# Mechanistic Studies of Active Site Mutants of *Thermomonospora fusca* Endocellulase E2†

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ABSTRACT: Endocellulase E2 from the thermophilic bacterium *Thermomonospora fusca* is a member of glycosyl-hydrolase family 6 and is active from pH 4 to 10. Enzymes in this family hydrolyze $\beta$-1,4-glycosidic bonds with inversion of the stereochemistry at the anomeric carbon. The X-ray crystal structures of two family 6 enzymes have been determined, and four conserved aspartic acid residues are found in or near the active sites of both. These residues have been mutated in another family 6 enzyme, *Cellulomonas fimi* CenA, and evidence was found for both a catalytic acid and a catalytic base. The corresponding residues in E2 (D79, D117, D156, and D265) were mutated, and the mutant genes were expressed in *Streptomyces lividans*. The mutant enzymes were purified and assayed for activity on three cellulosic substrates and 2,4-dinitrophenyl-$\beta$-D-cellobioside. Activity on phosphoric acid-swollen cellulose was measured as a function of pH for selected mutant enzymes. Binding affinities for each mutant enzyme were measured for two fluorescent ligands and cellotriose, and circular dichroism spectra were recorded. The results show that the roles of D117 and D156 are the same as those for the corresponding residues in CenA; D117 is the catalytic acid, and D156 raises the p$K_a$ of D117. No specific function was assigned to the CenA residue corresponding to D79, but in E2, this residue also assists in raising the p$K_a$ of D117 and is important for catalytic activity. The D265N mutant retained 7% of the wild-type activity, indicating that this residue is not playing the role of the catalytic base. Experiments were conducted to rule out contamination of the D265 enzymes by either wild-type E2 or an endogenous *S. lividans* CMCase.

The thermophilic bacterium *Thermomonospora fusca* produces six cellulases, E1−E6 (*1*). The X-ray crystal structures of the catalytic domains of both E2 and E4 have been determined (*2*, *3*). Endocellulase E2 is a 43 kDa protein with two globular domains connected by a flexible linker region. The X-ray crystal structure of the 30 kDa catalytic domain was determined at 1.8 Å resolution (*2*) and refined to 1.18 Å (*4*). E2 belongs to glycosyl hydrolase family 6 (*5*), and cleaves with inversion of stereochemistry. The inverting mechanism (*6*) has been proposed to proceed in a concerted fashion with a catalytic base abstracting a proton from a water molecule and the resulting hydroxide ion attacking the anomeric carbon, while a catalytic acid protonates the sugar leaving group.

The crystal structure revealed a catalytic cleft as opposed to the tunnel seen in the family 6 exocellulase, CBHII, from *Trichoderma reesei* (*7*). There are four aspartic acid residues conserved in all family 6 cellulases, which are in or near the active sites of E2 and CBHII. In E2, these residues are D79, D117, D156, and D265. D79 is on a loop 11 Å away from the proposed site of cleavage and is in a position quite different from that of the corresponding residue in CBH II. The other three residues are in similar positions in the two structures. D117 and D265 are on opposite sides of the catalytic cleft and were proposed to be the catalytic acid and catalytic base, respectively. D156 is buried underneath D117 and is proposed to raise the p$K_a$ of D117, which is needed since E2 is active up to pH 10.

The X-ray crystal structure of CBHII and some mutagenesis studies (*8*, *9*) indicate that CBHII D221 (the residue corresponding to E2 D117) functions as the catalytic acid and CBHII D175 (E2 D79) acts to raise the p$K_a$ of the catalytic acid. However, there was no evidence for a catalytic base. There has been considerable work done on another family 6 endocellulase, *Cellulomonas fimi* CenA (*10*). Although no crystal structure for this enzyme has been determined, site-directed mutagenesis data provided evidence for a catalytic acid, CenA D252 (E2 D117), a catalytic base, CenA D392 (E2 D265), and a third residue, CenA D287 (E2 D156), that aided the catalytic acid.

