Site-Directed Mutation of the Putative Catalytic Residues of Endoglucanase CenA from *Cellulomonas fimi*†

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ABSTRACT: The catalytic domains of β-1,4-glucanases can be grouped into families of related amino acid sequences. The endoglucanase CenA from *Cellulomonas fimi* is a member of family B. All enzymes from this family are believed to hydrolyze β-1,4-glucosidic bonds using a general acid−base catalytic mechanism resulting in inversion of anomeric configuration at the scissile bond. Three-dimensional structures for two cellulases from family B have been determined by X-ray crystallographic analysis. These structures show that there are four Asp residues which are in a position to function as acid catalyst, base catalyst, and/or transition state stabilizers. These aspartates are conserved in all members of family B. The roles of Asp216, Asp252, Asp287, and Asp392, the corresponding amino acids in CenA, were determined. These aspartates have been systematically replaced with alanine and glutamate via site-directed mutagenesis, and the resulting effect on activity, substrate specificity, and overall structure has been determined. Changes in overall structure were monitored using circular dichroism spectroscopy, and no significant differences between the wild-type and mutant proteins were found. Active site structure was also found to be intact as all proteins bound to a cellobiose affinity column. The Michaelis−Menten parameters of the enzyme were determined on 2,4-dinitrophenyl cellobioside as well as (carboxymethyl)-cellulose and phosphoric acid-swollen cellulose. Initial characterization of mutant proteins indicates that Asp252 and Asp392 are the acid and base catalysts, respectively, in CenA. Residue Asp287 appears to aid Asp252 in acid catalysis, and Asp216 is not absolutely required for catalysis.

Cellulases are capable of hydrolyzing β-1,4-glucosidic bonds and also serve to disrupt the crystalline structure of cellulose (Béguin et al., 1987). Enzymes which hydrolyze internal β-1,4 bonds are classed as endoglucanases; those releasing cellobiose units from the ends of cellulose chains are exoglucanases or cellobiohydrolases.

The Gram-positive soil bacterium *Cellulomonas fimi* produces an array of cellulases when grown on cellulose (Béguin et al., 1977). The genes encoding four endoglucanases (CenA, CenB, CenC, and CenD), two exoglucanases, (CbhA and CbhB), and one xylanase/exoglucanase (Cex) have been cloned in *Escherichia coli* and sequenced (Shen, unpublished data; Whittle et al., 1982; O'Neill et al., 1986; Wong et al., 1986; Owolabi et al., 1988; Coutinho et al., 1991; Meinke et al., 1993, 1994). These enzymes are all multidomain proteins containing discrete cellulose binding and catalytic domains.

Endoglucanase CenA comprises two domains, a catalytic domain and a cellulose binding domain, separated by a short linker sequence composed principally of prolyl and threonyl residues, the Pro-Thr linker (Wong et al., 1986). Alone, CenA has weak activity on crystalline cellulose but is relatively active on PAS-cellulose[1] and CM-cellulose (Meinke et al., 1993).

The catalytic domains from different glycoside hydrolases can be grouped into families of related amino acid sequences (Béguin, 1990; Gilkes et al., 1991; Henrissat, 1991). The catalytic domain of CenA is a member of family 6. This family also includes exoglucanase CBHII from *Trichoderma reesei* (Chen et al., 1987; Teeri et al., 1987) and endoglucanase E2 from *Thermomonospora fusca* (Lao et al., 1991).

Hydrolysis of a β-1,4-glucosidic bond occurs with retention or inversion of the anomeric configuration of the product released (Koshland, 1953). In all cases examined, members of a given family catalyze hydrolysis with the same stereoselectivity, suggesting that their active sites have similar topologies (Claeyssens & Henrissat, 1992; Gebler et al., 1992). Family 6 members tested, including CenA, are inverting enzymes. Such a reaction is believed to involve a direct displacement of the leaving group by water, via an oxocarbonium-like transition state, and is aided by both general acid and general base catalysis (Sinnott, 1990). A general acid catalyst protonates the leaving group as it departs, and a general base deprotonates the incoming water, in a concerted fashion. Aspartic acid and glutamic acid residues are the most likely candidates for these roles as they appear to be involved in hydrolysis by many glucosidases.

