The pKa of the General Acid/Base Carboxyl Group of a Glycosidase Cycles during Catalysis: A 13C-NMR Study of Bacillus circulans Xylanase†

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ABSTRACT: The 20 kDa xylanase from Bacillus circulans carries out hydrolysis of xylan via a two-step mechanism involving a covalent glycosyl–enzyme intermediate. In this double-displacement reaction, Glu78 functions as a nucleophile to form the intermediate, while Glu172 acts as a general acid catalyst during glycosylation, protonating the departing aglycone, and then as a general base during deglycosylation, deprotonating the attacking water. The dual role of Glu172 places specific demands upon its ionization states and hence pKa values. 13C-NMR titrations of xylanase, labeled with [δ-13C]glutamic acid, have revealed pKa values of 4.6 and 6.7 for Glu78 and Glu172, respectively. These agree well with the apparent pKa values obtained from a study of the pH dependence of kcat/Km and demonstrate that, at the enzyme’s pH optimum of 5.7, the nucleophile Glu78 is deprotonated and the general acid Glu172 initially protonated. Remarkably, the pKa for Glu172 drops to 4.2 in a trapped covalent glycosyl–enzyme intermediate, formed by reaction with 2′,4′-dinitrophenyl 2-deoxy-2-fluoro-β-xylobioside [Miao et al. (1994) Biochemistry 33, 7027–7032]. A similar pKa is measured for Glu172 when a glutamine is present at position 78. This large decrease in pKa of ~2.5 units is consistent with the role of Glu172 as a general base catalyst in the deglycosylation step and appears to be a consequence of both reduced electrostatic repulsion due to neutralization of Glu78 and a conformational change in the protein. Such “pKa cycling” during catalysis is likely to be a common phenomenon in glycosidases.

Hydrolysis of glycosidic bonds by glycosidases typically follows one of two possible mechanisms, distinguished by the observed stereochemical outcome (Koshland, 1953; Sinnott, 1990; McCarter & Withers, 1994). Inverting glycosidases hydrolyze the bond with net inversion of anomeric configuration via a mechanism involving general base-catalyzed attack of water on the anomeric center of the substrate, coupled with general acid-catalyzed cleavage of the glycosidic bond. The active site residues responsible for this acid/base catalysis have proven to be a pair of carboxylic acids (Glu or Asp) in essentially all cases investigated to date (Svensson, 1994; Davies & Henrissat, 1995; Withers & Aebersold, 1995). Retaining glycosidases employ a double-displacement mechanism in which a covalent glycosyl–enzyme intermediate is formed and hydrolyzed via oxocarbenium ion-like transition states, as shown for a xylanase in Figure 1. Again, a pair of carboxylic acids is involved, but in this case, one of these residues serves as a nucleophile in the first step, the formation of the intermediate, and then as a leaving group in the second hydrolysis step. The other carboxyl group is required to play a dual role, functioning as a general acid in the glycosylation reaction, and then as a general base to facilitate deglycosylation. Such a dual role places specific demands upon the enzyme to control the ionization state of the general acid/base residue at each step in catalysis. An understanding of how this control is effected is essential for a complete description of the mechanism of retaining glycosidases.

Studies of the pH dependence of the kinetic parameters of the double-displacement enzymatic reaction provide some insights into this question, and indeed, a considerable number of such analyses have been reported (Kempton & Withers, 1992; Tull & Withers, 1994). However, interpretation of kinetic data on its own is not trivial, having many pitfalls as described elsewhere (Knowles, 1976; Brocklehurst, 1994). Thus, it is critical to measure directly the pKa values of the catalytic residues in these enzymes. Perhaps the most reliable and unambiguous method for such measurements is to use NMR spectroscopy to monitor titrations of groups within the enzyme and thereby determine their individual ionization constants. This approach has, for example, confirmed the pKa values assigned to the catalytic carboxyls of native and chemically or genetically modified hen egg white lysozymes that were determined by potentiometric titrations or through the use of optical spectroscopy (Parsons
The postulated oxocarbenium transition states (Double-displacement mechanism of retaining glycosidases. The nucleophilic carboxylate attacks the glycosidic bond of the general base in the glycosyl-enzyme intermediate species. No study has been performed to measure the ionization constant of the general base in the glycosyl-enzyme intermediate species.

The xylanases from Bacillus circulans (BCX) and subtilis (BSX) provide an ideal system to address this key question. These highly related enzymes are small (20 kDa) retaining glycosidases, responsible for the degradation of xylan, a 1,4-linked polymer of xylose (Gebler et al., 1992). The three-dimensional structures of both native BCX and a catalytically inactive mutant enzyme-substrate/inhibitor complex have been determined by X-ray crystallography (Figure 1; Campbell et al., 1993; Wakarchuk et al., 1994). The kinetic properties of the wild type and numerous site-directed mutants of these xylanases toward natural and synthetic substrates have been well-characterized (Wakarchuk et al., 1992, 1994). The three-dimensional structures of both native BCX and a catalytically inactive mutant enzyme-substrate complex have been determined by X-ray crystallography (Figure 1; Campbell et al., 1993; Wakarchuk et al., 1994). Furthermore, the NMR spectrum of the B. circulans enzyme has been assigned extensively (Plesniak et al., 1996). The active site of BCX contains two glutamic acid residues, at positions 78 and 172, that are suitably disposed for catalysis (6.5 Å from Cα to Cα; McCarter & Withers, 1994; Davies & Henriissat, 1995). Fortuitously, these are the only glutamic acid residues in the enzyme and mutation of either severely disrupts catalysis. Chemical modification studies with the homologous Schizophyllum commune xylanase A indicated that a glutamic acid residue is the nucleophile (Bray & Clark, 1994) and that one of the essential acidic residues has an elevated pK_a, suggestive of it being the acid catalyst in the reaction (Bray & Clarke, 1990). Similar results have been reported for Bacillus pumilis xylanase (Ko et al., 1992). The specific assignment of the Glu78 as the nucleophile in BCX was substantiated by kinetic analyses of site-directed mutants modified at this position (Wakarchuk et al., 1994) and confirmed by detection of a trapped glycosyl-enzyme intermediate by electrospray mass spectroscopy, followed by sequencing of the purified glycopeptide (Miao et al., 1994). Therefore, Glu172 functions as the acid/base catalyst in BCX.

