

## Abstract

Previous studies of the low molecular mass family 11 xylanase from *Bacillus circulans* show that the ionization state of the nucleophile (Glu78, pK(a) 4.6) and the acid/base catalyst (Glu172, pK(a) 6.7) gives rise to its pH-dependent activity profile. Inspection of the crystal structure of BCX reveals that Glu78 and Glu172 are in very similar environments and are surrounded by several chemically equivalent and highly conserved active site residues. Hence, there are no obvious reasons why their apparent pK(a) values are different. To address this question, a mutagenic approach was implemented to determine what features establish the pK(a) values (measured directly by  $^{13}\text{C}$  NMR and indirectly by pH-dependent activity profiles) of these two catalytic carboxylic acids. Analysis of several BCX variants indicates that the ionized form of Glu78 is preferentially stabilized over that of Glu172 in part by stronger hydrogen bonds contributed by two well-ordered residues, namely, Tyr69 and Gln127. In addition, theoretical pK(a) calculations show that Glu78 has a lower pK(a) value than Glu172 due to a smaller desolvation energy and more favorable background interactions with permanent partial charges and ionizable groups within the protein. The pK(a) value of Glu172 is in turn elevated due to electrostatic repulsion from the negatively charged glutamate at position 78. The results also indicate that all of the conserved active site residues act concertedly in establishing the pK(a) values of Glu78 and Glu172, with no particular residue being singly more important than any of the others. In general, residues that contribute positive charges and hydrogen bonds serve to lower the pK(a) values of Glu78 and Glu172. The degree to which a hydrogen bond lowers a pK(a) value is largely dependent on the length of the hydrogen bond (shorter bonds lower pK(a) values more) and the chemical nature of the donor ( $\text{COOH} > \text{OH} > \text{CONH}_2$ ). In contrast, neighboring carboxyl groups can either lower or raise the pK(a) values of the catalytic glutamic acids depending upon the electrostatic linkage of the ionization constants of the residues involved in the interaction. While the pH optimum of BCX can be shifted from -1.1 to +0.6 pH units by mutating neighboring residues within the active site, activity is usually compromised due to the loss of important ground and/or transition state interactions. These results suggest that the pH optima of an enzyme might be best engineered by making strategic amino acid substitutions, at positions outside of the "core" active site, that electrostatically influence catalytic residues without perturbing their immediate structural environment.