FOR THE RECORD

Complete measurement of the pK_a values of the carboxyl and imidazole groups in *Bacillus circulans* xylanase

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Abstract: Electrostatic interactions in proteins can be dissected experimentally by determining the pK_a values of their constituent ionizable amino acids. To complement previous studies of the glutamic acid and histidine residues in *Bacillus circulans* xylanase (BCX), we have used NMR methods to measure the pK_a s of the seven aspartic acids and the C-terminus of this protein. The pK_a s of these carboxyls are all less than the corresponding values observed with random coil polypeptides, indicating that their ionization contributes favorably to the stability of the folded enzyme. In general, the aspartic acids with the most reduced pK_a s are those with limited exposure to the solvent and a high degree of conservation among homologous xylanases. Most dramatically, Asp 83 and Asp 101 have pK_a s < 2 and thus remain deprotonated in native BCX under all conditions examined. Asp 83 is completely buried, forming a strong salt bridge with Arg 136. In contrast, Asp 101 is located on the surface of the protein, stabilized in the deprotonated form by an extensive network of hydrogen bonds involving an internal water molecule and the neutral side-chain and main-chain atoms of Ser 100 and Thr 145. These data provide a complete experimental database for theoretical studies of the ionization behavior of BCX under acidic conditions.

Keywords: aspartic acid; electrostatics; NMR; pK_a ; xylanase

The ionizable side chains of the acidic and basic amino acids play important roles in establishing the structure, stability, and function of proteins (Creighton, 1993). Electrostatic interactions between charged groups may help stabilize the native conformation of a protein, as well as define the specificity and affinity of ligand or substrate binding. Ionizable groups often serve important enzymatic roles, functioning as electrophiles, nucleophiles, or general acid/base catalysts. To understand the contributions made by these

amino acid residues, it is necessary to determine experimentally their pK_a values and hence protonation states within a given protein. Such studies provide key insights into the possible catalytic functions of active site residues in enzymes, as well as direct measures of the contributions made by electrostatic interactions to the stability of proteins. By thermodynamic linkage, the free energy of stabilization of the native state of a protein relative to its unfolded state, due to the deprotonation of a charged group, is reflected directly by the difference in the pK_a values of that group in these two conformational states (Tanford, 1961). Accordingly, considerable experimental and theoretical efforts continue to be directed toward understanding the complex factors that dictate the pK_a s of amino acids in proteins and protein–ligand complexes (Yang et al., 1993).

Bacillus circulans xylanase (BCX) is a 20.4-kDa endo- β -(1,4)-xylanase belonging to the Family G or 11 of glycosidases (Gilkes et al., 1991; Henrissat & Bairoch, 1993). This enzyme catalyzes the hydrolysis of the polysaccharide xylan, a major constituent of plant biomass. BCX and several homologous xylanases from *Aspergillus*, *Bacillus*, *Thermomyces*, and *Trichoderma* sp. have been characterized extensively in terms of structure and enzymology (Campbell et al., 1993; Wakarchuk et al., 1994; Törrönen & Rouvinen, 1995; Krengel & Dijkstra, 1996; Schlacher et al., 1996). BCX is a net basic protein with two Glu, seven Asp, two His, seven Arg, and five Lys residues. Previously, we measured the pK_a s of the glutamic acids, which fortuitously are the two catalytic carboxyls, as well as the histidines in this protein. The pK_a of the nucleophile, Glu 78, is 4.6, whereas that of the general acid/base, Glu 172, is 6.7 in the free enzyme and 4.2 in a trapped glycosyl-enzyme intermediate (McIntosh et al., 1996). These values agree closely with the apparent pK_a s describing the pH dependence of the second-order rate constant, k_{cat}/K_m , for BCX toward xylobiosyl substrates. The pK_a of His 156, a surface residue, is relatively unperturbed at $\sim$ 6.5. In contrast, His 149 has a pK_a of <2.3, and thus is never ionized significantly within the folded protein (Plesniak et al., 1996a). This histidine is completely buried within the hydrophobic core of BCX and is involved in an extensive network of hydrogen bonding interactions. The structural importance of

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His 149 is confirmed by the destabilizing effect of amino acid substitutions at this position.