To determine the function of the four conserved Asp residues in E2, they were individually mutated to Ala, Asn, and Glu. These residues were chosen for the following reasons. Ala has the smallest side chain except for Gly (which has no side chain and can adopt $\phi$ and $\psi$ angles that are unusual for other residues) and thus best mimics removal of the side chain. Asn removes the carboxylic acid group but is the least disruptive in terms of size and thus allows examination of the removal of the carboxylic acid with minimal change in the size of the side chain. Glu retains the carboxylic acid but with altered size and geometry. The activities of each mutant enzyme and wild-type E2 were assayed on three cellulosic substrates. In addition, E2 and

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selected mutant enzymes were assayed on 2,4-dinitrophenyl-$\beta$-D-cellobioside (2,4-DNPCB)[1] which has a good leaving group and therefore does not require acid catalysis. Circular dichroism spectroscopy was used to test for gross structural changes in the mutant enzymes. In addition, the binding affinities of each enzyme were determined for $MU(Glc)_2$, $MU(Glc)_3$, and cellotriose. Finally, the $D_2O$ solvent isotope effect was measured to determine if proton transfer was kinetically significant. This study confirms the results of the CenA study for the catalytic acid-related residues, but there is a contradiction with regard to the catalytic base.

## MATERIALS AND METHODS

*Bacterial Strains and Growth Conditions*. The conditions for growth of *Escherichia coli* and *Streptomyces lividans* were as previously described (*11*).

*Construction of E2 Mutations*. All mutations were made by overlap PCR mutagenesis (*12*) except for D265E and D265V, where the Altered Sites *in vitro* Mutagenesis Kit was used (Promega, Madison, WI). Plasmid pSZ61 (*13*), containing the E2 gene and the pLT1 promoter, was used for all PCR mutagenesis, while plasmid pSZ13 (*13*) was used to create D265E and D265V. Mutagenic primers were synthesized by the Cornell Biotechnology Facility (*14*).

Mutant plasmids were isolated from *E. coli* transformants, and the E2 coding regions were sequenced at the Biotechnology Sequencing Facility (Cornell University). Some plasmids were found to contain additional mutations in the E2 coding sequence. The desired mutations were rescued by using restriction enzymes to cut away the sequence with the extra mutation, and then the wild-type sequence was ligated in with T4 DNA ligase.

Once a plasmid with only the desired mutation was isolated, it was transformed into *S. lividans* (*15*) and transformants were screened for stability as described previously (*14*).

*Production and Purification of E2 and E2 Mutant Proteins*. All enzymes were purified from 2 L cultures of *S. lividans* grown for 60 h at 30 °C in tryptic soy broth with 50 $\mu$g/mL thiostrepton as previously described (*16*). The purified enzyme fractions were concentrated with PTGC 10000 NMWL membranes (Millipore), and the protein concentration was determined by measuring the absorbance at 280 nm using an extinction coefficient of 81 800 $M^{-1}$ $cm^{-1}$ (*16*). The circular dichroism (CD) spectrum for each enzyme was recorded at 37 °C in 50 mM potassium phosphate at pH 7.0 as previously described (*13*).

*Fluorescence Binding*. The binding affinities of wild-type and mutant enzymes were determined, for $MU(Glc)_2$, $MU(Glc)_3$, and MUXylGlc, by titrating the enzyme into a solution of the ligand and measuring the decrease in fluorescence caused by binding. The binding affinities for cellotriose were measured by titrating cellotriose into a cuvette containing both enzyme and $MU(Glc)_2$ or MUXylGlc and measuring the increase in fluorescence caused by cellotriose displacing the fluorescent ligand. Fluorescence and displacement titrations were carried out using an Aminco SLM 8000C spectrofluorimeter under conditions where no significant hydrolysis occurred during the measurements, as described previously (*11*).

*CMC, SC, and FP Activity Assays*. All assays were carried out in triplicate. CMC and SC activities were assayed using six different amounts of wild-type enzyme (between 0.1 and 1.6 $\mu$g). For mutant enzymes, the amount that was added was selected to produce an amount of reducing sugars roughly equal to that produced by E2. All assays (400 $\mu$L volume) were incubated for 1 h at 50 °C with either 1% CMC or 0.2% SC in 50 mM sodium acetate buffer at pH 5.5. Activity was calculated as described previously (*13*). The D117 mutant activities on CMC and SC were calculated by comparison of the reducing sugars produced by 0.2 $\mu$g of E2 with that produced by 200 $\mu$g of the mutant enzyme under the conditions described above. The activities on FP were determined by incubating 40 $\mu$g of each enzyme with one 3.4 mg Whatman number 1 filter paper disk in 50 mM sodium acetate (pH 5.5) for 24 h at 50 °C. In all the assays, the amount of reducing sugar that was produced was determined using dinitrosalicylic acid (DNS) as described previously (*16*).

