The pK_a of the General Acid/Base Carboxyl Group of a Glycosidase Cycles during Catalysis: A ^{13}C-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 pK_a values. ^{13}C-NMR titrations of xylanase, labeled with [δ-^{13}C]glutamic acid, have revealed pK_a values of 4.6 and 6.7 for Glu78 and Glu172, respectively. These agree well with the apparent pK_a values obtained from a study of the pH dependence of k_{cat}/K_m 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 pK_a 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 pK_a is measured for Glu172 when a glutamine is present at position 78. This large decrease in pK_a 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 "pK_a 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 pK_a values of the catalytic residues in these enzymes. Perhaps the most reliable and unambiguous method for such measurements is to use NMR[1] spectroscopy to monitor titrations of groups within the enzyme and thereby determine their individual ionization constants. This approach has, for example, confirmed the pK_a 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

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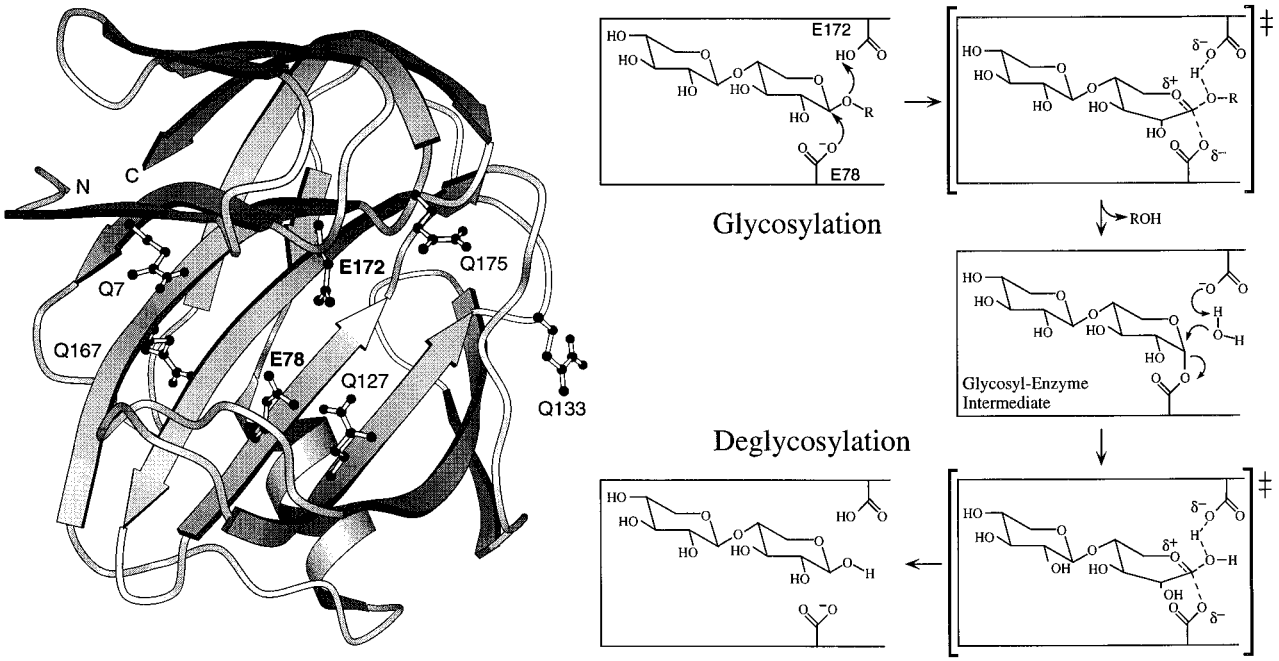


FIGURE 1: (Left) Molscript ribbon diagram of BCX showing the positions of the nucleophile Glu78 and acid/base catalyst Glu172, as well as the five glutamine residues (Kraulis, 1991). For clarity, the only protons drawn are the $H^{\epsilon 2}$ of the glutamine side chain amides. (Right) Double-displacement mechanism of retaining glycosidases. The nucleophilic carboxylate attacks the glycosidic bond of the β-1,4-linked xylose polymer to form a covalent glycosyl−enzyme intermediate, while the second carboxyl group serves as a general acid/base catalyst. The postulated oxocarbenium transition states (‡) for the glycosylation and deglycosylation reactions are indicated with square brackets.