Results and discussion: To complete the analysis of the ionization behavior of BCX under acidic conditions, we have measured the pK_a s of the remaining carboxyl groups within this protein. Shown in Figure 1 is the pH dependence of the chemical shifts of the γ - ^{13}C nuclei from the seven aspartic acids in BCX at 25 °C. The upfield change of ~ 3 ppm with decreasing pH observed for five of these residues is consistent with that expected for the protonation of a carboxyl group (Gu et al., 1994). The pK_a values of these side chains were determined by fitting of the NMR data to standard equations describing titrations involving one (Asp 11, 106, and 121) or two (Asp 4 and 119) ionizable groups (Shrager et al., 1972). In the latter case, the apparent or macroscopic pK_a associated with the aspartic acid is taken to correspond to the major change in chemical shift occurring near pH 3. Minor changes near neutral pH are attributed to perturbations of these residues due to the titrations of other ionizable groups in BCX.

In striking contrast to five of the aspartic acid residues in BCX, Asp 83 and Asp 101 have essentially constant γ - ^{13}C chemical shifts between pH 2.06 and 9.35 and thus do not titrate over this measured pH range. The pK_a s of these side chains must be either <2 or >9 . The downfield chemical shift (184.3 ppm) of Asp 101 is indicative of a deprotonated carboxylate, whereas the upfield chemical shift (176.8 ppm) of Asp 83 is characteristic of a protonated carboxylic acid. However, as is evident by the dispersion of aspartic acid γ - ^{13}C chemical shifts observed in BCX, the ion-

Fig. 1. pH Dependence of the γ - ^{13}C chemical shifts of the seven aspartic acid residues in BCX. Lines represent the best fit of the measured data points to the equations describing a titration involving one or two ionizable groups. These data were obtained from 1D ^{13}C -NMR spectra of isotopically labeled BCX, as described previously (McIntosh et al., 1996). Sample conditions were 25 mM sodium phosphate, 3 mM NaN_3 , and 10% $\text{D}_2\text{O}/90\%$ H_2O at 25 °C, and pH readings are uncorrected for the glass isotope effect. The labeled protein was prepared by expressing the gene encoding BCX in the *Escherichia coli* strain EA1 grown in a synthetic medium containing 250 mg/mL 99% L- $[\gamma$ - $^{13}\text{C}]$ aspartate (Muchmore et al., 1989; Sung et al., 1993). With lesions in the *AsnA* and *AsnB* genes, this strain does not convert metabolically aspartic acid to asparagine (Anderson et al., 1993). The assignments of the γ - ^{13}C resonances were obtained from a variation of the 3D CBCACO(CA)HA experiment that provides correlations between the $\text{C}^\alpha/\text{C}^\beta$, C^γ , and $\text{H}^{\beta/\beta'}$ of each Asp residue in a uniformly $^{13}\text{C}/^{15}\text{N}$ -labeled sample of BCX (Plesniak et al., 1996b).

ization states of these residues cannot be determined reliably based only on the data presented in Figure 1. To resolve this critical question, we also recorded the spectrum of the selectively labeled protein in 90% $\text{D}_2\text{O}/10\%$ H_2O buffer at pH_{obs} 6.09. Whereas the ^{13}C resonance of a neutral carboxylic acid experiences a two-bond isotope shift of ≈ -0.25 ppm upon deuteration, that of an ionized carboxylate remains unaffected (Ladner et al., 1975; Yamazaki et al., 1994). The absence of such a readily measurable isotope shift for either Asp 83 or Asp 101 demonstrates unambiguously that both residues exist in the *deprotonated* form within BCX, and thus must have pK_a values <2.0 (data not shown). Indeed, if we assume that changes in chemical shift are detectable at pHs within a unit of the pK_a of a carboxyl group, then estimates for the upper bounds for the pK_a s of Asp 83 and Asp 101 can be set at ~ 1.0 .