*SC Activity as a Function of pH*. All three D156 mutant enzymes as well as D79N, D265N, and E2 were assayed for activity over the pH range of 3−11. The reactions were carried out at 50 °C for 1 h with 0.2% SC. For pH values from 3 to 8, 100 mM citrate phosphate was used, while for pH values from 8 to 11, 100 mM glycine phosphate was used. The pH stabilities of E2, D156A, and D156N were tested from pH 2.3 to 12. Each enzyme was preincubated in 200 $\mu$L of the appropriate buffer at 50 °C. After 1 h, 10 $\mu$L aliquots of the preincubated enzyme were added to 190 $\mu$L of 1 M sodium acetate (pH 5.5) and assayed with 0.2% SC for 1 h at 50 °C and reducing sugars assessed as described above.

*2,4-DNPCB Assay*. Both $K_m$ and $k_{cat}$ were measured for E2 and D117A on 2,4-DNPCB, which was a gift from the laboratory of S. Withers (University of British Columbia, Vancouver, BC). A Zeiss PM6 Spektralphotometer was used to measure the absorbance at 400 nm since this machine has a narrow beam which allowed for the use of small volume cuvettes (reactions were carried out in 180 $\mu$L volumes to conserve substrate). Reactions were carried out at 50 °C in 50 mM sodium acetate (pH 5.8), and substrate blanks were carried out at 150, 200, 278, and 1000 $\mu$M. The rates, as a function of concentration, formed a straight line, and the slope was used to calculate the blank for all other 2,4-DNPCB concentrations. Assays were carried out using 1.57 $\mu$M enzyme and initial substrate concentrations of 20, 40, 80, 150, 200, and 500 $\mu$M for D117A; for the E2 experiments, substrate concentrations of 1000 and 2000 $\mu$M were also used. The change in absorbance at 400 nm measured every 5 min minus the blank was used as the activity at the substrate concentration at the beginning of each 5 min interval. The concentration of formed product was calculated using an extinction coefficient of 10 910 $M^{-1}$ (*17*) and was subtracted from the initial substrate concentration to determine the substrate concentration at the beginning of the next 5 min interval. For inverting enzymes, there is no transglycosylation so this approach is theoretically sound. To

[1] Abbreviations: CMC, carboxymethyl cellulose; SC, phosphoric acid-swollen cellulose; FP, Whatman number 1 filter paper; 2,4-DNPCB, 2,4-dinitrophenyl-$\beta$-D-cellobioside; DNP, dinitrophenyl; $MU(Glc)_2$, 4-methylumbelliferyl-$\beta$-D-cellobioside; $MU(Glc)_3$, 4-methylumbelliferyl-$\beta$-D-cellotrioside; MU(XylGlc), 4-methylumbelliferyl-$\beta$-D-xylosyl(1−4)-$\beta$-D-glucoside; CD, circular dichroism.

Table 1: Dissociation Binding Constants

| | $k_d$ | | | |
|---|---|---|---|---|
| enzyme | $MU(Glc)_2$ (nM) | $MU(Glc)_3$ (nM) | cellotriose ($\mu$M) | MU(XylGlc) (nM) |
| E2 | 400 | 53 | 130 | 3.8 |
| D79A | 300 | 35 | 77 | ND |
| D79E | 290 | 200 | *a* | ND |
| D79N | 740 | 47 | 90 | ND |
| D117A | 270 | 57 | 120 | ND |
| D117E | 210 | 17 | 250 | ND |
| D117N | 1500 | *a* | *a* | ND |
| D156A | 530 | 35 | 300 | ND |
| D156E | 1000 | 100 | 190 | ND |
| D156N | 390 | 80 | 100 | ND |
| D265A | ND[b] | >50000 | ND | 2800 |
| D265E | ND | ND | ND | 30000 |
| D265N | >50000 | 4500 | 140 | 840 |
| D265V | ND | ND | ND | 50000 |

[a] Curve does not fit the data. [b] Not determined.

demonstrate the validity of this approach, reactions were carried out so that the low substrate concentration of one run overlapped the high substrate concentration of another run.

