8396 *Biochemistry* **2009,** *48,* 8396–8404
DOI: 10.1021/bi900991h

BIOCHEMISTRY
including biophysical chemistry & molecular biology
Article

Mechanistic Analysis of the Blood Group Antigen-Cleaving *endo*-β-Galactosidase from *Clostridium perfringens*[†]

Fathima Aidha Shaikh,[‡] Mialy Randriantsoa,[§] and Stephen G. Withers*[,‡]

[‡]*Department of Chemistry, 2036 Main Mall, University of British Columbia, Vancouver V6T 1Z1, Canada, and* [§]*Centre de Recherches sur les Macromolécules Végétales (CERMAV - CNRS), affiliated with Joseph Fourier University, BP 53, 38041 Grenoble Cedex 9, France*

Received June 12, 2009; Revised Manuscript Received July 22, 2009

ABSTRACT: The A and B antigens are of vital importance in blood transfusion and organ transplantation. The specificity of EABase, an *endo*-β-galactosidase from *C. perfringens*, toward the cleavage of A and B trisaccharides from glycoconjugates is unique and holds significant potential for use in modifying blood group antigens on cell surfaces. The mechanism of this enzyme and others in its family (GH98) and the identities of its catalytic residues have not previously been experimentally determined. Direct ^{1}H NMR analysis of the hydrolysis of a synthetic substrate, DNP-β-A-trisaccharide, by EABase revealed that EABase is an inverting *endo*-β-galactosidase. Both activated and nonactivated substrates were used to kinetically characterize EABase and its mutants (E354A, D429A, D453A, E467A, and E506A) at pH 6.0, 37 °C. Hydrolysis of DNP-β-A-trisaccharide by EABase follows normal Michaelis−Menten kinetics with an apparent K_M of 64 ± 3 μM and a k_{cat} of 105 ± 5 min^{-1}. Mutation of two putative active site residues, D453 and E506, to alanine resulted in complete loss of activity, strongly suggesting that one or both of these residues functions as the base catalyst. The kinetic data also strongly suggest that E354 is the acid catalyst since the activity of the E354A mutant with nonactivated natural substrates is 1100-fold lower than that of the wild type enzyme, while its activity is only 10-fold lower when assayed with an activated aryl glycoside substrate (DNP-β-A-trisaccharide). Further support is obtained through comparison of pH profiles for the wild type and E354A mutants: mutation of the acid catalyst eliminates the basic limb from the bell-shaped pH-dependence of k_{cat}/K_M seen for the wild type enzyme.

Clostridium perfringens, a gram-positive pathogenic bacterium, is commonly found in soil, marine sediment, insects, and the intestines of mammals and humans (*1*, *2*). Infection by *C. perfringens* is known to cause food-poisoning and gas gangrene (*2*−*5*). Gas gangrene is caused by the release of enterotoxins and hydrolases by *C. perfringens* to destroy tissues in the host (*4*−*8*). One hydrolase of interest, which was isolated by Anderson et al. as a contaminant within commercial preparations of sialidases from *C. perfringens*, is EABase[1], an *endo*-β-galactosidase capable of liberating both the A and B trisaccharides from glycoconjugates (*9*). Because of the lack of significant amino acid sequence similarity to the other 97 CAZY glycoside hydrolase families identified at that time, the authors assigned EABase to a new glycoside hydrolase family, GH98 (*10*). The mechanism of enzymes within this family and the identities of the catalytic residues have yet to be determined experimentally (*9*). The specificity of EABase toward cleavage of A- and B-type trisaccharides from glycoconjugates is unique and holds significant potential for use in modifying blood group antigens on cell surfaces as well as for use as a synthetic tool.

The ABO blood groups differ through the identities of the sugar antigens on the surface of red blood cells: a terminal α-1,3-linked *N*-acetylgalactosamine within the sequence GalNAcα-1,3-(Fucα1,2)-Galβ- for the A-antigen or an α-1,3-linked galactose within the sequence Galα-1,3-(Fucα1,2)-Galβ- for the B-antigen, both of which are absent in the O-blood type (Fucα1,2-Galβ-) (*11*−*13*). Since all individuals have antibodies to the antigen that they lack, transfusing the incorrect blood type or an organ from a donor with the incorrect blood type results in the lysis of the incompatible blood cells and/or an immune response attacking the implanted organ, which can be fatal (*14*−*18*). Thus, these antigens are of vital importance in blood transfusion and organ transplantation. Current methods of modifying blood types involve the conversion of A- or B-types to type O through the use of glycosidases. The first example of this was the use of an α-galactosidase from coffee beans to convert blood type B cells to blood type O (*19*). The major limitations of this method included the large (and costly) quantities of enzyme required and the inability to convert blood type A cells to O. Liu et al. made a significant advance in the conversion of A/B-types to O through their production of

[†]This work was supported by the Natural Sciences and Engineering Research Council of Canada (NSERC), the Canadian Institutes for Health Research, and the Michael Smith Foundation for Health Research (MSFHR) F.S. acknowledges support for a doctoral fellowship from the Michael Smith Foundation for Health Research (MSFHR). S.G.W. acknowledges support from the Canada Research Chairs Program, the Canada Foundation for Innovation. and the B.C. Knowledge Development Fund.

*To whom correspondence should be addressed. Phone: (604) 822 3402. Fax: (604) 822 8869. E-mail: withers@chem.ubc.ca.

[1]Abbreviations: EABase, blood group antigen A/B cleaving *endo*-β-galactosidase; DNP-, dinitrophenyl-; CAZY, carbohydrate active enzymes; CD, circular dichroism; GH, glycoside hydrolase; GalNAc, *N*-acetylgalactosamine; Gal, galactose; NMR, nuclear magnetic resonance; PCR, polymerase chain reaction; TLC, thin layer chromatography; HBAH, parahydroxybenzoic acid hydrazide.