Finally, based upon the pH dependence of the amide ^{15}N chemical shift, of Trp 185 determined using a 2D ^1H - ^{15}N heteronuclear correlation experiment, the pK_a of the α -carboxyl group of this C-terminal residue is assigned tentatively to ~ 2.7 (data not shown; Bundi & Wüthrich, 1979). A summary of the measured pK_a values of all the carboxyl and imidazole groups in BCX is presented in Table 1.

The pK_a s of an aspartic acid side chain and a C-terminal α -carboxyl group in an unstructured polypeptide are approximately 4.0 and 3.3–3.8, respectively (Richarz & Wüthrich, 1978; Creighton, 1993). The pK_a s of all the aspartic acids and the C-terminus of BCX are less than these reference values, indicating that the environment of each in the native protein favors its deprotonation. Stated equivalently, the ionization of these acidic groups contributes to the stability of the folded protein relative to its unfolded state. Quantitatively, each decrease in the pK_a of a given residue compared to its value in the unfolded state by one unit corresponds to ~ 1.4 kcal/mol in free energy of stabilization of the native protein at 25 °C (Tanford, 1961; Anderson et al., 1990). The reduced pK_a s of the carboxyl groups may result from global electrostatic effects, due to the net positive charge of BCX under acidic conditions, combined with local electrostatic interactions including opposite charge pairing and hydrogen bonding. Offsetting these favorable effects is the screening of exposed charged groups by solvent counterions and the destabilization of ionizable groups upon burial within the low dielectric medium of the protein core. To help in the evaluation of these complex factors, a summary of the environments of the Asp, Glu, and His residues observed in the crystal structure of BCX is also given in Table 1.

With pK_a values below 2.0, Asp 83 and Asp 101 contribute most significantly to the stabilization of BCX. Indeed, because the stability of BCX decreases with pH such that this protein unfolds readily under strongly acidic conditions (Davoodi et al., 1995; Plesniak et al., 1996a), we conclude that these two aspartate groups are always ionized within the context of the native enzyme. Based on the crystal structure of BCX (Campbell et al., 1993), Asp 83 is buried entirely in the interior of the protein, forming hydrogen bonds with the positively charged guanido group of Arg 136 and the phenolic O $^\gamma$ s of Tyr 53 and Tyr 105 (Fig. 2). The structural importance of this ion pair is highlighted by the strict conservation of an Asp or Glu residue at position 83 and an Arg or Lys residue at position 136, respectively, in all Family G xylanases. It is intriguing to note that, in bacteriophage T4 lysozyme, Asp 10 has a $pK_a < 2$, yet also shows an unusual upfield γ - ^{13}C shift characteristic of a protonated carboxyl. This aspartate is buried within the protein, interacting with the side chains of two arginines and a tyrosine (Anderson et al., 1993). It is plausible that a strongly

Table 1. Measured pK_a values and structural characteristics of the carboxyl and imidazole groups in BCX

Residue	pK_a ^a	Hydrogen bonding partner ^b	Fractional solvent-accessible surface area ^c	Conservation ^d (%)
Asp 4	3.0	Thr 43 (N, O ^γ)	0.35	15
Asp 11	2.5	Gly 13 (N), Asn 35 (N), H ₂ O (244)	0.25	70
Asp 83	<2.0	Tyr 53 (O ^η), Tyr 105 (O ^η), <u>Arg 136</u> (N ^{η1})	0.00	100
Asp 101	<2.0	Ser 100 (O ^γ), Thr 145 (N, O ^{γ1}), H ₂ O (200)	0.08	85
Asp 106	2.7	<u>Arg 89</u> (N ^{η2})	0.15	80
Asp 119	3.2	<u>Arg 73</u> (N ^ε , N ^{η2})	0.43	50
Asp 121	3.6		0.97	15
Glu 78	4.6	Tyr 69 (O ^η), Gln 127 (N ^{ε2})	0.12	100
Glu 172	6.7	Asn 35 (N ^{δ2}), Tyr 80 (O ^η)	0.13	100
His 149	<2.3	Ser 130 (O ^γ), H ₂ O (200)	0.00	100
His 156	~6.5	Ala 152 (O)	0.33	35
W185 (α-C)	~2.7	<u>Ala 1</u> (α-N), Asn 52 (N)	0.00	—