*Solvent Isotope Effect*. CMC was dissolved in both $D_2O$ and $H_2O$ to a concentration of 1%. Anhydrous sodium acetate was added to yield a concentration of 50 mM, and the pH was measured with pH paper and confirmed to be the same for both the $D_2O$ and $H_2O$ samples. The assays used 0.235 and 0.783 $\mu$g of E2 for 1 h, 0.783 $\mu$g of E2 for 10, 15, 20, and 25 min, and 0.4 $\mu$g of E2 for 5, 10, and 15 min. The amount of reducing sugar was measured using the DNS reagent. In addition, assays were carried out using 200 $\mu$M 2,4-DNPCB as a substrate with either 105 $\mu$g of D79N, 12.4 $\mu$g of D117A, or 200 $\mu$g of D265N for 1 h at 50 °C.

## RESULTS

The CD spectra for all the mutant enzymes could not be distinguished from that of E2 (data not shown), showing that the mutant enzymes had no gross conformational changes with respect to wild-type E2.

*$MU(Glc)_2$, $MU(Glc)_3$, MU(XylGlc), and Cellotriose Binding.* The binding constants for $MU(Glc)_2$, $MU(Glc)_3$, and cellotriose were determined for all mutant enzymes except those for which the data did not fit the model or where the binding affinity was too weak to be measured (Table 1). The binding results show that the D79, D117, and D156 mutant enzymes, with the exception of D117N, have binding affinities essentially equal to those of the wild type. The data for D117N show that it has a 4-fold decrease in affinity for $MU(Glc)_2$, and the theoretical curve did not fit the data for $MU(Glc)_3$ and cellotriose. However, the raw data showed the same decrease in fluorescence after the addition of D117N to a $MU(Glc)_3$ solution that was seen for wild-type E2 which indicates that binding does occur. Collectively, these results indicate that the active sites of the D79, D117, and D156 mutant enzymes are not drastically altered and that the observed changes in activity are probably due to the altered bond-making or bond-breaking ability of the mutants rather than to altered binding properties.

In contrast, all of the D265 mutant enzymes exhibited a marked decrease, 100−1000-fold, in affinity for the MU ligands (Table 1), although the D265N enzyme had wild-type binding affinity for cellotriose. This suggests that the decreased activities of the D265 mutant enzymes could be due to decreased levels of substrate binding.

*CMC, SC, and FP Assays*. The activities of the different E2 mutant enzymes on CMC and SC were affected to varying degrees (Table 2). The D117 mutant enzymes exhibited the largest drop in activity; D117N exhibited a 5000-fold drop in activity on CMC and SC which was at the low end of the sensitivity of this assay. This is consistent with D117 being the essential catalytic acid. There were no significant differences between the activities of the mutant enzymes on CMC or SC, showing that the charge on CMC did not affect catalysis.

Surprisingly, enzymes in which the proposed catalytic base, D265, was mutated to Ala, Glu, or Asn retained from 2 to 10% of the wild-type activity. This demonstrates that this residue is not playing an essential role and thus D265 is probably not the catalytic base.

The D79 mutant enzymes exhibited an about 100-fold decrease in activity on CMC and SC. Clearly, this residue is important for activity despite the fact that the crystal structure of E2cd shows it to be in a loop 11 Å away from the site of bond cleavage (*2*).

D156E was the only mutation that had no effect on CMC and SC activity. It seems that a carboxylic acid side chain in this position is important for optimal activity since the D156A and D156N mutations both decrease activity, but the D156E mutation does not.

The absolute activity of E2 on FP is much lower than the activity on CMC and SC. All mutations of E2 studied here, except D156E, exhibit a much larger percent decrease in their CMC and SC activities than in FP activity which suggests that the rate-limiting step for crystalline cellulose degradation is different from that for amorphous cellulose. This step has been proposed to involve moving a cellulose strand into the active site (*13*). However, if hydrolysis is slowed enough, it could become the rate-limiting step even on FP. It is interesting to note that the D117N enzyme, which has the lowest FP activity of any E2 mutant enzyme, has absolute activities on CMC and SC only 2-fold higher than on FP, whereas E2 has a 1000-fold higher activity on SC than on FP.