Trapping of the glycosyl-enzyme intermediate was achieved by the use of a 2-deoxy-2-fluoro-β-xylobioside with a good leaving group, 2,4-dinitrophenolate. The presence of the fluorine substituent results in destabilization of the transition states for glycosylation and deglycosylation on two accounts. First, the great electronegativity of the fluorine inductively destabilizes the oxocarbenium ion-like transition states. Second, the limited hydrogen-bonding capabilities of the fluorine ensure that full transition state stabilization is not achieved (Street et al., 1992; Namchuck & Withers, 1995; Withers & Aebersold, 1995). In this way, both the formation and the hydrolysis of the intermediate are compromised. However, the presence of the 2,4-dinitrophenolate leaving group ensures that the formation of the intermediate is faster (t1/2 ~ 1 min) than its hydrolysis (t1/2 ~ days), and thus, the glycosyl-enzyme intermediate accumulates.

This study describes the use of 13C-NMR spectrometry combined with selective 13C labeling to determine the pK_a values of the two active site glutamic acid residues in wild type and mutant B. circulans and subtilis xylanases and in...
the trapped glycosyl—enzyme intermediate. The results are discussed in light of the pH dependence of the enzymatic reaction of retaining glucosidases and the dual role of the general acid/base carboxyl residue in catalysis.

MATERIALS AND METHODS

Protein Expression and Isotopic Labeling. The genes encoding BCX and BSX were cloned into the pCW plasmid system under control of an inducible tac promoter (Sung et al., 1993; Wakarchuk et al., 1994). BCX and BSX differ only through the replacement of threonine 147 by serine. These side chains are exposed on the surface of the protein, and the substitution has no effect on the activities or stabilities of the two proteins (Wakarchuk et al., 1992). Although the crystal structure of BCX was solved recently (Campbell et al., 1993), the clones for the xylanase mutants E78Q and E172Q were available in the BSX background. Accordingly, for these NMR and enzymological studies, the two xylanases are viewed as equivalent.

Unlabeled xylanase was produced as described previously (Sung et al., 1993). Xylanases, 13C-enriched in the side chain δ-carboxyl and -amides of the glutamate and glutamine residues, respectively, were prepared using the general transaminase-deficient Escherichia coli strain DL39 avrA::Tn5 (LeMaster & Richards, 1988). The bacteria were grown at 30 or 37 °C in a synthetic medium (Muchmore et al., 1990; Anderson et al., 1993) containing 300 mg/L 99% l-[δ-13C]glutamate (Tracer Technology, Cambridge, MA). This auxotrophic E. coli strain was utilized to reduce the possibility of dilution of the glutamate label by transamination reactions. Since suitable E. coli strains with lesions to prevent the metabolic interconversion of glutamate and glutamine are unavailable, unlabeled glutamine (400 mg/L) was also added to the growth media for the preparation of wild type BCX protein. Unfortunately, this did not prevent the conversion of the [δ-13C]glutamate to [δ-13C]glutamine, and thus, glutamine was omitted from the media in all subsequent protein preparations.

Protein Characterization. The xylanases were >90% pure as judged by SDS-PAGE and Coomassie staining. Wild type and mutant xylanases, E78Q and E172Q, were screened by proteolytic digestion using V-8 protease, which cleaves at carboxyl-containing residues, to confirm the number and positions of the glutamate residues in each protein. The molecular masses of the proteins were measured using electrospray mass spectroscopy, and the following results, with standard deviations, were obtained: wild type BCX, observed 20 395 ± 3.4 Da, expected 20 396 Da; [δ-13C]Glu- and -Gln-labeled wild type BCX, observed 20 400 ± 2.3 Da, expected 20 403 Da (assuming ~100% 13C enrichment of seven residues); [δ-13C]Glu- and -Gln-labeled E78Q BSX, observed 20 387 ± 3.6 Da, expected 20 388 Da; and [δ-13C]Glu- and -Gln-labeled E172Q BSX, observed 20 381 ± 2.8 Da, expected 20 388 Da. The deviations between the observed and expected molecular masses of the labeled proteins may reflect isotopic dilution of the [δ-13C]glutamate.