^aApparent pK_a s of the aspartic acid residues are determined from the data in Figure 1, and those of the glutamic acid were taken from McIntosh et al. (1996) (25 mM sodium phosphate, 3 mM NaN₃, and 10% D₂O/90% H₂O at 25 °C). Histidine pK_a s are from Plesniak et al. (1996a), and that of the C-terminus is from a HSQC-titration of ¹⁵N-labeled BCX (25 mM sodium acetate, 3 mM NaN₃, and 10% D₂O/90% H₂O at 30 °C). Errors are estimated to be ±0.1 units.

^bHydrogen bonds were determined from the crystal structure of BCX (PDB file 1XNB; Campbell et al., 1993) by visual inspection and by using the programs HBPLUS (McDonald & Thornton, 1994) and VADAR (Wishart et al., 1995). Only internal water molecules are listed. Positively charged groups are underlined.

^cFractional solvent-accessible surface areas are reported for selected atoms of the aspartic acid (C^γ, O^{γ1}, O^{γ2}), glutamic acid (C^δ, O^{δ1}, O^{δ2}), and histidine (C^γ, C^{δ2}, N^{δ1}, C^{ε1}, N^{ε2}) side chains in the PDB file 1XNB relative to those calculated for the same atoms in a fully extended Gly-X-Gly tripeptide. Calculations were performed with the program SURFCV using a probe radius of 1.4 Å (Sidharan et al., 1995).

^dResidue conservation is based on the aligned sequences of 14 Family G xylanases (Wakarchuk et al., 1994). Aspartic acid and glutamic acid residues are considered equivalent.

electron-withdrawing ionic hydrogen bond or an electric field effect from an adjacent charged guanido group leads to the upfield changes in the γ-¹³C chemical shifts of these two aspartates (Batch-

Fig. 2. Structural environment of Asp 83 and Asp 101 in BCX. Both of these residues have pK_a s < 2.0 and thus are negatively charged under all conditions examined. Asp 83 is buried, yet stabilized by a salt bridge with Arg 136. Asp 101 is nearby on the surface of BCX, forming hydrogen bonds to Ser 100, Thr 145, and an internal water molecule. His 149, which is also hydrogen bonded to this water molecule, has a pK_a < 2.3 and thus is neutral within the folded protein. The figure was generated from the PDB coordinate file 1XNB (Campbell et al., 1993) using the program SETOR (Evans, 1993).

elor et al., 1973; Gu et al., 1994). In support of this suggestion, the second and third most upfield carboxylate resonances in Figure 1 arise from Asp 106 and Asp 119, both of which also contact arginine side chains in BCX (Table 1).

Asp 101 is also a strong acid in this xylanase, yet, in contrast to Asp 83, is not adjacent to any positively charged groups. The carboxyl of Asp 101 is positioned at the surface of BCX, neither buried within its hydrophobic core nor exposed fully to the solvent. The charge of this side chain appears to be stabilized by hydrogen bonding to the amide and hydroxyl of Thr 145, the hydroxyl of Ser 100, and an internal water molecule (Fig. 2). Consistent with this interaction, the amide H^N resonance of Thr 145 is shifted strongly downfield to 10.58 ppm (Plesniak et al., 1996b). Furthermore, Asp 101, Ser 100, and the buried water are well conserved in homologous xylanases of known structure. Somewhat surprisingly, Asp 101 is hydrogen bonded to the same internal water molecule as is the buried, neutral His 149. This histidine has a pK_a < 2.3, and thus is never positively charged in native BCX, despite the presence of the adjacent negatively charged aspartate (Plesniak et al., 1996a). This emphasizes the complex balance between charge stabilizing and destabilizing interactions within a folded protein.