Of the family 6 enzymes, CBHII and E2 are the only members for which detailed three-dimensional structures have been described (Rouvinen et al., 1990; Spezio et al.,

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[1] Abbreviations: PAS-cellulose, phosphoric acid-swollen cellulose; CM-cellulose, (carboxymethyl)cellulose; DNPC, 2′,4′-dinitrophenyl β-D-cellobioside; DNP, dinitrophenyl; IPTG, isopropyl β-D-thiogalactopyranoside; CD, circular dichroism.

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1993). These structures do indeed show similar topologies, and many of the amino acids which are conserved throughout family 6 are located at the active sites of CbhII and E2. Conserved amino acids that are not near the active site probably play other important roles such as maintaining structure. There are four conserved aspartate residues in the active site of both proteins that could have catalytic roles. The function of these residues in catalysis has not been determined conclusively.

Another method of identifying the amino acids which are important for catalysis involves kinetic analysis of single-residue mutant forms of the enzyme. In this way, a direct and quantifiable assessment of the contribution of each individual amino acid toward catalysis can be made. This technique has been used for a number of β-glycosidases, including β-glucosidase from *Agrobacterium faecalis* (Trimbur et al., 1992; Withers et al., 1992), CelD from *Clostridium thermocellum* (Chauvaux et al., 1992), and Cex from *Cellulomonas fimi* (MacLeod et al., 1994; Tull et al., 1994). If an amino acid believed to be catalytically important is mutated without affecting activity significantly, it is not catalytically important. Unfortunately, the reverse is not always true. The discovery of a mutation which causes a protein to have lower activity does not conclusively prove that the changed amino acid is catalytically important. The activity could be lower because the changed amino acid is involved in enzyme stabilization, substrate binding, etc. Alone, this technique can only be used to rule out noncatalytic residues and suggest putative catalytic residues.

To determine their participation in hydrolysis by CenA, Asp216, Asp252, Asp287, and Asp392 were changed to alanine and glutamic acid. Catalytic parameters for each of the mutants and for wild-type CenA were determined using a substrate which does not need acid catalysis, DNPC, as well as with substrates which do need acid assistance for efficient cleavage, CM-cellulose and PAS-cellulose. Structural integrity was monitored using circular dichroism spectroscopy and by evaluation of binding to a cellobiose affinity column.

MATERIALS AND METHODS

Strains, Plasmids, Culture Conditions. *Escherichia coli* strains JM101 (Vieira & Messing, 1988) and RZ1032 (Kunkel et al., 1987), phage M13mp18::*cen*A (Shen et al., 1991), and plasmid pTugKS (Graham et al., 1994) were described previously. Plasmid pTZ18R::*cen*A.N was obtained from D. Nordquist. Plasmid pTugKS::*cen*A.N was constructed by isolating the *Nhe*I−*Hin*dIII fragment from pTZ18R::*cen*A.N and inserting it into pTugKS. All strains harboring plasmids were grown in Luria broth (Miller, 1972) supplemented with 100 μg of ampicillin/mL (pTZ18R::*cen*A.N) or 50 μg of kanamycin A/mL (pTugKS::*cen*A.N).

DNA Techniques and Mutagenesis. Isolation of all plasmid and M13 RF DNA was according to Sambrook *et al.* (1989). Single-stranded DNA was isolated as described previously (Trimbur et al., 1992). Uracil-containing, single-stranded DNA was generated in RZ1032 as host. Site-directed mutants were generated by the method of Kunkel (Kunkel et al., 1987) using T7 DNA polymerase.

Mutation of Asp252 to alanine and glutamate was carried out using the redundant, phosphorylated oligonucleotide primer [pATCCTCGAGCCC(GA)(AC)(CG)GCGCTCGCG-CAG] and single-stranded pTZ18R::*cen*A.N template DNA. The remaining mutations were generated using single-stranded M13mp18::*cen*A template DNA and the following phosphorylated oligonucleotide primers: (i) pATCCCGGGC-CGCGCCTGCGGATCCCACTCCGGCGGT; (ii) pATC-CCGGGCCGCGAGTGCGGATCCCACTCCGGCGGT; (iii) pCGCGTCTACATCGCCGCGGGGCATGC-GAAGTGGCTG; (iv) pCGCGTCTACATCGAGGCGGGC-CACGCG; (v) pCCCGGCGAGTCCGCGGGGCGCGTG-CAAC; and (vi) pCCCGGCGAGTCCGAGGGCGCAT-GCAACGGCGGCCCG. Some of these primers incorporated unique restriction sites into the DNA without altering the amino acid coding sequence. After *in vitro* mutagenesis, the double-stranded DNA was transformed into JM101. Transformants were screened either for reduced CM-cellulase activity using Congo Red staining or by restriction site analysis.