Enzymatic Characterization. Kinetic parameters for the hydrolysis of ONPxb by wild type BCX were measured as a function of pH at 25 °C, as outlined previously (Lawson et al., 1996). Three different buffer systems, each containing 50 mM NaCl, were used to cover the pH ranges of 3.0–5.0 (20 mM succinic acid), 5.0–7.0 (20 mM MES), and 7.0–8.5 (20 mM HEPES). The Michaelis–Menten parameters and apparent pK, values describing the dependence of kcat/Km upon pH were determined with the program GraFit (Leatherbarrow, 1990).

13C-NMR. The 13C-enriched xylanase samples were dialyzed into 25 mM sodium phosphate, 3 mM NaCl, and 10% D2O/90% H2O, at pH* ~7.5. The samples contained 0.5–0.75 mM protein in 1.7–2.5 mM of buffer. One-dimensional 13C-NMR spectra were recorded at 25 °C with a Varian UNITY spectrometer using a 10 mm broad-band probe operating at 125.7 MHz for carbon. The apparent T1 lifetimes of the glutamate and glutamine δ-carboxyls in wild type BCX ranged from 2.3 to 3.7 s, as measured with a fast inversion recovery experiment (not shown). For comparison, the T1 of the δ-13C of free glutamate is 11.5 s at pH* 2.15 and 25 °C. On the basis of these lifetimes, the 13C spectra of the proteins were recorded using an 8.8 µs (55°) pulse, and collecting 1024 complex points over a spectral width of 4000 Hz, with an acquisition time of 0.256 s and a recycle delay of 1.0 s. Broad-band WALTZ-16 H decoupling, at a field strength of 840 Hz and centered at ~2.5 ppm for protons, was applied during the acquisition period only. Generally, 500–3000 transients were collected per spectrum. The data were processed with FELIX 2.3 ( Biosym Inc.) using mild exponential line broadening and 16-fold zero-filling. Chemical shifts were referenced to an external sample of DSS at 0.0 ppm.

The 13C-NMR spectra of wild type BCX and E172Q BSX modified with DNP2FXb were recorded as outlined above. The 13C-labeled wild type BCX was initially 0.8 mM in 1.7 mL of 25 mM sodium phosphate, 3 mM NaCl, and 10% D2O, at pH* 5.81 at 25 °C. Solid DNP2FXb (1.7 mg, or 2.2 mM final) was added directly to the protein sample. Upon mixing, the solution turned yellow due to the released 2,4-dinitrophenol group. The covalent modification of BCX was complete within approximately 15 min, as judged by 13C-NMR measurements, and was stable for a period of several days. The inhibition of the enzyme was confirmed by >99% loss of activity toward the substrate analog ONPxb, by mass spectrometry (observed 20 672 ± 1.8 Da, expected 20 669 Da) and by 15N-NMR. A similar procedure was used with 13C-labeled E172Q BSX, except that the protein was initially 1.3 mM at pH* 7.51.

pK, Measurements. The pK, values of the glutamic acid residues were determined by measuring the 13C-NMR spectra of the [δ-13C]Glu- and -Gln-labeled proteins as a function of pH* at 25 °C. The pH* of the sample was recorded in the 10 mm NMR tube using a microelectrode (Ingold). The pH was decreased by the addition of microliter aliquots of 0.2 N HCl and, if necessary, raised by dialysis against alkaline sample buffer to avoid possible aggregation resulting from the direct addition of base. The pK, values were determined by a nonlinear least squares fitting of the observed chemical shifts as a function of pH to the equations for titrations involving one or two ionizable groups (Shrager et al., 1972) using the programs PlotData (Triumf, University of British Columbia) and NonLin (Johnson, 1994). The reported standard errors reflect the precision of the data fitting and do not include the possible errors in the sample pH that are estimated to be ±0.1 unit. A detailed discussion of the data analysis will be provided elsewhere. In this manuscript, the term “apparent pK,” or simply “pK,” refers to the phenomenological ionization constant describing the depen-
dence of enzyme activity or chemical shift upon pH, whereas “microscopic” and “macroscopic” pK\textsubscript{a} refer to explicit models such as the deprotonation of a single or two coupled acidic groups (Edsell & Wyman, 1958).

RESULTS

Enzymatic Analysis. The enzymatic activity of wild type BCX against the soluble substrate analog ONPXb was measured as a function of pH at 25 °C. As shown in Figure 2A, k\textsubscript{cat}/K\textsubscript{M} follows a classic bell-shaped curve with maximal activity near pH 5.7. The pH dependence of k\textsubscript{cat}/K\textsubscript{M} can be fit adequately to a model involving two ionizable catalytic residues with apparent pK\textsubscript{a} values of 4.6 and 6.7 in the free enzyme. On the basis of previous studies of BCX and retaining glycosidas in general, we attribute the lower pK\textsubscript{a} to Glu78, which must be deprotonated to function as a nucleophile, and the higher pK\textsubscript{a} to Glu172, which must remain protonated to act as a general acid (Wakarchuk et al., 1992, 1994; Miao et al., 1994).