Of the five aspartic acids in BCX that show measurable pH titrations, those that are well conserved in Family G xylanases (Asp 11 and 106) have pK_a s < 3, whereas those that are less conserved (Asp 4, 119, and 121) have pK_a s ≥ 3. The latter three also have the most exposed carboxyls of all the aspartic acids in BCX. This is not unexpected because residues involved in stabilizing the native fold of a protein are often maintained across a family of related proteins and often form the core of their structures. Both Asp 106 and Asp 119 are hydrogen bonded to arginine

guanido groups in the crystal structure of BCX. However, the more exposed of these, Asp 119, has a pK_a of 3.2, suggesting that solvent screening may reduce the stabilizing effect of this salt bridge. In contrast, neither Asp 4 nor Asp 11 is located in the immediate proximity of a charged side chain. The depressed pK_a s of these two residues are attributed to hydrogen bonding interactions with neutral polar atoms. The side chain of Asp 121 is entirely exposed to the solvent, accounting for its relatively unperturbed pK_a of 3.6. Lastly, in the crystal structure of BCX, the α -carboxyl of Trp 185 forms a salt bridge to the α -amino group of Ala 1, presumably leading to its reduced pK_a of 2.7. Consistent with this interaction, heteronuclear relaxation studies reveal that the termini of BCX are well ordered in solution and do not display rapid local motion typical of residues near the ends of many proteins (L.P. McIntosh, unpubl. obs.).

In summary, we have measured the pK_a s of all the carboxyl and imidazole groups in BCX and thereby helped define the roles that each plays in establishing the pH-dependence stability of this protein. This study provides a database for future experimental and theoretical studies aimed at understanding the factors that determine the precise pK_a values of these residues within the folded and unfolded states of xylanases.