 Published on Web 07/24/2009

recombinant glycosidases, from *B. fragilis* and *E. meningosepticum*, which cleave both A and B monosaccharides (GalNAc and Gal) efficiently while leaving the blood cell intact and fully functional (*20*). The engineering of highly efficient enzymes that are capable of transferring the A and B antigens onto red blood cells or modifying pre-existing antigens on the cells would help overcome the shortage of donated blood by allowing interconversion between blood types. Such approaches would also be useful in the synthesis of artificial blood or in the generation of related antigens on other cell types (*11*, *12*). EABase was shown by Anderson et al. to be capable of hydrolyzing the intact oligosaccharide from intact cell surfaces. The unique substrate specificity of EABase, therefore, makes it an attractive target for use in the transfer of complex and biologically relevant sugars onto cell surfaces, either with the native enzyme or through the use of mutant forms (glycosynthases) engineered for this purpose. Such enzymes would overcome the need for a series of glycosyltransferases or a series of glycosynthases which transfer a single sugar at a time onto the cell surface (*21*). Instead, an intact oligosaccharide could be transferred in one step.

Glycosynthases are generated by mutating the nucleophilic residue of a retaining hydrolase to a non-nucleophilic residue, thereby eliminating hydrolytic activity (*22*−*24*). When glycosyl fluorides of anomeric configuration opposite to that of the natural substrate are used as donors with these mutant enzymes, they efficiently catalyze the transfer of this sugar onto suitable acceptors (*22*−*24*). While the majority of glycosynthases have been derived from retaining enzymes, a couple of examples of inverting glycosynthases have been recently published in which the catalytic base was mutated, as had been predicted (*25*−*27*). In order to develop a glycosynthase from EABase, the catalytic mechanism and catalytic residues of this enzyme need to be identified.

EABase has been assumed to act via a retaining mechanism on the basis of a paper by Rigden who used amino acid sequence information to make predictions (*28*). Through this, with no experimental data, the catalytic nucleophile and acid/base residues in the case of a retaining mechanism were suggested to be E354, D467, and D429 (*28*). The aims of this article are to experimentally determine the mechanism of EABase through direct ^{1}H NMR monitoring of the enzymatic reaction and to identify the catalytic residues by kinetic analysis of mutants that have been modified at their key conserved carboxylic acids. In doing so, a convenient chromogenic substrate for the kinetic assay for such GH98 *endo*-β-galactosidases is synthesized by chemical modification of an oligosaccharide synthesized in engineered bacterial cells.

EXPERIMENTAL PROCEDURES

Materials, Bacterial Strains, Plasmids, and Media. *E. coli* BL21(DE3) cells were purchased from Novagen, and *C. perfringens* 10543 genomic DNA was obtained from ATCC. *Pwo*, *Pfu*, and EXPAND high DNA polymerases and T4 DNA ligase (and Rapid DNA ligation kit) were obtained from Roche and Fermentas. Restriction endonucleases XhoI and NheI were obtained from New England BioLabs. Gel-extraction and plasmid-DNA-purification kits were from Qiagen. The plasmids used were pCRBlunt (Zero Blunt PCR Cloning Kit, Invitrogen) and pET21a(+) (Novagen, Madison, WI). 2,4-Dinitrophenyl β-D-galactopyranoside (DNPGal) was synthesized according to previous protocols (*29*, *30*). HisTrapFF columns and MonoQ ion exchange columns were from GE Lifesciences. All other reagents were from Sigma-Aldrich Co., and all biochemical reagents for described molecular biological procedures were obtained from New England BioLabs (Mississauga, ON, Canada) and Boehringer Mannheim (Laval, QQ, Canada). Isopropyl β-D-thiogalactoside (IPTG) was obtained from Rose Scientific (Edmonton, Canada). A^+-porcine gastric mucin (PGM) (Type II) was obtained from Sigma Aldrich Co. A-Trisaccharide (GalNAcα-1,3-(Fucα1,2)-Galβ-) was synthesized within an engineered *E. coli* cell line as described previously (*31*).

DNA Manipulation. Primers were synthesized by Integrated DNA Technologies (IDT) (Iowa State). DNA sequencing was performed by the Nucleic Acid and Protein Service (NAPS) Unit at the University of British Columbia using an Applied Biosystems PRISM 377 automated sequencer. Bacterial transformations (in either electrocompetent or chemically competent cells) were performed according to standard protocols. All DNA fragments were purified using agarose gel with SYBR Safe (Invitrogen) DNA gel stain added to visualize DNA.

The DNA fragments were extracted from the gel using QIAquick DNA gel purification kits (Qiagen, CA). Plasmids were prepared using single-colony overnight cultures and purified using Qiaprep Spin Miniprep kits (Qiagen, CA). Restriction endonucleases were supplied by Fermentas and used with the 1× Tango buffer (Fermentas) optimal for double digest with NheI and XhoI.

Cloning of the EABase Gene from C. perfringens into an Expression Vector. The gene coding for EABase was amplified by PCR from genomic *C. perfringens* DNA (ATCC 10543) using primers containing restriction sites NheI and XhoI as follows: EABase-fw (5′-GGT ATG GAA GTT TAT GCT AGC TTG GAA GAA AGC AG-3′) and EABase-rv (5′-CCG CTC GAG CTT AAT TAC AAT ATC AAA ATC-3′). Standard PCR conditions were used for 30 cycles with EXPAND high-fidelity polymerase, and the 2.3 kbp insert was digested (NheI/XhoI) and ligated into pET-21a vector similarly digested and also treated with shrimp alkaline phosphatase. Plasmids obtained from selected observed colonies were submitted for sequencing to the NAPS unit at UBC, and plasmids containing the full length gene with no errors were used in subsequent experiments.