\textsuperscript{13}C-NMR Spectra of Xylanase. The \textsuperscript{13}C-NMR spectrum of labeled wild type BCX is shown in Figure 2B. Seven \textsuperscript{13}C peaks are observed, arising from the δ-carbons of the two glutamate (Glu78 and -172) and five glutamine (Gln7, -127, -133, -167, and -175) residues in the protein. The peaks were assigned specifically using a variation of the three-dimensional CBCACO(CA)HA experiment that provided correlations between the C/\textsuperscript{13}C, C/\textsuperscript{15}N, and H/\textsuperscript{15}N resonances of each Glu and Gln residue in the sample of uniformly \textsuperscript{13}C-enriched BCX (supporting information; Kay, 1993). This allowed us to classify the side chain carbonyl peaks using the previously determined H- and \textsuperscript{13}C-NMR assignments of BCX (Plesniak et al., 1996). These assignments were confirmed by the selective substitutions of Glu for Glu78 or Glu172 (Figure 2E,F).

pH Dependence of the \textsuperscript{13}C-NMR Spectrum of BCX. The pK\textsubscript{a} values of Glu78 and Glu172 in wild type BCX were determined from the pH dependence of the \textsuperscript{13}C-NMR spectrum of this labeled protein (Figure 2B,D). With decreasing pH, the peaks assigned to these two glutamate side chains shift upfield by ~2.8 ppm (tabulated in the supporting information). This upfield shift is consistent with that expected for the protonation of a carboxylate group (Batchelor et al., 1975; Rabenstein & Sayer, 1976; Gu et al., 1994). Also, as noted previously, the chemical shifts of ionized Glu carboxylates are generally downfield from those of the Gln carbonyls, while the protonated carboxylic acids have chemical shifts similar to those of the amides (Anderson et al., 1993). The pronounced pH-dependent chemical shifts of the two glutamates undoubtedly reflect the protonation/deprotonation of their own side chain carboxylates, as opposed to indirect effects arising from the ionization of adjacent residues. Therefore, the apparent pK\textsubscript{a} values of Glu78 and Glu172 are 4.6 ± 0.02 and 6.7 ± 0.02, respectively (Figure 3). The resonance from Glu172 is also exchange broadened at pH conditions near its pK\textsubscript{a}. This may result from retarded kinetics of protonation of the side chain due to its structural environment within the protein or from a conformational change coupled to ionization that occurs on a time scale of intermediate chemical exchange.

Closer inspection of the data in panels B and D of Figure 2 reveals that both Glu78 and Glu172 follow biphasic titrations. In addition to the major change of ~2.8 ppm, Glu78 shifts upfield by an additional 0.33 ppm with an apparent midpoint pK\textsubscript{a} of 6.5 ± 0.2, while Glu172 shows a comparable upfield shift of 0.44 ppm corresponding to an apparent pK\textsubscript{a} of 4.6 ± 0.1. The reciprocal correlation of the two apparent pK\textsubscript{a} values determined for Glu78 and Glu172 suggests strongly that the chemical shifts of these residues are coupled to one another in a pH-dependent manner. Consistent with this conclusion, monophasic titrations are observed in the BSX variants with single glutamate residues (Figure 2E,F).

As discussed by Shrager et al. (1972), two ionizable groups may show coupled or biphasic spectral changes during a pH titration if (i) the chemical shift or (ii) the microscopic pK\textsubscript{a} of one group is dependent upon the ionization state of the other. In the first case, the chemical shift of the carboxyl may be influenced directly by electric fields that perturb the shielding of the \textsuperscript{13}C nucleus or indirectly through conformation changes that, for example, alter hydrogen-bonding interactions. In the absence of detailed structural information, the expected magnitudes and directions of these possible pH-dependent chemical shift perturbations are difficult to predict (Batchelor et al., 1975; Gu et al., 1994). However, inspection of the crystallographic structure of BCX reveals that the δ-carbons of the two catalytic residues are separated by 6.5 Å, and thus, these glutamates should interact electrostatically, as in the classical example of a dibasic acid (Edsell & Wyman, 1958). This corresponds to case (ii), in which the microscopic pK\textsubscript{a} of one glutamic residue is dependent upon the ionization state of the other, such that the full chemical shift change due to protonation occurs only upon the neutralization of both groups.

An analysis of the pH dependence of the \textsuperscript{13}C-NMR spectra of the wild type enzyme using a model of two coupled ionization equilibria, yet independent chemical shifts for Glu78 and Glu172 (Shrager et al., 1972), yields the microscopic pK\textsubscript{a} values summarized in Scheme 1. From a simultaneous fit of the two titration curves, the pK\textsubscript{a} of Glu78 is 4.63 ± 0.03 when Glu172 is neutral and 5.79 ± 0.08 when it is negatively charged. Similarly, the pK\textsubscript{a} of Glu172 is 5.50 ± 0.10 when Glu78 is neutral and 6.66 ± 0.04 when it is charged. Therefore, by this model, the mutual electrostatic repulsion between the carboxylate groups elevates each pK\textsubscript{a} by ~1.2 units. The lower ionization pathway in Scheme 1 is favored significantly, and thus, the microscopic pK\textsubscript{a} values of 4.63 (Glu78) and 6.66 (Glu172) are essentially equal to the macroscopic or apparent pK\textsubscript{a} values assignable to these two groups (Edsell & Wyman, 1958).