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References

- Anderson DE, Becktel WJ, Dahlquist FW. 1990. pH-induced denaturation of proteins: A single salt bridge contributes 3–5 kcal/mol to the free energy of folding of T4 lysozyme. *Biochemistry* 29:2403–2408.
- Anderson DE, Lu J, McIntosh LP, Dahlquist FW. 1993. The folding, stability and dynamics of T4 lysozyme: A perspective using nuclear magnetic resonance. In: Clore GM, Gronenborn AM, eds. *NMR of proteins*. London: MacMillan Press. pp 258–304.
- Batchelor JG, Prestegard JH, Cushley RJ, Lipsky SR. 1973. Electric field effects in the ^{13}C nuclear magnetic resonance spectra of unsaturated fatty acids. A potential tool for conformational analysis. *J Am Chem Soc* 95:6358–6364.
- Bundi A, Wüthrich K. 1979. Use of amide ^1H -NMR titration shifts for studies of polypeptide conformation. *Biopolymers* 18:299–311.
- Campbell R, Rose D, Wakarchuk W, To R, Sung W, Yaguchi M. 1993. A comparison of the structures of the 20 kD xylanases from *Trichoderma harzianum* and *Bacillus circulans*. In: Suominen P, Reinikainen T, eds. *Proceedings of the second TRICEL symposium on Trichoderma reesei cellulases and other hydrolyases*. Espoo, Finland, 1993. Helsinki: Foundation for Biotechnological and Industrial Fermentation Research. pp 63–77.
- Creighton TE. 1993. *Proteins: Structures and molecular properties*, 2nd ed. New York: W.H. Freeman & Co.
- Davoodi J, Wakarchuk WW, Campbell RL, Carey PR, Surewicz WK. 1995. Abnormally high pK_a of an active-site glutamic acid residue in *Bacillus circulans* xylanase—The role of electrostatic interactions. *Eur J Biochem* 232:839–843.
- Evans S. 1993. SETOR: Hardware lighted three-dimensional solid model representations of macromolecules. *J Mol Graphics* 11:134–138.
- Gilkes NR, Henrissat B, Kilburn DG, Miller RC Jr, Warren RAJ. 1991. Domains in microbial beta-1,4-glycanases: Sequence conservation, function, and enzyme families. *Microbiol Rev* 55:303–315.
- Gu Z, Zambrano R, McDermott A. 1994. Hydrogen bonding of carboxyl groups in solid-state amino acids and peptides: Comparison of carbon chemical shielding, infrared frequencies, and structures. *J Am Chem Soc* 116:6368–6372.
- Henrissat B, Bairoch A. 1993. New families in the classification of glycosyl hydrolases based on amino acid sequence similarities. *Biochem J* 293:781–788.
- Krengel U, Dijkstra BW. 1996. Three-dimensional structure of the endo-1,4- β -xylanase I from *Aspergillus niger*: Molecular basis for its low pH optimum. *J Mol Biol* 263:70–78.
- Ladner HK, Led JJ, Grant DM. 1975. Deuterium isotope effects on ^{13}C chemical shifts in amino acids and dipeptides. *J Magn Reson* 20:530–534.
- McDonald IK, Thornton JM. 1994. Satisfying hydrogen bonding potential in proteins. *J Mol Biol* 238:777–793.
- McIntosh LP, Hand G, Johnson PE, Joshi MD, Körner M, Plesniak LA, Ziser L, Wakarchuk WW, Withers SG. 1996. 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. *Biochemistry* 35:9958–9966.
- Muchmore DC, McIntosh LP, Russell CB, Anderson DE, Dahlquist FW. 1989. Expression and ^{15}N labeling of proteins for proton and nitrogen-15 NMR. *Methods Enzymol* 177:44–73.
- Plesniak LA, Connelly GP, Wakarchuk WW, McIntosh LP. 1996a. Characterization of a buried neutral histidine residue in *Bacillus circulans* xylanase: NMR assignments, pH titration, and hydrogen exchange. *Protein Sci* 5:2319–2328.
- Plesniak LA, Wakarchuk WW, McIntosh LP. 1996b. Secondary structure and NMR assignments of *Bacillus circulans* xylanase. *Protein Sci* 5:1118–1135.
- Richarz R, Wüthrich K. 1978. Carbon-13 NMR chemical shifts of the common amino acid residues measured in aqueous solutions of the linear tetrapeptides H-Gly-Gly-X-L-Ala-OH. *Biopolymers* 17:2133–2141.
- Schlacher A, Holzman K, Hayn M, Steiner W, Schwab H. 1996. Cloning and characterization of the gene for the thermostable xylanase XynA from *Thermomyces lanuginosus*. *J Biotechnol* 49:211–218.
- Shrager RI, Cohen JS, Heller SR, Sachs DH, Schechter AN. 1972. Mathematical models for interacting groups in nuclear magnetic resonance titration curves. *Biochemistry* 11:541–547.
- Sidharan S, Nicholls A, Sharp KA. 1995. A rapid method for calculating derivatives of solvent accessible surface areas of molecules. *J Comp Chem* 16:1038–1044.
- Sung WL, Luk CK, Zahab DM, Wakarchuk W. 1993. Overexpression and purification of the *Bacillus subtilis* and *Bacillus circulans* xylanases in *Escherichia coli*. *Protein Express Purif* 4:200–216.
- Tanford C. 1961. *Physical chemistry of macromolecules*. New York: J. Wiley & Sons.
- Törrönen A, Rouvinen J. 1995. Structural comparison of two major endo-1,4-xylanases from *Trichoderma reesei*. *Biochemistry* 34:847–856.
- Wakarchuk WW, Campbell RL, Sung WL, Davoodi J, Yaguchi M. 1994. Mutational and crystallographic analyses of the active site residues of the *Bacillus circulans* xylanase. *Protein Sci* 3:467–475.
- Wishart DS, Willard L, Sykes BS. 1995. VADAR—Volume, area and dihedral angle reporter. Edmonton, Alberta: University of Alberta.
- Yamazaki T, Nicholson L, Wingfield P, Stahl SJ, Kaufman JD, Eyermann CJ, Hodge CN, Lam PSY, Prabhakar KJ, Chang CH, Weber PC, Torchia DA. 1994. NMR and X-ray evidence that the HIV protease catalytic aspartyl groups are protonated in the complex formed by the protease and a non-peptide cyclic urea-based inhibitor. *J Am Chem Soc* 116:10791–10792.
- Yang AS, Gunner MR, Sampogna R, Sharp K, Honig B. 1993. On the calculation of pK_a s in proteins. *Proteins Struct Funct Genet* 15:252–265.