Site-Directed Mutagenesis. Two methods, the Quikchange method and the two step, 4-primer method, were used in the generation of mutants. The only method that was able to generate D453A and E506A was the two-step, four primer method, most likely due to the self-complementarity of the primers used and possible hairpin formation of primers that had a T_m of 65−68 °C. For the E506A mutant, pET-21aWT was used as the template and two separate PCR reactions containing the following primer combinations in the first amplification: E506Afw, 5′-ATA TAA TTT TG**C** ACA TCC AGC AT-3′ with T7term and 5′-ATG CTG GAT GT**G** CAA AAT TAT ATA CAC-3′ (the nucleotide mutations are shown in bold, and the amino acid change is underlined). The amplified fragments from this were used as templates in the second PCR reaction using the T7 and T7term primers. This generated a 2.5 kbp full length gene, which was then subcloned into pET-21a vector using the NheI and XhoI restriction sites. A similar protocol was followed with D453A. The E354A mutant was generated using the Quikchange method with the following primers (E354Afw, 5′-GGT GTA TTT AGT ACA GCA AAT TAT TGG GTT TGG-3′ and E354Arev, 5′-CTG TCC AAA CCC AAT AAT TTG CTG TAC TAA ATA CAC C-3′) using pET-21aWT as the template. DpnI digestion of the wild type template followed by transformation into *E. coli* R1360 yielded colonies containing the mutation.

Expression and Purification of Recombinant Wild Type EABase and Mutants. All enzymes were expressed in *E. coli* BL21(DE3) cells. For the production of each enzyme, a single colony was inoculated into 50 mL of LB medium containing 100 μg/mL ampicillin and grown overnight. From this overnight culture, 5 mL was inoculated into 1 L of LB medium containing 100 μg/mL ampicillin. Cells were induced with 0.5 mM IPTG once they reached an OD of 0.7 and harvested after 4 h by centrifugation at 5000*g* for 30 min. The cell pellet was resuspended in 50 mL of lysis buffer (20 mM Tris at pH 7.9, 0.5 M NaCl, and 20 mM imidazole) and lysed using a French press. EDTA-free protease inhibitor was added after the lysis step, and the lysed cell mixture was centrifuged at 15000*g* for 30 min. The filtered supernatant was added to a 1.0 mL HisTrapFF column and eluted using a gradient of 0.05−0.30 M imidazole; with the enzyme eluting as a defined peak at 0.15 M imidazole. Fractions containing EABase were concentrated and dialyzed against 10 mM Tris at pH 7.5 overnight. The dialyzed protein was applied to a MonoQ column and eluted with a NaCl gradient of 0−300 mM NaCl. A broader peak containing EABase (eluted at 0.1 M NaCl) was concentrated and run on sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS−PAGE) to afford >95% pure protein. Fresh columns were used for each mutant to avoid cross-contamination. The enzymes were concentrated and buffer exchanged into 20 mM sodium acetate at pH 6.0.

CD Spectroscopy. CD spectra were recorded on a JASCO spectropolarimeter in a 0.1 cm cuvette. Enzyme concentrations were standardized to be in the range of 0.2−0.3 μg/μL in 20 mM sodium acetate buffer (pH 6). Samples were analyzed in scanning mode from 230 to 197 nm. Sample cuvettes were cleaned extensively with water and buffer between runs and a blank run consisting of 20 mM sodium acetate (pH 6.0) was performed after cuvettes were washed between different enzyme samples to ensure the CD spectra obtained were free of contamination.

TLC Analysis of Enzymatic Cleavage of Natural Substrates. TLC-scale enzyme assays were performed on a 20 μL scale at 37 °C in 20 mM sodium acetate buffer (pH 6.0). For TLC analysis using blood group A^+ porcine gastric mucin (A^+-PGM) (type II, Sigma), 25 μg of PGM and 5 μL of 1 mg/mL EABase were used. Two microliters of the reaction mixture was spotted onto a silica gel-coated TLC plate (EMD Chemicals, Inc.) and the plate was run using a 2:1:1, v/v/v 1-butanol/acetic acid/water solvent system. The plate was visualized using a stain made from 2 g of ammonium molybdate in 100 mL of 90:10 H_2O/H_2SO_4.

Synthesis of DNP-β-A-trisaccharide (*2,4-dinitrophenyl 2-acetamido-2-deoxy-α-D-galactopyranosyl-(1→3)-[α-L-fucopyranosyl-(1→2)]-β-D-galactopyranoside*). A-Trisaccharide was converted to its 2,4-dinitrophenyl glycoside using approaches that were previously described for the synthesis of other glycosides containing the DNP leaving group (*29*, *30*). The A-trisaccharide (0.1 mmol) was acetylated by stirring it in a 20% v/v acetic anhydride/pyridine mixture at 0 °C for 1 h and subsequently stirring it overnight at 20 °C. The solvents were evaporated, and the resulting residue was dissolved in ethyl acetate (30 mL) and washed with 1 M HCl (1×30 mL), saturated $NaHCO_3$ (2×30 mL), and brine (30 mL), dried over $MgSO_4$, and concentrated. The anomeric acetate was removed by treatment with 1.2 equivalents of hydrazine acetate in dry DMF at 55 °C for 3 h (until the starting material was no longer visible by TLC). The reaction mixture was cooled to room temperature, concentrated, dissolved in ethyl acetate (50 mL), and washed with saturated $NaHCO_3$ (2×30 mL) and brine (30 mL), dried over $MgSO_4$ and concentrated. In the next step, 1.2 equivalents of dinitrofluorobenzene (DNFB) and 1.5 equivalents of 1,4-diazabicyclo-[2.2.2]octane (DABCO) in dry DMF (10 mL) were added to the protected trisaccharide hemiacetal and the reaction mixture stirred overnight at 20 °C. The solvent was evaporated under reduced pressure and the product purified by flash column chromatography (petroleum ether/ethyl acetate) to give the protected DNP-β-A-trisaccharide product. ^{1}H NMR: (400 MHz, $CDCl_3$): δ 8.90 (d, J = 2.3 Hz, 1 H, Ar−H), 8.49 (dd, 1 H, Ar−H), 7.58 (d, J = 9.0 Hz, 1 H, Ar−H); 5.19 (d, $J_{1,2}$ = 7.9 Hz, 1 H, H1′).