*2,4-DNPCB Activity*. The data for E2 fit the Michaelis−Menten equation quite well (Figure 1A). The data for the D117A enzyme are more variable (Figure 1B), and this variability may affect the $K_m$ value; however, it is clear that it does not affect $k_{cat}$ since the data points at 500 $\mu$M are tightly grouped and are not significantly higher than the points at 200 $\mu$M. Despite the 5000-fold decrease in CMC and SC activities, the activity of the D117A enzyme on 2,4-DNPCB is essentially equal to that of E2 (Table 2). This substrate has an excellent leaving group (2,4-DNP) with a p$K_a$ of 3.96 (*18*) and therefore does not require acid catalysis. Michaelis−Menten parameters are typically determined at less than 5% substrate conversion levels; however, the data presented here represent in some cases >50% substrate conversion. There are two lines of argument which justify this method. The first is that inverting enzymes have no reverse reaction. The second is that activity at a given substrate concentration was essentially the same regardless of the initial concentration. For example, the activity around

Table 2: Activities of E2 Wild-Type and Mutant Enzymes

| enzyme | CMC (%) | SC (%) | FP (%) | 2,4-DNPCB[a] $k_{cat}$ ($min^{-1}$) |
|---|---|---|---|---|
| E2 | 100 (433 $min^{-1}$) | 100 (750 $min^{-1}$) | 100 (0.77 $min^{-1}$) | 1.1 |
| D79A | 1.3 | 0.7 | 42 | |
| D79E | 0.4 | 0.6 | 38 | |
| D79N | 3.9 | 2.0 | 55 | 0.14 |
| D117A | 0.03 (0.13 $min^{-1}$) | 0.02 (0.15 $min^{-1}$) | 9 (0.07 $min^{-1}$) | 1.2 |
| D117E | 0.06 (0.26 $min^{-1}$) | 0.06 (0.45 $min^{-1}$) | 17 (0.13 $min^{-1}$) | 0.3 |
| D117N | 0.02 (0.09 $min^{-1}$) | 0.02 (0.15 $min^{-1}$) | 5 (0.04 $min^{-1}$) | 0.3 |
| D156A | 2.2 | 1.0 | 67 | 0.5 |
| D156E | 130 | 120 | 84 | 1.3 |
| D156N | 10 | 8.6 | 74 | 0.5 |
| D265A | 2.5 | 2.0 | 26 | |
| D265E | 2.0 | 1.6 | 35 | |
| D265N | 11 | 7.0 | 56 | 0.05[b] |
| D265V | 0.1 | 0.2 | 18 | |
| D79N/D265N | 0.23 | 0.14 | 27 | |

[a] All 2,4-DNPCB activities except for those of the wild type and D117A were calculated at a single substrate concentration (1000 $\mu$M). [b] Data only reflect the specific activity rather than $k_{cat}$ under the given conditions since it is very likely that D265N is not saturated at 1000 $\mu$M 2,4-DNPCB.