Covalent Modification of Wild Type BCX with DNP2FXb. The mechanism-based inhibitor (or “slow substrate”) DNP2FXb forms a long-lived covalent glycosyl−enzyme intermediate with Glu78 in wild type BCX (Miao et al., 1994). Such covalent modification of xylanase is evident by the marked change in the \textsuperscript{13}C-NMR spectrum of the isotopically enriched protein upon reaction with this compound. As illustrated in Figure 2, the \textsuperscript{13}C chemical shifts of Glu78 and -172 and Gln7, -127, and -175 all differ relative to those in the native protein. A new upfield-shifted peak at 175.3 ppm is attributed to the covalently modified Glu78. Even after extensive dialysis to remove unreacted inhibitor, the \textsuperscript{13}C-NMR spectrum of the modified BCX remained unchanged. However, consistent with previous kinetic studies of the reactivation process, slow hydrolysis of the glycosyl−enzyme adduct did occur over a period of several
Figure 2: (A) Dependence of $k_{cat}/K_m$ upon pH for hydrolysis of ONPXb by BCX at 25 °C, fit to the kinetic expression for two ionizable groups with apparent $pK_a$ values of 4.6 and 6.7 (line). (B) $^{13}$C-NMR spectra of wild type BCX recorded as a function of pH at 25 °C. The peaks corresponding to Glu78 and Glu172 are highlighted in black to emphasize the titrations of these two residues. (C) $^{13}$C-NMR spectra of wild type BCX covalently modified with DNP2FXb at position 78. The assignments of the spectra of the modified protein are extrapolated from the those in panel B, and E78* denotes the resonance from the glycosylated Glu78. The peaks corresponding to Glu172 are shown in solid black to emphasize the titration of this residue. (D) The apparent $pK_a$ values describing the pH dependence of the $\delta$-$^{13}$C chemical shifts of the two Glu and five Gln residues in native BCX (circles) and Glu172 in the glycosyl–enzyme intermediate (triangles; labeled E172*) were determined by fitting the data from the spectra in panels A and B to the equations for titrations involving one or two ionizable groups. (E and F) $^{13}$C-NMR spectra of E172Q and E78Q BSX, respectively, recorded as a function of pH at 25 °C. The assignments are extrapolated from those determined for the wild type protein, and the peaks from Glu78 and Glu172 are shown in solid black. In E78Q BSX, although Glu172 appears to deviate from a monophasic titration, curve fitting to the equation for a biphasic titration does not yield a reliable second apparent $pK_a$. 

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in the spectrum of the mutant xylanase, while a new peak, arising from the introduced glutamine side chain, was detected (Figure 2E,F). In addition, the resonances of Glu78, -127, and -175 were perturbed by the mutations, as expected due to their sensitivity to the ionization states of Glu78 and -172 in the wild type protein.

The chemical shift of Glu78 in the E172Q protein, with Glu172 mutated to glutamine, follows a monophasic titration with a pKₐ of 5.0 ± 0.01 (Δδ = −3.93 ppm). The pKₐ of this catalytic residue is thus elevated by ~0.4 unit relative to wild type xylanase. This result is unexpected if Glu78 and Glu172 are coupled solely by electrostatic interactions, because, by Scheme 1, the pKₐ of Glu78 should remain near 4.6 in the presence of a neutral side chain at position 172. Therefore, the introduction of Glu172 may perturb the environment of Glu78. In a reciprocal manner, the resonance of Glu78 shifts downfield by 0.5 ppm with an apparent pKₐ of 5.1 ± 0.02, indicating that its δ-¹³C chemical shift is dependent upon the ionization state of Glu78.

The substitution of Glu78 to Gln dramatically alters the pKₐ of Glu72. In the mutant E78Q, Glu172 follows a titration curve with a pKₐ of 4.2 ± 0.02 (Δδ = −3.81 ppm). Again, exchange broadening of the δ-¹³C resonance of Glu172 is observed. This pKₐ is essentially identical to that measured for Glu72 in BCX covalently modified with DNP2FXb. Therefore, amidation or glycosylation of Glu78 has the same net effect on the ionization equilibrium of Glu172. Both of these modifications eliminate the negative charge on Glu78 and thus are expected to lead to a reduction in the pKₐ of Glu72. However, on the basis of Scheme 1, the microscopic pKₐ of Glu72 in the presence of a neutral protonated Glu78 in wild type BCX is 5.50. This is significantly higher than the pKₐ of ~4.2 in either the mutant or covalently modified wild type xylanase, suggesting that the coupling between the two catalytic glutamic acids is not purely electrostatic. Conformational changes in the active site may also help dictate the ionization behavior of Glu172. Note that the resonance of Glu78 shifts downfield (Δδ = +0.51 ppm) with an apparent pKₐ of 4.2 ± 0.04, indicating that its δ-¹³C chemical shift is sensitive to the ionization state of Glu78.

Covalent Modification of E172Q Xylanase with DNP2FXb. The inhibitor DNP2FXb will also react with E172Q BSX. This mutated xylanase contains only the nucleophilic residue Glu78 yet will form a covalent intermediate with the inhibitor since the activated 2,4-dinitrophenol leaving group does not require general acid-catalyzed assistance. The ¹³C-NMR spectrum of E172Q shows changes upon complex formation with DNP2FXb similar to those observed for the wild type protein (supporting information). However, this modified protein has no titratable glutamate residues, and thus, its ¹³C-NMR spectrum is essentially independent of pH. This important control measurement confirms the assignment of the single titrating peak in the covalently modified wild type protein to the general base catalyst Glu172. Furthermore, it demonstrates that the pH dependence of the chemical shifts of the glutamine residues in the wild type and mutant proteins (vide infra) results from the ionization of the catalytic glutamic acids and not from other unidentified residues.