Deprotection was achieved by suspending the fully acetylated DNP-β-A-trisaccharide (20 μmol) in dry methanol (3 mL), which was cooled to 0 °C, under nitrogen. Distilled acetyl chloride (0.6 mL) was added dropwise to the stirring solution, and the mixture was stirred at 0 °C and allowed to stand at 4 °C for three days, with monitoring by TLC. The solvent was evaporated under reduced pressure, and the residue was purified over a reverse phase C18 Sep-Pak column and subsequently lyophilized. ^{1}H NMR: (600 MHz, D_2O): δ 8.79 (d, J = 2.3 Hz, 1 H, Ar−H), 8.43 (dd, 1 H, Ar−H), 7.62 (d, J = 9.2 Hz, 1 H, Ar−H); 5.30 (d, $J_{1,2}$ = 7.73 Hz, 1 H, H1′), 5.01 (d, $J_{1,2}$ = 3.67 Hz, 1 H, H1″), 5.02 (d, $J_{1,2}$ = 3.72 Hz, 1 H, H1‴) ESI-MS: calcd for $[C_{26}H_{37}N_3O_{19}+Na]^+$ 718.20; found, 718.41.

Enzyme Kinetics. The reaction progress of the EABase-catalyzed hydrolysis of DNP-A-trisaccharide was monitored at 37 °C using a continuous spectrophotometric assay on a Cary-4000 spectrometer (Varian Inc.) connected to a circulating water bath. Unless otherwise indicated, assays were performed in 20 mM sodium acetate buffer at pH 6.0 in cells of 1 cm path length. Prewarmed 200 μL cuvettes were loaded with an appropriate volume of water, buffer, and substrate at concentrations from 0.1 to 5 times K_M and incubated for 6 min before data were acquired. Spontaneous hydrolysis of substrates was monitored prior to the addition of enzyme. A final amount of 90 ng of EABase was added to each cuvette, and the change in absorption was monitored at 405 nm. The apparent kinetic parameters were generated by GraFit 4.0 (Erithacus Software). An absorption coefficient of 8584 M^{-1} cm^{-1} was determined for 2,4-dinitrophenolate in 20 mM sodium acetate buffer at pH 6.0 at 37 °C. Similar procedures were used for the kinetic analysis of mutants and similar enzyme amounts (~90−100 ng) were used with the following exceptions: D453A and E506A were tested using amounts ranging from 100 ng−100 μg.

The reaction progress of the EABase-catalyzed hydrolysis of A-pentasaccharide was monitored using a stopped reducing sugar assay. The stop (quenching) solution (HBAH solution) (50 mL) consisted of 4% NaOH, 0.1 M *p*-hydroxybenzoic acid hydrazide (HBAH), 0.1 M sodium sulfite, 50 mM trisodium citrate dihydrate, and 20 mM calcium chloride. To reaction mixtures (0.5 mL) containing buffer and substrate at concentrations varying from 0.1 to 5 times K_M was added wild type EABase (20 μg), and the reactions were incubated at 37 °C. Controls which did not contain EABase were also run. Aliquots (50 μL) were removed at 15 min intervals and added to 100 μL of the HBAH solution. These samples were then boiled for 12 min, cooled to room temperature, and their absorbance at 420 nm was recorded by adding 100 μL of this solution to 900 μL of water in a 1 cm plastic cuvette. The measured absorbance was plotted as a function of time, and the initial rates were used to generate the apparent kinetic parameters using GraFit 4.0 (Erithacus Software).

```
EABase  342   SKYSALQGVFSTENYWVWTDNVESNAAEYLKLSAKYGGYFIWSEQNNGGSIEKAFGSNGK  401
1       571   QKYPNLHGIFSTENYWIWANDIENKAADYLKVSAKNGGYFIWAEQNNGSAIEKAFGKNGK  630
2       548   QKYPNLHGIFSTENYWIWANDIK                                      570
3       548   QKYPNLHGIFSTENYWIWANDIENKAADYLKVSAKNGGYFIWAEQNNGSAIEKAFGKNGK  607
4       146   QKYSVLKGVLNIENYWIYNNQLAPHSAKYLEVCAKYGAHFIWHDH                190

EABase 402   TVFKEAVEKYWENFIFMYKNTPQAEGNDAPTSSYMTGLWLTDYAYQWGGLMDTWKWYETG  461
1      631   IAFQKSVDKYWKNLIFMFKNTPAAEGNDSTTESYMKGLWLSNHTYQWGGLMDTWKWYETG  690
3      608   IAFQKSVDKYWKNLIFMFKNTPAAEGNDSTTESYMKGLWLSNHTYQWGGLMDTWKWYETG  667
4      203   TFF-EASQKYHKNLVLATKNTPIRD--DAGTDSIVSGFWLSGLCDNWGSSTDTWKWWEKH  259

EABase 462   KWKLFESGNIGKTQGNRQWLTEPEALLGIEAMNIYLNGGCVYNFERPAYTYGVRNEESPL  521
1      691   KWKLFASGNIGKSQGDRQWLTEPESMLGEEALGIYLNGGVVYNFEHPAYTYGVNNKESLL  750
3      668   KWKLFASGNIGKSQGDRQWLTEPESMLGEEALGVYLNGGVVYNFERPAYTYGVNNKESLL  727
4      260   YTNTFETG---RARDMRSYASEPESMIAMEMMNVYTGGGTVYNFEC               302
```

FIGURE 1: Sequence comparisons among selected GH 98 enzymes. Putative catalytic aspartates and glutamates are highlighted in bold and underlined. Boxes surround regions where 4 or more amino acids are conserved adjacent to putative catalytic residues. Organisms are as follows: 1, [*Streptococcus pneumoniae* CGSP14]; 2, [*Streptococcus pneumoniae* G54]; 3, [*Streptococcus pneumoniae* SP3-BS71]; 4, [*Streptococcus pneumoniae* TIGR4].