Positive mutants were sequenced by the procedure of Tabor and Richardson (1987). Deaza-7-dGTP and deza-7-dATP replaced dGTP and dATP, respectively, and the reaction was carried out at 43 °C. Once sequenced, DNA fragments containing the appropriate mutations were isolated and inserted into wild-type pTZ18R::*cen*A.N. A *Nhe*I−*Hin*dIII fragment was then isolated and inserted into pTugKS::*cen*A, a high-expression vector.

Protein Production and Purification. Mutants were grown in Terrific broth (Sambrook et al., 1989) containing 50 μg/mL kanamycin and 0.1 mM IPTG (Sigma, St. Louis, MO). Cultures were grown at 37 °C for 18−24 h by which time CenA had leaked into the medium (Guo et al., 1988). The protein was precipitated from the clarified supernatant with 50% $(NH_4)_2SO_4$. Proteins were recovered by centrifugation (10 000 rpm), and the pellet was resuspended in binding buffer (5 mM imidazole, 500 mM NaCl, and 20 mM Tris-HCl, pH 7.9). After further centrifugation (40 000 rpm), the clarified supernatant was loaded onto a pET Ni^{2+} column (Novagen, Markham, Ontario). The column was washed with wash buffer (60 mM imidazole, 500 mM NaCl, and 20 mM Tris-HCl, pH 7.9). Bound proteins were eluted with a gradient of imidazole (0−500 mM) in Tris-HCl/NaCl buffer (20 mM/500 mM, pH 7.5). The fractions were screened for CenA by SDS−PAGE using a 12% gel. Fractions containing CenA were combined, and EDTA was added to 10 mM. The protein was concentrated by ultrafiltration using a PM10 membrane (Amicon, Beverly, MA), and the buffer was exchanged with potassium phosphate buffer (50 mM, pH 7) by dialysis. The concentrated protein solution was centrifuged at 40 000 rpm for 30 min and then filtered through a 0.2 μm filter (Gelman Sciences, Ann Arbor, MI).

Protein purity was assessed by SDS−PAGE using a 12% gel (Bio-Rad, Richmond, CA). The concentration of CenA was determined by measuring the absorbance at 280 nm using an extinction coefficient of 2.64 mL mg^{-1} cm^{-1} (Gilkes et al., 1992). Mutant protein concentrations were measured using the BCA protein determination kit (Pierce, Rockford, IL), with wild-type CenA as the standard.

Kinetic Characterization. Kinetic parameters for the hydrolysis of 2,4-DNPC were obtained as described previously (Kempton & Withers, 1992) in 50 mM potassium phosphate buffer at pH 7. Activity on CM-cellulose was measured using 0.4% CM-cellulose in sodium citrate buffer (50 mM, pH 7), at 37 °C. Activity on PAS-cellulose was determined using 10 mg of PAS-cellulose/mL in citrate buffer at 37 °C.

Reducing sugars were quantified with the *p*-hydroxybenzoic acid hydrazide reagent (Lever, 1972).

Cellobiose Affinity Chromatography. A cellobiose affinity column was prepared by linking *p*-aminobenzyl-thiocellobioside to Sepharose 4B (Tomme et al., 1988). Proteins were loaded onto the column in potassium phosphate buffer (50 mM, pH 7). After the column was washed with 50 mM phosphate buffer, bound proteins were eluted with 100 mM cellobiose in phosphate buffer.

Circular Dichroism Spectroscopy. CD spectra were recorded with a Jasco J-720 spectropolarimeter controlled with J-700 software. The spectra were obtained at a protein concentration of 2.2 μM in 1.25 mM potassium phosphate buffer, pH 7, 25 °C, using a 1 mm path length. They were recorded 3 times from 250 to 200 nm, at a scan rate of 20 nm/min using a 2.0 s response and a sensitivity of 10 mdeg.