& Raftery, 1972a,b; Inoue *et al*., 1992; Bartik *et al*., 1994). However, in the investigations of retaining glycosidases reported to date, pK_a values have only been obtained for the active site nucleophile and general acid in the free enzyme and or noncovalent enzyme−substrate/inhibitor complexes. 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 β-1,4-linked polymer of xylose (Gebler *et al*., 1992). 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 C^{δ} to C^{δ}; McCarter & Withers, 1994; Davies & Henrissat, 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 ($t_{1/2} \sim 1$ min) than its hydrolysis ($t_{1/2} \sim$ days), and thus, the glycosyl−enzyme intermediate accumulates.

This study describes the use of ^{13}C-NMR spectrometry combined with selective ^{13}C 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

[1] Abbreviations: $\Delta\delta$, the change in chemical shift upon titration from high to low pH; BCX, *Bacillus circulans* xylanase; BSX, *Bacillus subtilis* xylanase; DNP2FXb, 2′,4′-dinitrophenyl 2-deoxy-2-fluoro-β-xylobioside; DSS, sodium 2,2-dimethyl-2-silapentane-5-sulfonate; E72Q and E172Q, the substitution of glutamine for glutamic acid 72 or 172, respectively; 2FXb, 2-fluoro-β-xylobioside; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; MES, 2-(*N*-morpholino)ethanesulfonic acid; NMR, nuclear magnetic resonance; ONPXb, *o*-nitrophenyl β-xylobioside; pH*, the measured pH without correction for isotope effects; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; Xyl, xylose.

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, ^{13}C-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 *avt*A:: 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-^{13}C]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-^{13}C]glutamate to [δ-^{13}C]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-^{13}C]Glu- and -Gln-labeled wild type BCX, observed 20 400 ± 2.3 Da, expected 20 403 Da (assuming ∼100% ^{13}C enrichment of seven residues); [δ-^{13}C]Glu- and -Gln-labeled E78Q BSX, observed 20 387 ± 3.6 Da, expected 20 388 Da; and [δ-^{13}C]-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-^{13}C]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_a values describing the dependence of k_{cat}/K_m upon pH were determined with the program GraFit (Leatherbarrow, 1990).

^{13}C-NMR. The ^{13}C-enriched xylanase samples were dialyzed into 25 mM sodium phosphate, 3 mM NaN_3, and 10% D_2O/90% H_2O, at pH* ∼7.5. The samples contained 0.5−0.75 mM protein in 1.7−2.5 mL of buffer. One-dimensional ^{13}C-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 T_1 lifetimes of the glutamate and glutamine δ-carbonyls 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 T_1 of the δ-^{13}C of free glutamate is 11.5 s at pH* 2.15 and 25 °C. On the basis of these lifetimes, the ^{13}C 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 ^{1}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 0.00 ppm.

The ^{13}C-NMR spectra of wild type BCX and E172Q BSX modified with DNP2FXb were recorded as outlined above. The ^{13}C-labeled wild type BCX was initially 0.8 mM in 1.7 mL of 25 mM sodium phosphate, 3 mM NaN_3, and 10% D_2O, 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 ^{13}C-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 ^{19}F-NMR. A similar procedure was used with ^{13}C-labeled E172Q BSX, except that the protein was initially 1.3 mM at pH* 7.51.

pK_a Measurements. The pK_a values of the glutamic acid residues were determined by measuring the ^{13}C-NMR spectra of the [δ-^{13}C]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_a 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_a" or simply "pK_a" refers to the phenomenological ionization constant describing the depen-

dence of enzyme activity or chemical shift upon pH, whereas "microscopic" and "macroscopic" pK_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_{cat}/K_m follows a classic bell-shaped curve with maximal activity near pH 5.7. The pH dependence of k_{cat}/K_m can be fit adequately to a model involving two ionizable catalytic residues with apparent pK_a values of 4.6 and 6.7 in the free enzyme. On the basis of previous studies of BCX and retaining glycosidases in general, we attribute the lower pK_a to Glu78, which must be deprotonated to function as a nucleophile, and the higher pK_a to Glu172, which must remain protonated to act as a general acid (Wakarchuk *et al*., 1992, 1994; Miao *et al*., 1994).