A similar procedure was followed for the E354A mutant using 200−500 μg of protein.

pH Dependence of k_{cat}/K_M. The stability of the enzyme at each pH value over the assay time was first examined by adding enzyme (at the same concentrations used in the study below) to a tube containing buffer at the appropriate pH and incubated at 37 °C. After 15 min, 20 μL of the mixture was removed for assay by adding to another preincubated solution containing 80 μM DNP-A-trisaccharide in a final concentration of 20 mM sodium acetate at pH 6.0 buffer. Subsequent measurements of k_{cat}/K_M were only performed at those pH values where the enzyme retained more than 90% activity over the 10-min period. The buffers used are as follows: pH 4.0−6.0, 20 mM sodium acetate/acetic acid; pH 6.5−8.0, 20 mM phosphate; pH 8.0−9.0, 20 mM Tris-HCl; pH 9.5−10, glycine/NaOH.

The k_{cat}/K_M values for the hydrolysis of DNP-A-trisaccharide at each pH were determined by the substrate depletion method, following reaction time-courses at low ($\leq 0.2 \times K_M$) substrate concentrations, as follows. For both the wild type EABase and the E354A mutants, a solution of DNP-A-trisaccharide (12 μM and 6 μM, respectively) and the appropriate buffer were preincubated at 37 °C. A final concentration of 1−5 μg of enzyme was added to each cuvette, and the release of dinitrophenolate was monitored continuously at 405 nm for 5−10 min at which time 5−7 half-lives had passed. After the reaction was judged to be complete, the pH of the resulting mixture was checked to ensure that no significant change in pH had occurred. The resulting change in absorbance as a function of time was fitted to a first order rate equation using GraFit 4.0, yielding values for the pseudo-first order rate constant at each pH value as follows. At low substrate concentrations ([S] $\ll K_M$), the reaction rates are given by the following equation:

$$v = k_{cat}[E]_o[S]/K_M$$

The k_{obs} values correspond to $k_{cat}[E]_o/K_M$. Thus, division of the obtained rate constants by the enzyme concentration gives the k_{cat}/K_M values. By analyzing the bell-shaped k_{cat}/K_M versus pH plots using GraFit 4.0, two apparent pK_a values of ionizable groups were assigned.

Determination of Stereochemistry by NMR Spectroscopy. ^{1}H NMR spectrometry was performed on a Bruker 600AV at 600 MHz using 5 mm tubes. Experiments were conducted at ambient temperature using a water suppression protocol. EABase was buffer-exchanged with 10 centrifugations into 99.9% D_2O using an Amicon centrifugal ultrafilter with a molecular weight cutoff of 10 000 (Amicon), and DNP-A-tri (3.4 mg) was lyophilized twice from 99.9% D_2O. Sodium acetate buffer (20 mM, pH 6.0) was lyophilized three times in 99.9% D_2O. The NMR tube was filled with 0.3 mL of 20 mM sodium acetate buffer at pH 6.0 containing 16 mM DNP-β-A-trisaccharide. EABase (0.20 mg) was added after initial spectra of DNP-β-A-trisaccharide were obtained. After the addition of EABase, spectra were acquired approximately every 4 min, with a final ^{1}H NMR of the same reaction mixture being measured the following morning after overnight equilibration at 20 °C.

RESULTS

Cloning and Expression of Wild Type EABase. To facilitate the characterization of EABase from *C. perfringens* with the goals of obtaining large quantities of protein readily and generating variants of this enzyme easily, the gene encoding the enzyme was cloned into pET21-a and expressed as follows. Primers containing the coding region of the gene starting at the *N*-terminal peptide and containing the NheI and XhoI restriction sites were used for PCR amplification of the *C. perfringens* genome (ATCC 10543), yielding a single product of ~2.3 kb which was fully sequenced and shown to contain the full length open-reading frame. The EABase gene product was subcloned into pET21-a vector using the XhoI and NheI sites and transformed into *E. coli* BL21(DE3) cells. Plasmids were extracted and subsequently fully sequenced from colonies grown overnight. These were all shown to contain the full length gene encoding EABase, starting with the *N*-terminal peptide. A single colony was induced in 2 L of LB broth as described in Experimental Procedures, and the enzyme containing a His_6 tag was purified by nickel-affinity chromatography and Mono-Q ion-exchange. The gene in this plasmid consisted of a 2325 bp open reading frame encoding a protein consisting of 774 amino acids including the linkers and His_6 tag; the calculated theoretical molecular weight of this protein is M_r = 88147.3 Da. The purified enzyme was resolved by SDS−PAGE and migrated as a band with an approximate MW of 88 kDa. The approximate yield of protein was 15 mg protein from 1 L of LB medium.