pH-Dependent Chemical Shifts of the Glutamine Residues. The ¹³C-NMR chemical shifts of several nonionizable glutamine residues in BCX are pH-dependent, providing confirmation of the pKₐ values assigned to Glu78 and
Glu172, as well as insights into possible conformational changes associated with the ionization of the xylanase (Figure 2). Most strikingly, in the wild type BCX, Gln7 shifts downfield by over 2.5 ppm with decreasing pH, reporting a biphasic titration with apparent pKₐ values of 4.5 ± 0.02 (Δδ = +2.09 ppm) and 6.6 ± 0.08 (Δδ = +0.47 ppm). Similar behavior is seen with the mutants E172Q and E78Q, as well as the covalently modified wild type protein, corresponding to apparent monophasic pKₐ values of 5.0 ± 0.01 (Δδ = +2.71 ppm), 4.0 ± 0.04 (Δδ = +0.88 ppm), and 4.2 ± 0.08 (Δδ = +0.19 ppm), respectively. On the basis of the similarity of these apparent pKₐ values to those measured for the glutamate residues in the given proteins, it is likely that the chemical shift of the δ-carbonyl of Gln7 is dependent on the ionization states of Glu78 and Glu172. However, this glutamine is ∼10 Å (C⁰–C⁰) removed from either catalytic glutamic acid carbonyl and is at least 12 Å away from the γ-carboxyls of any of the seven aspartic acids in BCX. Thus, it is unlikely that the pH dependence of the chemical shift of Gln7 is due to a direct electric field effect with any titratable group in the protein that has a pKₐ below ∼8. Inspection of the crystal structure of BCX reveals that the side chain of Gln7 is stacked over the plane of the aromatic ring of Tyr166 on the edge of the active site cleft of BCX and is also hydrogen-bonded to the phenolic oxygen of Tyr5. Furthermore, Tyr166 may be weakly hydrogen-bonded to Tyr69, which in turn is strongly hydrogen-bonded to Glu78. Therefore, we postulate that the unusual upfield shift of Gln7 is due to an aromatic ring current effect and that its striking pH dependence may reflect changes in this effect associated with conformational perturbations of the active site coupled to the protonation of Glu78 and, to a lesser extent, Glu172. Less dramatically, the resonance of Gln175 is also slightly dependent upon the ionizations of two groups with apparent pKₐ values of 4.5 ± 0.3 (Δδ = +0.04 ppm) and 6.6 ± 0.07 (Δδ = +0.18 ppm) in wild type BCX. This residue, located on the same β-strand as Glu172, may be sensitive to the ionization states of the catalytic glutamic acids, possibly through conformational perturbations similar to those postulated for Gln7.

The δ-carbonyl of Gln127 shifts upfield with decreasing pH (Figure 2). In the wild type xylanase, Gln127 follows a titration with apparent pKₐ values of 4.5 ± 0.04 (Δδ = −0.76 ppm) and 6.6 ± 0.10 (Δδ = −0.32 ppm). The amide of Gln127 is hydrogen-bonded directly to the carboxyl of Glu78, and thus, it is expected that the glutamine would be sensitive to the ionization of Glu78. Consistent with this, in the E172Q variant, Gln127 reports a monophasic titration with an apparent pKₐ of 5.0 ± 0.01 (Δδ = −1.01 ppm), which is equal to the pKₐ of Glu78 in the same protein. Furthermore, in E78Q BSX or the covalently modified xylanases, both of which lack a glutamic acid at position 78, the chemical shift of Gln127 is essentially pH-independent. Thus, the δ-¹³C resonance frequency of Gln127 would appear to depend directly on the ionization of Glu78 and may show an apparent pKₐ of ∼6.6 in the wild type protein due to the interaction of Glu78 with Glu172.

Finally, the δ-¹³C chemical shift of Gln167 changes slightly with an apparent pKₐ of 3.0, whereas that of Gln133 is essentially independent of pH over the range studied. Neither of these glutamines is close to the catalytic glutamic acids (Figure 1).

**DISCUSSION**

**Correlating pKₐ Values with the Enzymatic Mechanism**

Using selective isotopic labeling and ¹³C-NMR, we have measured directly the pKₐ values of the catalytic glutamic acid residues in wild type and mutant *B. circulans* and *s. subtilis* xylanases, and in a trapped glycosyl–enzyme intermediate (Figure 3). Combined with a kinetic analysis of the pH dependence of the activity of BCX, these measurements provide key insights into the enzymatic mechanism of xylanases and glycosidases in general. As summarized schematically in Figure 1, the double-displacement reaction of BCX can be broken into two consecutive steps involving glycosylation to produce a covalent glycosyl–enzyme intermediate followed by deglycosylation to regenerate the native enzyme. The distinct catalytic roles played by the active site glutamic acid residues in each of these steps depend intimately upon their ionization states and hence pKₐ values.

