. However, such a group is not essential for substrates with good leaving groups needing no protonic assistance (pK_a < 5.5). General base catalysis appears to be less important for this enzyme than for other glycosidases such as *A. faecalis* β-glucosidase. Removal of the general acid/base catalyst decreased k_{cat} values, for substrates with good leaving groups (pK_a < 5.5) by 2000-fold for *A. faecalis* β-glucosidase (Wang et al., 1995), as opposed to only 50−125-fold for *B. circulans* xylanase. Despite the rate reduction, the mechanism employed by *B. circulans* xylanase remained unchanged as indicated by the similarity of the Brønsted plots, by the low K_m values observed using 2,5-DNPX_2, consistent with the accumulation of a xylobiosyl-enzyme intermediate, and by the trapping of the 2-fluoroxylobiosyl-enzyme intermediate in the Glu172Gln mutant (McIntosh et al., 1996).

The precise placement of the acid/base catalyst in *B. circulans* xylanase is not critical since either shortening or lengthening this carboxyl side chain results in approximately the same modest decrease in k_{cat}/K_m values (1000-fold for the natural substrate, xylan, and 3−23-fold for the aryl xylobiosides). This is in sharp contrast with the positional requirements of the catalytic nucleophile Glu78. Shortening the nucleophilic side chain (Glu78Asp) decreased k_{cat}/K_m values at least 1600-fold for the aryl xylobiosides and much more for xylan, whereas increasing the length (IAA-Glu78Cys) decreased the k_{cat}/K_m values for the aryl xylobiosides by 16−100-fold (Lawson et al., 1996; Wakarchuk et al., 1994). Thus, as expected, the positional requirements for proton transfer are less demanding than those for carbon−oxygen bond formation.

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