Synthesis of Substrates for EABase. Two chromogenic substrates for the kinetic analysis of EABase were synthesized. The first was Gal-α(1,3)-Gal-β-DNP, synthesized enzymatically by the incubation of donor UDPGal and acceptor DNP-Gal (synthesized according to previously published protocols) (*32*)

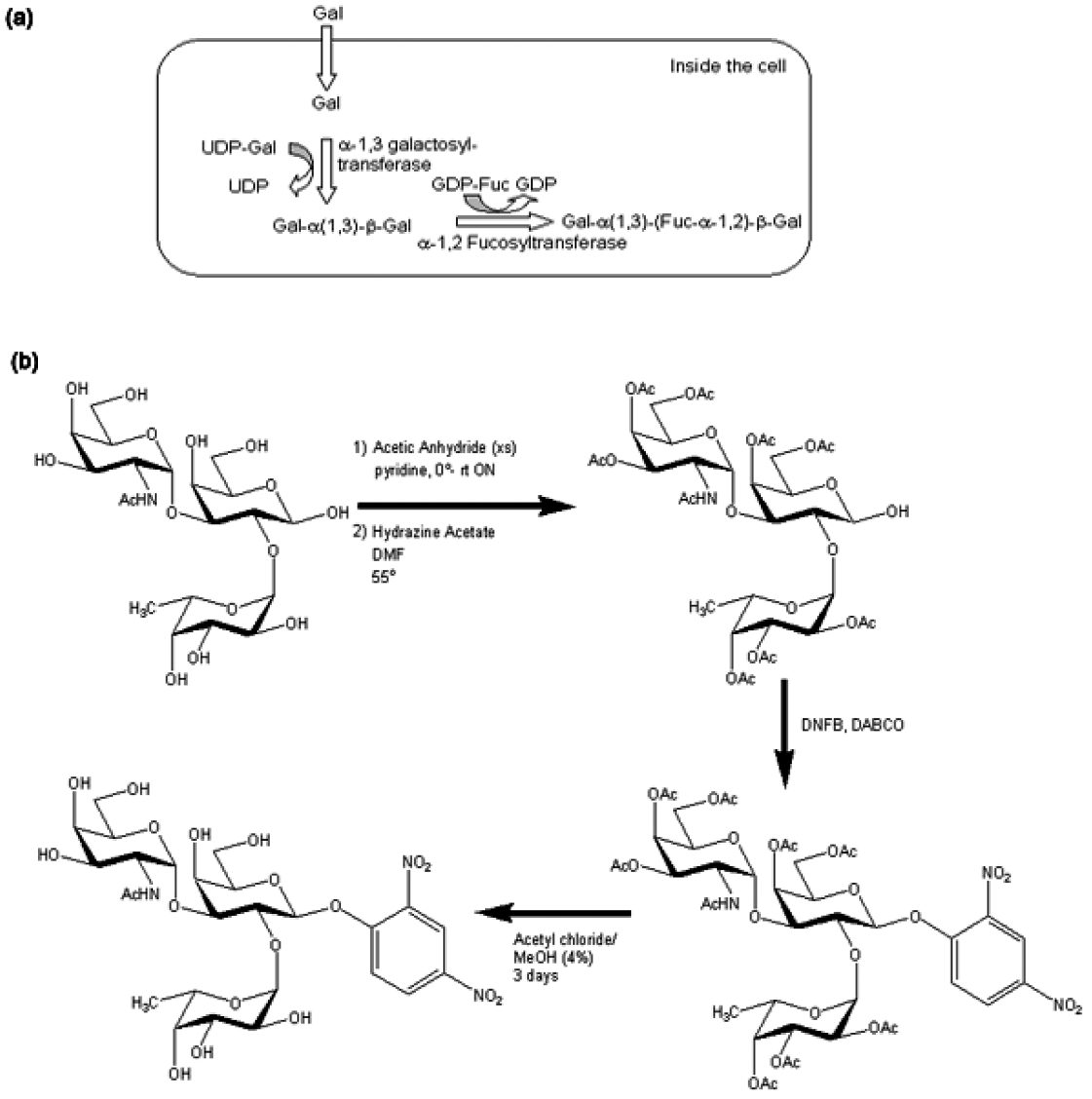

FIGURE 2: Schematic of synthetic routes toward a chromogenic substrate for kinetic analysis of EABase: (a) cell-based synthesis of A-trisaccharide and (b) chemical synthesis of DNP-A-trisaccharide.

with bovine α(1,3)-Gal transferase (*33*, *34*). The product was purified by C18 reverse-phase Sep-Pak column chromatography.

Initial attempts to synthesize the DNP-*β*-A-trisaccharide focused upon the feeding of DNP-Gal as a donor to metabolically engineered *E. coli* cells (which express *H. pylori* α(1,2)-fucosyltransferase and α(1,3)-galactosyltransferase) (*31*). Unfortunately, this was not successful: hydrolytic release of the dinitrophenyl moiety likely caused cell death. As an alternative approach, a sample of A-trisaccharide synthesized in the *in vivo* cell system was chemically converted to its 2,4-dinitrophenyl glycoside as described in Experimental Procedures (*32*, *35*). A scheme of the synthesis is provided in Figure 2.

Characterization of the Expressed EABase. The activity of the purified enzyme was initially tested by TLC analysis of the products formed from the incubation of EABase with blood group A^+ porcine gastric mucin (A^+PGM). The substrate requirement for EABase was further probed by incubating it with Gal-α(1,3)-DNP-*β*-Gal, a substrate containing an activated leaving group but lacking the α(1,2)-fucose residue on the terminal galactose. TLC, UV−vis spectrophotometric, and mass spectrometric analyses revealed that this substrate was not cleaved by EABase, confirming the requirement for a 2′-fucose for activity. Kinetic parameters of $K_M = 64 \pm 3$ *μ*M and a $k_{cat} = 105 \pm 5$ min^{-1} for hydrolysis of DNP-A-trisaccharide by EABase were determined spectrophotometrically (405 nm) (Table 1).

Table 1: Kinetic Parameters for Wild Type EABase and Mutants with DNP-A-trisaccharide[a]

	k_{cat} (min^{-1})	K_M (*μ*M)	k_{cat}/K_M (*μ*M^{-1} min^{-1})
WT	105 ± 5	64 ± 3	1.6 ± 0.1
E354A	10 ± 1	31 ± 3	0.32 ± 0.02
D453A	ND	ND	ND
E506A	ND	ND	ND
E467A	93 ± 5	59 ± 4	1.6 ± 0.1
D429A	81 ± 4	61 ± 3	1.3 ± 0.1

[a] ND: no hydrolysis detected.