(i) **Glycosylation and General Acid Catalysis.** In the absence of bound substrate, the apparent or macroscopic pKₐ values of Glu78 and Glu172 in wild type BCX are 4.6 and 6.7, respectively. The latter value supports the pKₐ of 6.8 reported for Glu172 on the basis of a FT-IR spectroscopic analysis of wild type and mutant BCX (Davoodi et al., 1995). Similarly, in the homologous xylanase A from *Schizophyllum commune*, Bray and Clark (1990) identified by chemical modification an ionizable group with an apparent pKₐ of 6.6 that is critical to the activity of the enzyme. The pKₐ values measured with ¹³C-NMR for the two catalytic glutamic acids in BCX also agree closely with those characterizing the pH dependence of kcat/Km for this xylanase. By standard kinetic formalisms describing enzymatically catalyzed reactions, kcat/Km is the second-order rate constant for the reaction of free enzyme with substrate. Therefore, the simplest interpretation of these results is that near pH 5.7, where the enzyme is maximally active, Glu78 exists predominately in the ionized state in order to act as a nucleophile, whereas Glu172 remains protonated to serve as a general acid or proton donor.

At this time, we have not measured the pKₐ values of the catalytic residues in the Michaelis complex. However, at 40 °C, the pH dependence of the first-order rate constant for the turnover of the enzyme–substrate complex, kcat, roughly parallels that of kcat/Km (apparent pKₐ values of 4.0 and 7.4, and 4.6 and 6.4, respectively; not shown). Since glycosylation is the rate-limiting step for hydrolysis of ONPXb by BCX, the apparent pKₐ values characterizing kcat correspond to ionization events in the noncovalent enzyme–substrate complex (Lawson et al., 1996). This comparison suggests that the pKₐ values of the active site glutamic acids are perturbed only slightly by substrate binding.

(ii) **Deglycosylation and General Base Catalysis.** Hydrolysis of the glycosyl–enzyme intermediate requires general base catalysis from the same group that functions initially as the general acid catalyst during the glycosylation reaction (Figure 1). This implies an important shift in the pKₐ of the carboxyl group during the catalytic cycle in order that it may serve more effectively as a proton donor and then acceptor. Experimental access to the pKₐ of Glu172 in its general base form was provided through the availability of a stable, yet catalytically competent, glycosyl–enzyme intermediate formed by the reaction of BCX with DNP2FXb (Miao et al., 1994).
As shown unambiguously in Figure 2, the $pK_a$ of Glu172 drops by $\sim 2.5$ units from 6.7 to 4.2 in wild type BCX upon glycosylation of Glu78. We postulate that, under optimal pH conditions for the activity of BCX, this $pK_a$ change allows Glu172 to remain deprotonated after serving as a general acid catalyst until the deglycosylation step of the hydrolysis reaction is initiated. During this step, the glutamate then acts as a general base to abstract a proton from water and facilitate nucleophilic attack on the bound xylosyl moiety. If the $pK_a$ of Glu172 had been fixed near 6.7, protonation would occur rapidly after glycosylation to regenerate the neutral glutamic acid, which could not function effectively as a general base catalyst. This study therefore provides clear experimental evidence that during catalysis the $pK_a$ of Glu172 "cycles" about the pH optimum of BCX in order to match its dual role as a general acid and then general base.

Such $pK_a$ values are well-precedented for acid/base-catalyzed hydrolysis of glycosides. In bacteriophage T4 lysozyme, an inverting glycosidase that cleaves peptidoglycan via a single-displacement mechanism (Kuroki et al., 1995), the $pK_a$ of the catalytic Asp20 is 3.6 while that of Glu11 is 5.4 (Anderson et al., 1993). In this mechanism, Asp20 functions as a general base to assist the nucleophilic attack of water on the glycosidic bond, while the general acid Glu11 concomitantly donates a proton to the aglycone leaving group. Thus, in both T4 lysozyme and BCX, the general base carboxylates have $pK_a$ values of approximately 4 and the general acids about 2 units higher. These $pK_a$ values are clearly insufficient for independent protonation of an acetal or deprotonation of water, respectively. They are, however, consistent with concerted events of proton transfer and bond rearrangements, since the $pK_a$ values at the transition states could be shifted enormously. These enzymes have apparently selected acid/base groups for which $pK_a$ values can be adjusted around the desired optimum pH for activity and which can assist in the stabilization of an oxocarbenium ion-like transition state by providing partial negative charge as bond cleavage occurs.

**Structural and Electrostatic Determinants of the $pK_a$ Values of the Catalytic Glutamic Acids**

Structural and enzymatic analyses of numerous retaining glycosidases have clearly revealed a common feature of two active site aspartic or glutamic acid residues involved in catalysis. In the prototypical retaining glycosidase, hen egg white lysozyme, Asp52, with a $pK_a$ of $\sim 3.7$, is negatively charged to stabilize the oxocarbenium ion electrostatically or possibly covalently, while the general acid Glu35 has a $pK_a$ of $\sim 6.2$ (Parsons & Raftery, 1972a; Inoue et al., 1992; Bartik et al., 1994). The $pK_a$ of Glu35 may be elevated to $\sim 6.5$ in the presence of noncovalent inhibitors, while Asp52 remains unperturbed (Parsons & Raftery, 1972b). Numerous studies have focused on dissecting the factors that establish the $pK_a$ values of the two catalytic residues in this protein. In the presence of ethylated Asp52 or the mutation of Asp52 to Asn, the $pK_a$ of Glu35 is reported to be 5.2 and 5.5, respectively (Parsons & Raftery, 1972a; Inoue et al., 1992).

Thus, the electrostatic repulsion of Asp52 carboxylate elevates the effective microscopic $pK_a$ of Glu35 by $\sim 1$ unit in the native enzyme. Several groups have concluded that the hydrophobic environment of Glu35, combined with unfavorable interactions with the macrodipole of an $\alpha$-helix, also contribute to the high $pK_a$ of this catalytic residue (Spassov et al., 1989; Inoue et al., 1992).