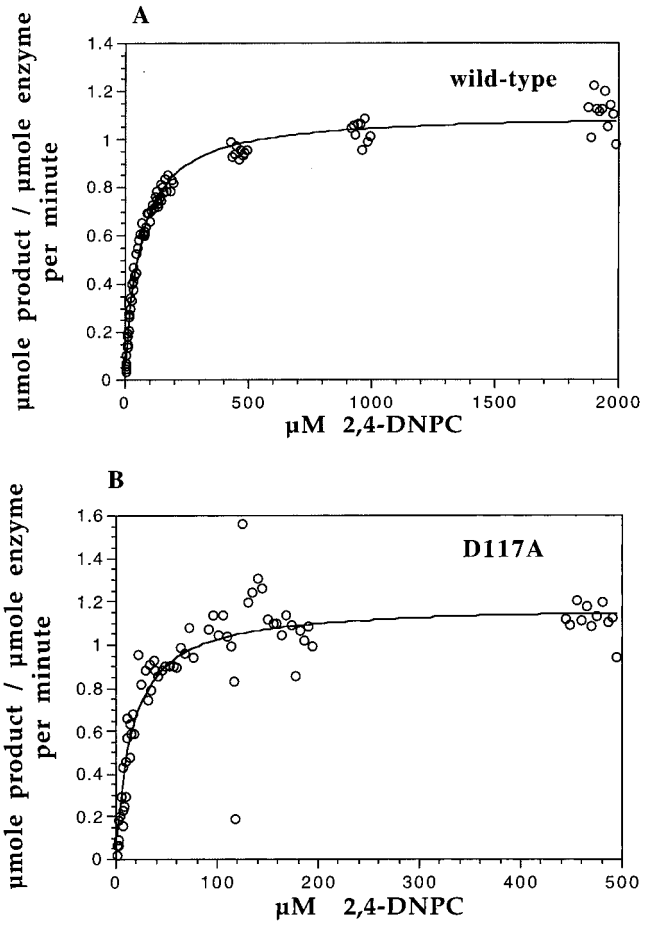


FIGURE 1: (A) Michaelis−Menten curve for wild-type E2 on 2,4-DNPCB. The solid line is the mathematical curve fit which gives values of 1.1 $min^{-1}$ and 59 $\mu$M for $k_{cat}$ and $K_m$, respectively. (B) Michaelis−Menten curve for D117A on 2,4-DNPCB. The solid line is the mathematical curve fit which gives values of 1.2 $min^{-1}$ and 15 $\mu$M for $k_{cat}$ and $K_m$, respectively.

130 $\mu$M was the same whether the reaction started with 150 or 200 $\mu$M 2,4-DNPCB.

The activities of some other mutant enzymes were tested at a 2,4-DNPCB concentration of 1000 $\mu$M (Table 2). The D117E, D117N, and all three D156 mutant enzymes retained a higher percentage of activity on 2,4-DNPCB than on CMC or SC, consistent with their roles in acid catalysis. The D79N enzyme had 14% of the wild-type activity on 2,4-DNPCB

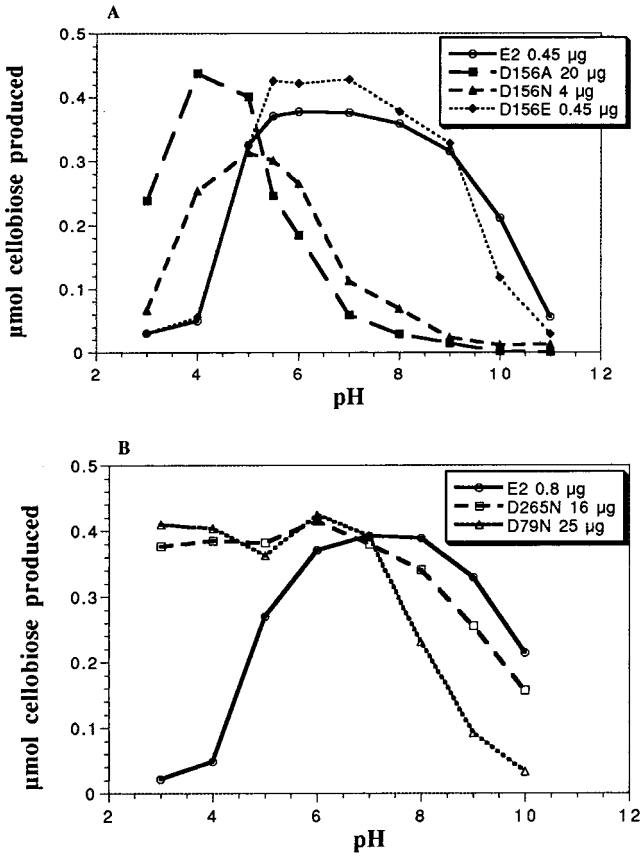


FIGURE 2: Activity on swollen cellulose as a function of pH. The reactions were carried out for 60 min.

compared with only 4% on CMC. The only mutant enzyme tested that had a lower percent activity on 2,4-DNPCB than on CMC was the D265N enzyme, 5 and 11%, respectively.

*pH Profile.* Since D156 was proposed to raise the p$K_a$ of D117, one would expect that mutating this residue would decrease the activity at high pH. Consistent with this role for D156, the broad pH range of E2 is narrowed to a peak centered around pH 4 for the D156A enzyme and pH 5 for the D156N enzyme. However, D156E exhibits only a slight shift at pH 10 (Figure 2A). The changes in activity as a function of pH for the D156A and D156N enzymes are not due to decreased stability since both of these enzymes are stable up to pH 11 (Figure 3). Our interpretation of these