Stereochemical Outcome of Hydrolysis by EABase. ^{1}H NMR spectroscopy was used to determine the anomeric stereochemistry of the initially formed products from the EABase-catalyzed hydrolysis of the synthetic substrate DNP-*β*-A-trisaccharide. Figure 3a shows a series of ^{1}H NMR spectra following the time course of the reaction from the point prior to enzyme addition ($t = 0$) to 68 min after the addition of enzyme. An expansion of the anomeric proton region of the ^{1}H NMR

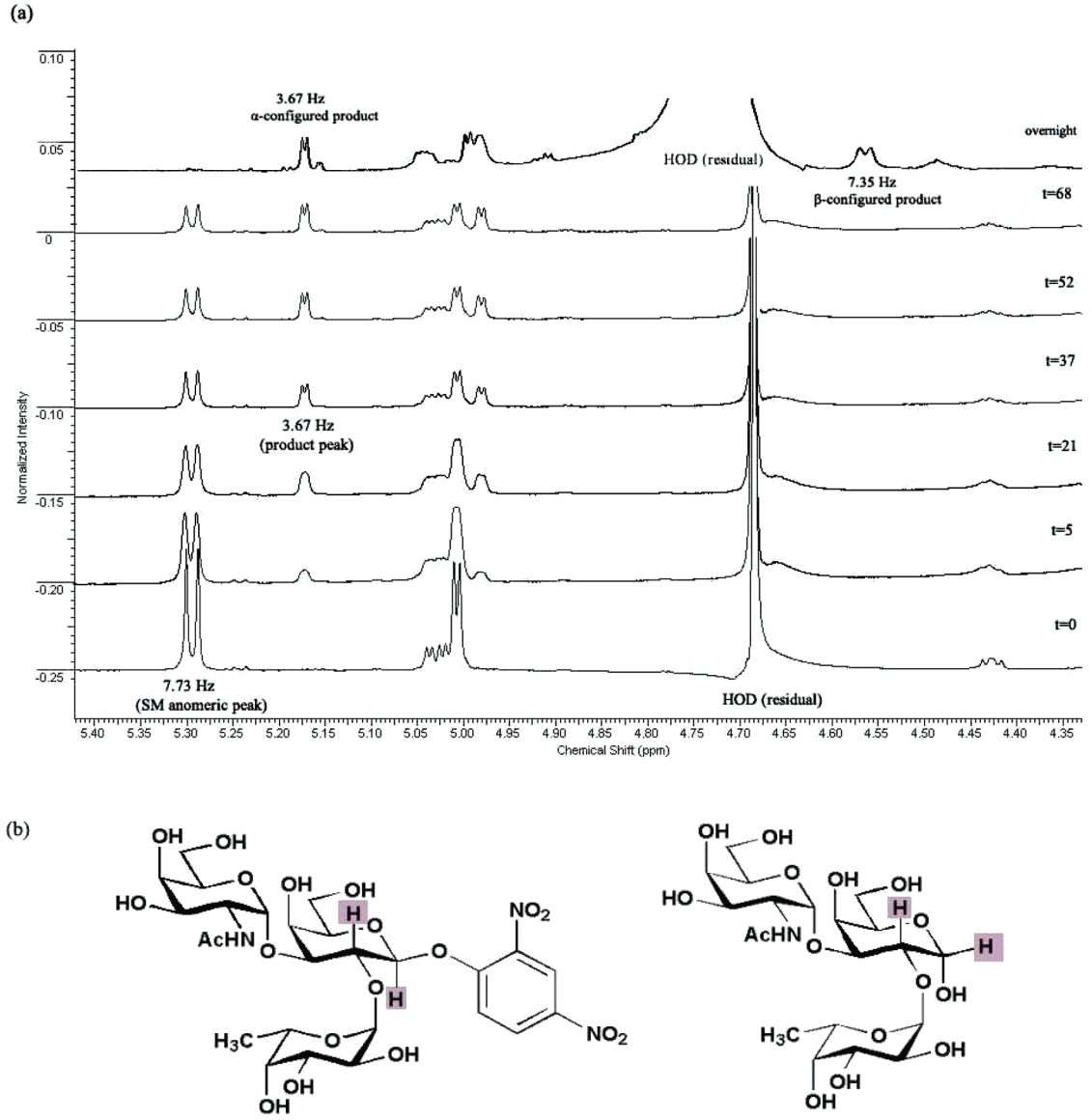

FIGURE 3: Determination of stereochemical outcome by ^{1}H NMR spectrometry. (a) ^{1}H NMR spectra of reaction mixtures containing DNP-A-trisaccharide to which EABase has been added, recorded after times indicated; (b) structure of the starting material, DNP-A-trisaccharide, with the H1 and H2 protons shaded in purple (left) and the structure of the product formed in the alpha configuration with H1 and H2 protons shaded in purple (right).

spectrum of the DNP-β-A-trisaccharide substrate is shown at t = 0 min. The doublet at δ 5.30 ppm (J = 7.7 Hz) corresponds to the anomeric proton of the β-galactosyl moiety, and the large peak at δ 4.68 ppm arises from residual HOD. Spectra taken at time intervals reveal a steady decrease in the intensity of the peak from the substrate anomeric proton δ 5.30 ppm (J = 7.7 Hz) as enzyme-catalyzed hydrolysis occurs. This is accompanied by the gradual appearance of a peak at δ 5.18 ppm, J = 3.2 Hz due to the anomeric proton of the initially formed product. This J value is in the range of H1−H2 coupling constants typically found for an α-configured sugar. Over this time-course, no signal was observed in the region between δ 4.65−4.50 ppm where the anomeric proton from the β-configured product would be expected to show.