^{13}C-NMR Spectra of Xylanase. The ^{13}C-NMR spectrum of labeled wild type BCX is shown in Figure 2B. Seven ^{13}C peaks are observed, arising from the δ-carbonyls 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^{β}/C^{γ}, C^{δ}, and $H^{\gamma/\gamma'}$ resonances of each Glu and Gln residue in the sample of uniformly ^{13}C-enriched BCX (supporting information; Kay, 1993). This allowed us to classify the side chain carbonyl peaks using the previously determined ^{1}H- and ^{13}C-NMR assignments of BCX (Plesniak *et al*., 1996). These assignments were confirmed by the selective substitutions of Gln for Glu78 or Glu172 (Figure 2E,F).

pH Dependence of the ^{13}C-NMR Spectrum of BCX. The pK_a values of Glu78 and Glu172 in wild type BCX were determined from the pH dependence of the ^{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_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_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 or midpoint pK_a of 6.5 ± 0.2, while Glu172 shows a comparable upfield shift of 0.44 ppm corresponding to an apparent pK_a of 4.6 ± 0.1. The reciprocal correlation of the two apparent pK_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 tiration if (i) the chemical shift or (ii) the microscopic pK_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 ^{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_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 ^{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 (Schrager *et al*., 1972), yields the microscopic pK_a values summarized in Scheme 1. From a simultaneous fit of the two titration curves, the pK_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_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_a by ~1.2 units. The lower ionization pathway in Scheme 1 is favored significantly, and thus, the microscopic pK_a values of 4.63 (Glu78) and 6.66 (Glu172) are essentially equal to the macroscopic or apparent pK_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 ^{13}C-NMR spectrum of the isotopically enriched protein upon reaction with this compound. As illustrated in Figure 2, the ^{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 ^{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