RESULTS AND DISCUSSION

Mutagenesis and Isolation of Mutants. The mutations at Asp252 were made using a redundant oligonucleotide primer. Possible mutants were screened for halos on CM-cellulose plates, and colonies with reduced halos, indicative of reduced activity, were picked. Colonies with reduced halos corresponded to Asp252Glu. Colonies with no halos were found to be either deletion mutants or the Asp252Ala mutant. Mutations at Asp216, Asp287, and Asp392 were made with individual oligonucleotide primers containing unique restriction sites and M13mp18::cenA single-stranded template DNA. Appropriate mutations were screened by digestion of putative mutant double-stranded DNA with appropriate restriction enzymes. DNA sequencing showed that only the single, desired mutation was present in all mutants.

Protein Production and Purification. Although all cultures were grown in a similar manner, the mutant proteins were expressed at quite different levels compared to the wild-type protein. The Asp392Glu mutant protein was not obtained, possibly because it was unstable in *E. coli*. Previously, CenA was purified by cellulose affinity chromatography using 8 M guanidinium chloride for desorption. Although no loss in activity occurred with the wild-type protein under these conditions, mutant proteins could be affected. For this reason, another purification scheme was utilized. Purification of CenA was greatly facilitated using the N-terminal histidine tag encoded by the pTug vector. This allowed for one-step purificataion on a pET Ni^{2+} column with elution of the protein under nondenaturing conditions. No significant differences in behavior during purification could be detected. Each protein ran as a single band on SDS−PAGE (>95% purity by inspection), at the same position as the native enzyme. CenA is a modular enzyme; the catalytic domain retains activity when separated from the N-terminal cellulose binding domain by proteolysis (Gilkes et al., 1988, 1989). The histidine tag should not affect the activity of CenA because it was attached to the cellulose binding domain at the N-terminus of the enzyme. The kinetic parameters on DNPC for CenA, with and without a histidine tag, were identical (unpublished data).

Structural Studies. CD spectra (Figure 1) of all the mutants were essentially indistinguishable from that of the native enzyme, indicating that no gross conformational changes resulted from the substitutions. Integrity of the active site structure was investigated by passing the various

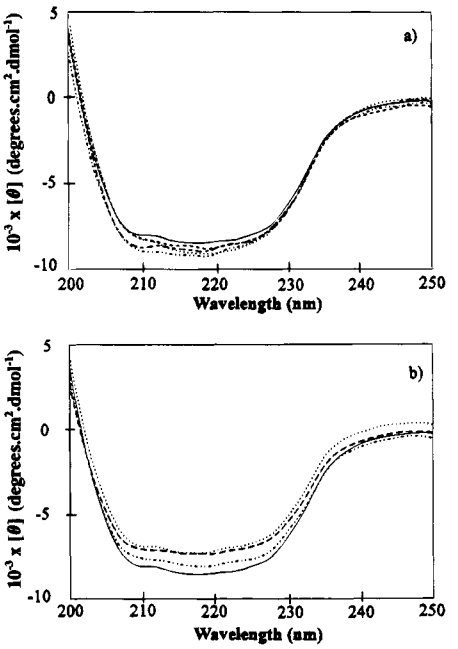

FIGURE 1: CD spectra of CenA and mutants. The data are expressed in terms of mean residual ellipticity (θ). (a) Wild type CenA (—), Asp216Ala (— • —), Asp252Ala (- - -), Asp287Ala (▪▪), and Asp392Ala (— - —). (b) Wild type CenA (—), Asp216Glu (— • —), Asp252Glu (— —), Asp287Glu (▪▪).

mutant proteins through a cellobiose affinity column. CenA was found to bind tightly to the column and could only be eluted with 100 mM cellobiose. All of the mutant proteins also bound to the affinity column and could be removed with 100 mM cellobiose. The cellulose binding domain from CenA did not absorb to the column. Therefore, it was the catalytic domain that bound to the affinity column, presumably through the active site. CBHII also binds to this affinity matrix (P. Tomme, personal communication), and cellobiose inhibits E2 by binding to the active site (Spezio et al., 1993). The active sites of the CenA mutants appeared to be relatively unaltered.