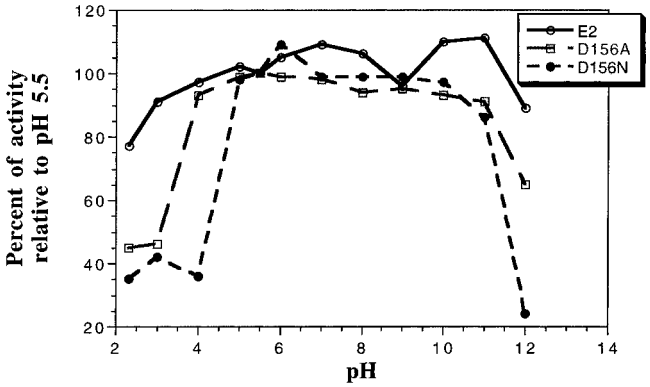


FIGURE 3: pH stability curve for E2, D156A, and D156N. Enzymes were incubated for 1 h at the indicated pH and then assayed as usual at pH 5.5.

results is that D156, due to its proximity to D117, alters the p$K_a$ of D117, allowing D117 to remain protonated at pH values of up to 10. A similar but less pronounced effect is seen with the D79N enzyme (Figure 2B). On the basis of these data, it seems that D79 also alters the p$K_a$ of D117 which is consistent with the results for CBHII (*8*, *9*). However, to do this, the loop containing D79 must move from its position in the crystal structure. When either of two Gly residues in the loop, G86 or G87, were mutated to Ala (*13*), the activities of the mutated proteins decreased markedly, suggesting that the flexibility provided by these Gly residues is important. The pH effect combined with the low activity of the D79 mutants is further evidence that loop mobility is essential for E2 activity. NMR experiments are underway in an attempt to identify the position of D79 after loop movement. The pH activity profile of the D265N enzyme (Figure 2B) is similar to that of E2 at the high pH values, but activity remains constant below pH 5, unlike E2 activity which decreases dramatically. This is also seen with the D79N enzyme.

It is possible that both D79 and D265 could act to hold a water molecule in place as was seen for E4 (*3*). If this were the case, then either one could function as the catalytic base so that in the D79 mutants D265 takes over as the base and, conversely, in the D265 mutants D79 takes over as the base. To test this hypothesis, a D79N/D265N double mutant enzyme was created. The results (Table 2) indicate that D79 and D265 act independently, since on SC the D265N enzyme has 7% and the D79N enzyme has 2% of the wild-type activity while the double mutant has 0.14% of the wild-type activity (2% of 7% is 0.14%). The activity of the double mutant on CMC (0.23%) was also close to the expected value (0.43%) if the residues are independent. Clearly, this double mutant does not give the 20000-fold decrease in rate that was seen in the mutant enzymes of the proposed catalytic base in CenA (*10*).

Since D265 was proposed to be the catalytic base (*2*), the high activity of the D265 mutants was unexpected. One possible explanation is that the D265 mutant enzyme preparations are contaminated with a *S. lividans* CMCase. The D265A, D265N, D79A, and D79N enzymes were assayed by native PAGE along with a sample known to contain the CMCase. The gel was run in duplicate, and one gel was stained with Coomassie Blue and the other tested on a CMC overlay. In all cases, the Coomassie-stained protein band migrated at the same position as an activity band that was well away from the *S. lividans* CMCase band (data not shown). The pH curve for the contaminated sample was also measured and showed a steady decrease in SC activity between pH 6 and 3 (data not shown); however, both D79N and D265N exhibit constant activity in this pH range. In addition, the D265N gene was recently cloned into the pET vector and expressed in *E. coli*. The *E. coli* D265N mutant had the same activity as the *S. lividans*-produced enzyme (data not shown).

*Solvent Isotope Effect*. The inability to find a residue which acts as the catalytic base suggested that proton transfer might not be rate-limiting. A comparison, in $H_2O$ versus $D_2O$, of the CMC activity of E2 and the 2,4-DNPCB activities of D79N, D117A, and D265N yielded isotope effects ($K_H/K_D$) in the range of 1.3 (data not shown). Primary deuterium isotope effects are in the range of 3−7.5 for cleavage of a deuterium−oxygen bond (*19*). The lack of a substantial solvent isotope effect indicates that something other than proton transfer is rate-limiting.