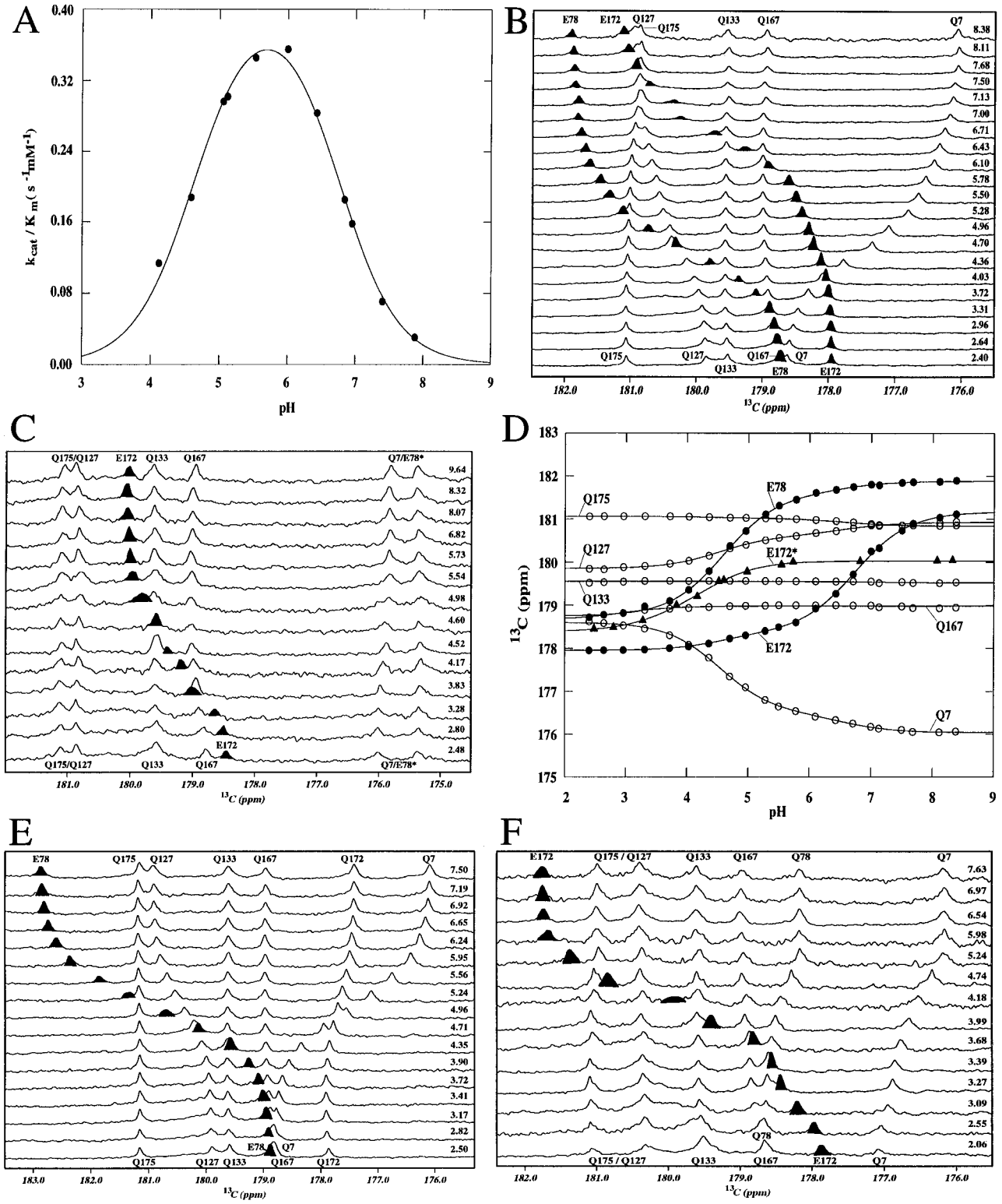


FIGURE 2: (A) Dependence of k_{cat}/K_m upon pH for hydrolysis of ONPXb by BCX at 25 °C, fit to the kinetic expression for two ionizable groups with apparent pK_a values of 4.6 and 6.7 (line). (B) ^{13}C-NMR spectra of wild type BCX recorded as a function of pH at 25 °C. The peaks corresponding to Glu78 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 δ-^{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.

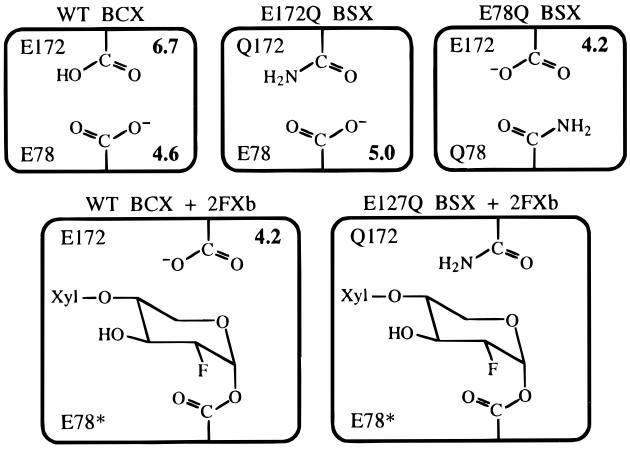
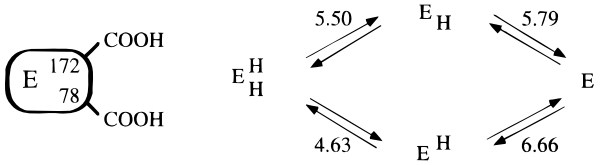


FIGURE 3: Summary of the pK_a values of Glu78 and Glu172 in the wild type, mutant, and glycosylated forms of BCX and BSX. The predominant protonation states at the optimum pH of 5.7 for enzymatic activity are indicated. In the case of the wild type protein, the numbers listed in bold beside Glu78 and Glu172 correspond to the macroscopic pK_a values describing the major chemical shift change exhibited by these residues. For the mutant and glycosylated proteins, the Glu residues show monophasic titrations, and thus, the numbers correspond to microscopic ionization constants.

Scheme 1



days (Miao *et al*., 1994). To confirm further the presence of the covalently bonded inhibitor, the mass spectrum (see Materials and Methods) and ^{19}F-NMR spectrum of the modified BCX were recorded after dialysis. A single broad resonance, split by $^2J_{H-F} = 47$ Hz, was detected at −205.6 ppm relative to $CFCl_3$. This resonance is assigned to the ^{19}F nucleus of the 2-deoxy-2-fluoro-α-xylobiosyl−enzyme complex.

The ^{13}C-NMR spectrum of the covalently modified protein was recorded as a function of pH (Figure 2C). In contrast to the native protein, only a single peak shows a distinct upfield shift of 1.59 ppm with decreasing pH. This peak is assigned to Glu172, as Glu78 is involved in a glycosidic linkage and thus is no longer titratable. In addition, as observed with native BCX, the resonance of Glu172 is exchange broadened during its titration. However, in contrast to the unmodified protein, the chemical shift of Glu172 now follows a monophasic titration curve with a pK_a of 4.2 ± 0.02 (Figure 2D). Remarkably, the pK_a of this catalytic residue is reduced by ∼2.5 units due to the formation of the glycosyl−enzyme intermediate.

pK_a Values of BSX Mutants with Single Glutamic Acid Residues. The ^{13}C-NMR spectra of *B. subtilis* xylanases with either Glu78 or Glu172 mutated to glutamine were recorded as a function of pH. This served both to confirm the assignments of the δ-carbonyl resonances in the wild type protein and to examine the interactions between these catalytic groups. These substitutions, involving isosteric side chains, were chosen to have minimal effects on the structures of the proteins. In each case, a single peak, corresponding to that of the eliminated glutamate side chain, was missing 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 Gln7, -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 pK_a of 5.0 ± 0.01 ($\Delta\delta = -3.93$ ppm). The pK_a 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 pK_a of Glu78 should remain near 4.6 in the presence of a neutral side chain at position 172. Therefore, the introduction of Gln172 may perturb the environment of Glu78. In a reciprocal manner, the resonance of Gln172 shifts downfield by 0.5 ppm with an apparent pK_a of 5.1 ± 0.02, indicating that its δ-^{13}C chemical shift is dependent upon the ionization state of Glu78.