A key question prompted by this study is what features of BCX establish the $pK_a$ values of Glu78 and Glu172 in the free and glycosyl–enzyme form of this protein? On the basis of the analysis of Scheme 1, the microscopic $pK_a$ values of Glu78 and Glu172 are 4.63 and 5.50, respectively, in the presence of a neutral catalytic partner. Inspection of the crystal structure of the protein, determined at pH 7.2, reveals no simple reason as to why the $pK_a$ of Glu172 is elevated by $\sim 1$ unit relative to that of Glu78 (Campbell et al., 1993). Both Glu78 and -172 are essentially buried in the protein, with only their side chain carboxyl groups exposed to the solvent (each having $\sim 15$ Å$^2$ of accessible surface area), and both are hydrogen-bonded to a tyrosine ring and a primary amide group. As pointed out previously, the side chain of Glu78 does lie 7.0 Å from the guanido group of Arg112 (C$\delta$–C$\delta$) (Wakarchuk et al., 1994; Davoodi et al., 1995); however, at 8.4 Å, the distance from Glu172 to this cationic side chain is essentially the same. Regardless of the reason, the fact remains that the $pK_a$ of Glu78 is lower than that of Glu172, and thus, the former group is preferentially ionized with increasing pH. On the basis of Scheme 1, the microscopic $pK_a$ of Glu172 is elevated to 6.7 in the presence of the negatively charged Glu78. Therefore, the electrostatic interaction between Glu78 and Glu172 may raise the effective $pK_a$ of the general acid group by $\sim 1.2$ units, corresponding to an energetic coupling of 2.303RT/(ApK_a) $\approx 1.6$ kcal/mol at 25 °C. By this same argument, elimination of the negative charge at position 78 due to the formation of the glycosyl–enzyme intermediate must reduce the $pK_a$ of Glu172. Thus, cycling of the $pK_a$ of the acid/base catalyst is in part intrinsic to the double-displacement mechanism, paralleling the cycling of the nucleophilic group between its ionized and glycosylated states.

Further inspection of the data in Figure 2 reveals that the simplified Scheme 1 does not fully account for the effects of mutations or covalent modification of BCX on the $pK_a$ values of the catalytic carboxyls. Specifically, mutation of Glu172 to a neutral Gln results in a slight elevation of the $pK_a$ of Glu78 to 5.0. More dramatically, mutation of Glu78 to Gln or the glycosylation of this residue leads to the dramatic reduction of the $pK_a$ of Glu172 to $\sim 4.2$. Solely on the basis of the electrostatic interactions implied by Scheme 1, the expected $pK_a$ of Glu172 in the latter two proteins is 5.5. Thus, the neutralization of the charge on Glu78 is only partially responsible for the reduction of the $pK_a$ of Glu172, as required for its activity as a general base. What additional factors help establish the $pK_a$ of this glutamate in the glycosyl–enzyme intermediate?

It appears likely that changes in the structure of the active site of the enzyme accompany the mutation or chemical modification of Glu78, as well as the protonation of Glu78 and/or Glu172. Evidence for this conclusion is provided by the pH-dependent chemical shifts of Gln7 and Gln175, residues that are located within the active site of BCX, yet do not directly interact with either catalytic group. In addition, the $^1$H and $^{15}$N chemical shifts of an unusually large number of amides in BCX also change markedly near pH 6.5, indicating that the backbone structure of the protein is perturbed by the protonation of Glu172 (A. Hedberg, L. A. Plesniak, and L. P. McIntosh, unpublished data). The exchange broadening of the $\delta$-$^{13}$C resonance of Glu172 near
the midpoint of its titration may also result from this postulated conformational transition. Furthermore, as seen in Figure 2, the chemical shifts of the glutamic acid and glutamine groups at high and low pH, and the changes in these shifts upon protonation of BCX, also differ between the wild type, mutant, and covalently modified xylanases. This implies differences in the environments of the side chain carbonyls in the various proteins. Finally, Törrönen et al. (1994, 1995) have reported the crystallographic structures of the homologous Trichoderma reesei xylanase II at pH 5.0 and 6.5. A striking conclusion of their thorough study is that the position of the side chain of the general acid/base glutamate differs markedly under the two pH conditions and that the overall structure of the active site of the enzyme is perturbed due to changes in an extensive network of hydrogen-bonded residues. It is reasonable to suggest that similar structural perturbations occur in BCX with changing of the pH or the amidation or glycosylation of Glu78. These conformational perturbations, combined with the effect of the electrostatic interaction between Glu78 and Glu172, may serve to establish the precise pK_a of the general acid/base residue during the hydrolysis of xylan. We are currently exploring this hypothesis by studying experimentally and theoretically the effects of the mutation of residues adjacent to positions 78 and 172 on the structure, activity, and pK_a values of BCX.

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SUPPORTING INFORMATION AVAILABLE

One table summarizing the pK_a values and chemical shift changes for the Glu and Gln residues observed upon protonation and two figures showing the assignments of the 13C0 resonances in wild type BCX and the 13C-NMR spectrum of E127Q BSX modified with DNP2Fxb (5 pages). Ordering information is given on any current masthead page.

REFERENCES


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