## DISCUSSION

The results of this study confirm some of the conclusions drawn from the CenA study and contradict others (*10*). The roles of D117 and D156 fit well with the results for CenA; however, the roles of D79 and D265 do not. D117 and its counterpart in CenA, D252, have clearly been shown to function as the catalytic acid because mutants of these residues have greatly decreased activity (>5000-fold) on substrates which require acid catalysis and near wild-type activity on a substrate which does not require acid catalysis (2,4-DNPCB). The residue corresponding to D117 in the family 6 cellulase, *T. reesei* CBHII, D221, has also been proposed to function as the catalytic acid (*7*). When this residue was mutated to Ala, the enzyme lost all activity on $MU(Glc)_3$, $MU(Glc)_4$, cellotriose, and cellotetraose (*8*); thus, it seems clear that this conserved residue is the catalytic acid.

The role of D156 was proposed to be that of raising the p$K_a$ of D117 (*2*). Results of the CenA study suggest that D287 (E2 D156) acts to raise the p$K_a$ of D252 (E2 D117). The pH activity curves presented in this paper demonstrate that this is the case for E2. Of the D156 mutants, D156A exhibits the largest drop in activity at pH 5.5 and the most dramatic shift in the pH curve. This is due to the fact that the Ala side chain carries no negative charge to affect the neighboring D117. D156N, which has more activity than D156A at pH 5.5 and has a pH optimum 1 full pH unit higher, also does not have a negative charge. However, the amide side chain of Asn has an oxygen which could carry a partial negative charge, making it more effective than Ala. Finally, D156E, which does have a negative charge, has essentially wild-type activity on all substrates tested and exhibits only a slight change in the pH profile of SC activity.

The apparent p$K_a$ of D117 is about 10. This is very high for an aspartic acid; however, there is precedence for large shifts in Asp p$K_a$ values. An Asp in reduced thioredoxin from *E. coli* has a p$K_a$ of >9 (*20*), and an Asp in ketosteroid isomerase has a p$K_a$ of 9.5 (*21*).

The D79N pH curve provides an additional explanation for the large perturbation in the p$K_a$ of D117 (Figure 2B). This residue despite its position 11 Å away from the site of

bond cleavage in the crystal structure is clearly important for full activity. D79N exhibits a shift in its pH activity curve similar to but less pronounced than those of D156A and D156N. If the loop on which D79 resides moves during substrate binding, D79 could come into a position such that D117 is between D79 and D156, stabilizing its protonation. In support of this idea, the residue corresponding to D79 in CBHII, D175, is proposed to raise the p$K_a$ of D221 (E2 D117).

The anomalous results of the D265 mutants need to be explained. The corresponding residue in CenA, D392, when mutated to Ala gave a decrease in activity of 4 orders of magnitude for both CMC and 2,4-DNPCB, suggesting that it is the catalytic base; however, binding data for the CenA mutants were not reported. Mutation of E2 D265 to Ala decreased its activity by only 2 orders of magnitude. As mentioned in the Results, it was shown in several ways that the mutant D265 enzymes were not contaminated with an *S. lividans* CMCase. In addition, a set of CMC and SC assays was carried out in both 0.05 M BisTris buffer (pH 5.5) and our standard 0.05 M NaAc buffer (pH 5.5) which produced indistinguishable results (data not shown), thus eliminating the possibility that the acetate ions in the buffer could partially replace D265. The reported activities were determined at a set substrate concentration and are not true measures of $k_{cat}$ because determinations of $K_m$ and $k_{cat}$ on CMC and SC are not possible due to the nonlinear kinetics with these substrates. The data for the binding of the D265 mutant enzymes to the MU ligands strongly suggest that D265 plays an important role in binding. This could well account for the lowered activities of the mutant enzymes, but the activities are too high for D265 to be the catalytic base.

One way to reconcile the results of this study with those of the CenA study is to conclude that, despite their classification into the same cellulase family, E2 and CenA have somewhat different reaction mechanisms. The CenA D392E mutant was made but was unstable and could not be isolated, and construction of a CenA D392N mutant was not reported. This would be expected to be the most stable of the D392 CenA mutants, and it could provide helpful evidence concerning the role of D392 in CenA. One other possibility is that despite binding to a *S*-cellobiose affinity column and a wild-type CD spectra, the D392A CenA mutant enzyme is not very stable and the loss of activity is due to inactivation. From the three-dimensional structures, it would appear that there are no residues other than D265 in E2 or its equivalent in CBH II (D401) that are positioned to function as a catalytic base and that at least some family 6 cellulases may actually lack this aid to catalysis.

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