The substitution of Glu78 to Gln dramatically alters the pK_a of Glu172. In the mutant E78Q, Glu172 follows a titration curve with a pK_a of 4.2 ± 0.02 ($\Delta\delta = -3.81$ ppm). Again, exchange broadening of the δ-^{13}C resonance of Glu172 is observed. This pK_a is essentially identical to that measured for Glu172 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 pK_a of Glu172. However, on the basis of Scheme 1, the microscopic pK_a of Glu172 in the presence of a neutral protonated Glu78 in wild type BCX is 5.50. This is significantly higher than the pK_a 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 Gln78 shifts downfield ($\Delta\delta = +0.51$ ppm) with an apparent pK_a of 4.2 ± 0.04, indicating that its δ-^{13}C chemical shift is sensitive to the ionization state of Glu172.

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 ^{13}C-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 ^{13}C-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 ^{13}C-NMR chemical shifts of several nonionizable glutamine residues in BCX are pH-dependent, providing confirmation of the pK_a 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 pK_a values of 4.5 ± 0.02 ($\Delta\delta$ = +2.09 ppm) and 6.6 ± 0.08 ($\Delta\delta$ = +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 pK_a values of 5.0 ± 0.01 ($\Delta\delta$ = +2.71 ppm), 4.0 ± 0.04 ($\Delta\delta$ = +0.88 ppm), and 4.2 ± 0.08 ($\Delta\delta$ = +0.19 ppm), respectively. On the basis of the similarity of these apparent pK_a 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 ($C^{\delta}-C^{\delta}$) 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 pK_a 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 pK_a values of 4.5 ± 0.3 ($\Delta\delta$ = +0.04 ppm) and 6.6 ± 0.07 ($\Delta\delta$ = +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 pK_a values of 4.5 ± 0.04 ($\Delta\delta$ = −0.76 ppm) and 6.6 ± 0.10 ($\Delta\delta$ = −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 pK_a of 5.0 ± 0.01 ($\Delta\delta$ = −1.01 ppm), which is equal to the pK_a 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-^{13}C resonance frequency of Gln127 would appear to depend directly on the ionization of Glu78 and may show an apparent pK_a of ~6.6 in the wild type protein due to the interaction of Glu78 with Glu172.

Finally, the δ-^{13}C chemical shift of Gln167 changes slightly with an apparent pK_a 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 pK_a Values with the Enzymatic Mechanism

Using selective isotopic labeling and ^{13}C-NMR, we have measured directly the pK_a values of the catalytic glutamic acid residues in wild type and mutant *B. circulans* and *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 pK_a values.

(i) Glycosylation and General Acid Catalysis. In the absence of bound substrate, the apparent or macroscopic pK_a values of Glu78 and Glu172 in wild type BCX are 4.6 and 6.7, respectively. The latter value supports the pK_a 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 pK_a of 6.6 that is critical to the activity of the enzyme. The pK_a values measured with ^{13}C-NMR for the two catalytic glutamic acids in BCX also agree closely with those characterizing the pH dependence of k_{cat}/K_m for this xylanase. By standard kinetic formalisms describing enzymatically catalyzed reactions, k_{cat}/K_m 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 predominantly 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 pK_a 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, k_{cat}, roughly parallels that of k_{cat}/K_m (apparent pK_a 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 pK_a values characterizing k_{cat} correspond to ionization events in the noncovalent enzyme−substrate complex (Lawson *et al*., 1996). This comparison suggests that the pK_a 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 pK_a 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 pK_a 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 ~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 xylobiosyl 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 ~3.7, is negatively charged to stabilize the oxocarbenium ion electrostatically or possibly covalently, while the general acid Glu35 has a pK_a of ~6.2 (Parsons & Raftery, 1972a; Inoue *et al*., 1992; Bartik *et al*., 1994). The pK_a of Glu35 may be elevated to ~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 ~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 α-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 ~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 ~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 ($C^{\delta}-C^{\zeta}$) (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 ~1.2 units, corresponding to an energetic coupling of $2.303RT(\Delta pK_a)$ = ~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 ~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 δ-^{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 $^{13}C^{\delta}$ resonances in wild type BCX and the ^{13}C-NMR spectrum of E127Q BSX modified with DNP2FXb (5 pages). Ordering information is given on any current masthead page.

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