Kinetic Studies. A number of amino acids are conserved in the catalytic domains of family 6 cellulases, probably because they play important roles in catalysis or in maintaining correct structure. Glutamic and aspartic acids appear to be involved in the mechanisms of many glucosidases, and among the 27 carboxylic residues present in the catalytic domain of CenA, 8 are conserved in all family 6 proteins. Of these, four amino acids, Asp216, Asp252, Asp287, and Asp392, are possibly involved in catalysis, based on the location near the active site of the corresponding residues in E2 from *Thermomonaspora fusca* (Spezio et al., 1993) and CBHII from *Trichoderma reesei* (Rouvinen et al., 1990). Although located near the active site, the exact function of each residue cannot be determined by structural data alone.

Hydrolysis by inverting glycosidases such as CenA requires both acid and base catalysis. The general base catalyst removes a proton from water, thus making it more nucleophilic, as the water attacks the anomeric center of the glucoside. In a concerted process, proton transfer occurs from the acid catalyst to the glycosidic oxygen as the glycosidic linkage is cleaved, thereby facilitating bond cleavage through stabilization of the leaving group. Because these catalytic residues are mechanistically important, their modification will affect the rate of catalysis. The extent to

Table 1: Michaelis–Menten Parameters for the Hydrolysis of 2′,4′-Dinitrophenyl β-D-Cellobioside by CenA

	CenA	D216A	D216E	D252A	D252E	D287A	D287E	D392A
k_{cat} (min^{-1})	22	1.2	0.050	18	1.1	0.60	8.8	<0.0010
K_m (mM)	0.17	0.38	0.11	0.010	0.030	0.10	0.14	
k_{cat}/K_m	130	3.2	0.50	1400	34	5.9	62	
x-fold decrease in k_{cat}		18	440	1.2	20	37	2.5	>22000

Table 2: Activities for the Hydrolysis of (Carboxymethyl)cellulose and Phosphoric Acid-Swollen Cellulose by CenA

	CenA	D216A	D216E	D252A	D252E	D287A	D287E	D392A
CM-cellulose[a]								
k_{cat} (min^{-1})	1700	13	1.2	0.0078	0.60		530	0.050
x-fold decrease in k_{cat}		130	1400	220000	2900	17000	3.0	34000
PAS-cellulose[b]								
k_{cat} (min^{-1})	640	0.50	0.40	ND[c]	0.40	0.050	470	0.020
x-fold decrease in k_{cat}		1300	1600	ND	1600	13000	1.4	32000

[a] As micromoles of glucose equivalents released per minute per micromole of enzyme at 0.4% CMC and determined using HBAH reagent. [b] As micromoles of glucose equivalents released per minute per micromole of enzyme at 1.0% PASC and determined using HBAH reagent. [c] ND, not determined.

which the rate is affected will depend on the substrate being hydrolyzed. Substrates having aglycones of high pK_a value, and, therefore, poor leaving group ability, will be affected greatly by removal of the acid catalyst. Those with aglycones of low pK_a, which need little or no protonic assistance for departure, will be affected very little, if at all. This has been shown in the case of a "retaining" glycosidase (MacLeod et al., 1994). By contrast, removal of the base catalyst should have an effect on all substrates as deprotonation of water is crucial for catalysis.

Michaelis–Menten parameters for hydrolysis of DNPC by each of the mutated proteins, as well as by wild-type CenA, are presented in Table 1. The activities of each protein on CM-cellulose and PAS-cellulose are presented in Table 2.

The k_{cat} value for Asp252Ala on CM-cellulose, a substrate with a sugar leaving group and thus requiring acid catalysis, was reduced at least (2×10^5)-fold relative to wild type. This was by far the largest decrease in activity observed. Whether the residual activity is from the mutant or is due to contaminating wild-type protein could not be determined; thus, the value determined must be considered as a minimum estimate. Because the activity was so low on CM-cellulose, this mutant was not assayed on PAS-cellulose. In contrast to CM-cellulose, the k_{cat} value of Asp252Ala with DNPC is almost the same as that of the wild-type enzyme. This is consistent with a role of this residue as general acid catalyst. Interestingly, the K_m value for DNPC with this mutant was considerably lower than with wild type. The origin of this improved binding is unclear, though it is tempting to attribute it to improved interactions of the aromatic leaving group with a less polar active site. The X-ray structures for both CBHII and E2 also show that the corresponding residues, Asp221 and Asp117, respectively, are located in a position to act as the general acid (Rouvinen et al., 1990; Spezio et al., 1993). In E2, the carboxyl oxygen of Asp117 is 4.5 Å from the hydroxyl group on the reducing end of cellobiose. Mutagenesis experiments on Asp221 in CBHII show that this residue is essential for activity on CM-cellulose, as all activity was lost when it was mutated to alanine (Rouvinen et al., 1990).

The mutant Asp392Ala showed the second largest decrease in activities. The k_{cat} values for this mutant on CM-cellulose and PAS-cellulose were reduced about (3×10^4)-fold, respectively, while virtually no activity could be detected when it was assayed with DNPC. Because this residue is important for activity both on substrates having very poor leaving groups (CM-cellulose and PAS-cellulose) and on substrates with excellent leaving groups (DNPC), it is likely that it acts as the general base catalyst in the proposed mechanism. The incoming water molecule is not very nucleophilic and therefore cannot displace the leaving group on its own. The general base is required to deprotonate the incoming water molecule, thus making it more nucleophilic. The X-ray crystal structures of both CBHII and E2 show that the residues corresponding to Asp392, Asp401, and Asp265, respectively, are in a position to act as general base. In E2, Asp265 is 5.0 Å from the anomeric carbon on the reducing end of cellobiose and 5.7 Å from the hydroxyl group, allowing enough space for a water molecule. This residue is also found to form a salt bridge with Arg221 and is therefore very likely in the deprotonated form (Spezio et al., 1993).

The third largest decrease in activity upon mutation was that for Asp287Ala, where k_{cat} values were reduced (2×10^4)- and (1×10^4)-fold on CM-cellulose and PAS-cellulose, respectively. This residue is obviously important for activity on these substrates but is not as crucial as Asp252 or Asp392. The k_{cat} value on DNPC was reduced only 37-fold while the K_m value remained virtually unchanged. Because there is a much larger decrease in activity on substrates requiring acid catalysis, it is likely that this residue in some way aids the acid catalyst. Perhaps this is done by providing the correct environment to raise the pK_a of Asp252, thus keeping it protonated and able to act as an acid. Indeed, the structure for E2 reveals that the corresponding residue, Asp156, is only 3.8 Å from the putative acid, Asp117, and thus may well play a role in modulating the placement or the acidity of the acid catalytic group. This residue appears too buried to act as the acid catalyst itself as it is 9.6 Å from the hydroxyl group on the reducing end of cellobiose (Spezio et al., 1993).

The k_{cat} values for Asp216Ala were reduced on all substrates tested but only by 18-, 132-, and 1380-fold on DNPC, CM-cellulose, and PAS-cellulose, respectively. Because the mutants retain considerable activity, it is unlikely that this residue is directly involved in catalysis. Mutation of the corresponding residue in CBHII was found to reduce

the rate of hydrolysis of cellotriose some 5-fold, which is comparable to the effect in CenA (Rouvinen et al., 1990). The K_m value of this mutant on DNPC is approximately the same as for the wild-type enzyme, which may indicate that Asp216 is unimportant in binding this substrate. In the structure of CBHII, the corresponding amino acid, Asp175, is positioned near the acid catalyst, where it may serve to raise the pK_a of Asp221 (the acid catalyst). In the structure for E2, the corresponding amino acid, Asp79, is 11 Å away from the acid catalyst, Asp117, and is therefore unlikely to have such an effect upon it. Certainly, the catalytic properties of the Asp216Ala mutant of CenA are more consistent with an indirect role for this residue, as suggested by the structure of E2. It may also be that the structure for E2 changes when an actual substrate is bound, thus bringing Asp79 as close to Asp117 as it is in CBHII. Further analysis may reveal a role for this amino acid, which is likely to be important as it is conserved throughout the family.

Mutation of Asp216, Asp252 or Asp287 to Glu decreases the k_{cat} for all substrates. The extra methylene group could shift the carboxyl group so that it is no longer in a position to function correctly. It may also cause steric congestion as there may not be enough room in the enzyme to accommodate the larger side chain. However, the decrease in activity of Asp287Glu is quite small on all substrates, suggesting that there is more space in this area of the active site to accommodate this change.

The use of substrates with different leaving groups allows elucidation of the roles of the conserved aspartate residues in the active site of CenA. Asp252 and Asp392 are the acid and base catalyst, respectively. Asp287 plays a role in hydrolysis, probably by serving to increase the pK_a of Asp252. Asp216 is not required for catalysis on any of the substrates tested and is likely not catalytically important.

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