However, ^{1}H NMR analysis of the same sample, using the same water suppression protocol, after overnight equilibration at ambient temperature, clearly revealed a resonance at δ 4.52 ppm (J = 7.3 Hz) which is in the range of H1−H2 coupling constants typically found for the β-configured product (Figure 3a). These results confirm that the anomeric proton peak of the β-configured trisaccharide was not hidden under the residual HOD peak or as a result of the water suppression protocol. Mutarotation of the α-configured trisaccharide to the β-configured trisaccharide appears to be very slow at ambient temperatures and under the conditions used for this ^{1}H NMR experiment.

As further confirmation that the α-anomer is the first formed product, a linear plot of the intensity of the anomeric peaks of the product and substrate as a function of time (Figure 4) shows that the rate of substrate depletion equals the rate of formation of the α-anomer of the trisaccharide product (slopes are identical: 0.015/min), showing that no other products were initially formed.

These results show that the product released by EABase is the α-configured trisaccharide and thus that this enzyme uses an inverting mechanism. These results are contrary to the initial predictions by Rigden (*28*).

Amino Acid Sequence Alignments with Other Family 98 Hydrolases. Alignment of the full gene sequence of EABase with those of other Family 98 hydrolases reveals several

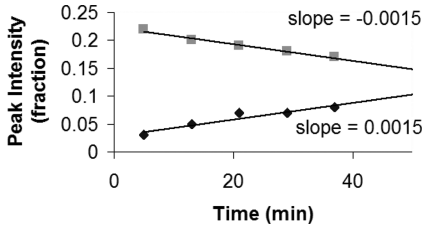

FIGURE 4: Correlation between decrease in intensity of substrate peak with time (negative slope) and increase in intensity of product peak with time (positive slope).

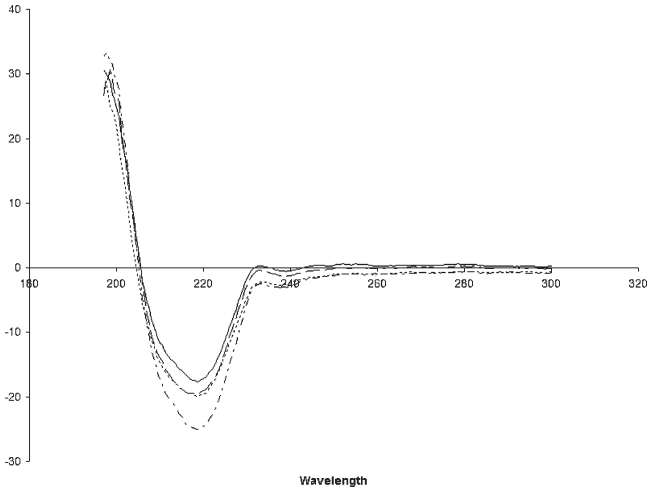

FIGURE 5: CD Spectra of wild type enzyme (- - -), E354A (—), D453A (——), and E506A (–- –-). Similar concentrations of protein were used to obtain spectra (between 1 and 3 μg).

conserved aspartate and glutamate residues within the predicted catalytic domain region (Figure 1). Indeed, aspartate and glutamate residues are involved as active site residues in the majority of glycosidase mechanisms. Of these, three (E354, D453, and E506) are surrounded by three to four additional conserved residues suggesting that they might be those that are most likely to serve in catalysis. Rigden has suggested that this enzyme follows a retaining mechanism and predicted the catalytic residues to be E354, D429, and E467 (*28*). D429 is conserved; however, the region flanking the residue is not; additionally, E467 is not fully conserved, which suggests that it may not play a role in catalysis. The five residues that were chosen for mutation to alanine (E354, D429, D453, E467, and E506) were based on a combination of Rigden's predictions and on the residues in the most conserved regions.

Cloning and Expression of Mutants of EABase. Site directed mutagenesis was used to generate alanine mutants of putative catalytic residues. Mutagenesis using the Quikchange method yielded colonies for the E354A, E467A, and D429A mutants. DNA sequencing results showed that some of the colonies contained the single desired mutation (a few colonies contained undigested wild type plasmid). No colonies were obtained for the D453A and E506A mutations using the Quikchange method despite numerous attempts, presumably because of unwanted secondary structure formation in the polynucleotide. For these mutants, the 4-primer method was used. Sequencing of selected colonies showed that they contained the single desired mutations.

The conformational integrity of the mutant proteins was verified by circular dichroism spectroscopy. As seen in Figure 5, CD spectra for all mutants are essentially identical to those of the wild type in all cases, therefore confirming that all the mutants were folded correctly.

Table 2: Kinetic Parameters for Wild Type EABase and Mutants with A-Pentasaccharide[a]

	k_{cat} (min^{-1})	K_M (μM)	k_{cat}/K_M (μM^{-1} min^{-1})
WT	4.1 ± 0.2	384 ± 42	$(1.1 \pm 0.1) \times 10^{-2}$
E354A	$(3.7 \pm 0.1) \times 10^{-3}$	ND	ND

[a] ND: not determinable.

Kinetic Characterization of Mutants of EABase. Inverting glycosidases proceed through a concerted, single-displacement mechanism in which a carboxylic acid residue acts as a base and another as an acid. The base residue deprotonates water as it attacks the anomeric center, thereby making it more nucleophilic. In concert with this, the acid residue transfers a proton to the glycosidic oxygen, facilitating the cleavage of the glycosidic linkage via stabilization of the leaving group. Since these residues are critical to the mechanism, we expect their removal to have a significant effect on catalysis. Removal of the acid catalyst should slow the hydrolysis of substrates with poor leaving groups much more than substrates with good (activated) leaving groups ($pK_a < 7$) which need little protonic assistance. However, removal of the base residue should substantially slow hydrolysis of all substrates.

Activated Substrate. Michaelis−Menten kinetic parameters for the hydrolysis of DNP-β-A-trisaccharide by wild type EABase and each of the five mutants are summarized in Table 1. The mutants E506A and D453A were completely inactive when assayed with DNP-β-A-trisaccharide, even when assayed at high concentrations of substrate and enzyme. Confirmation that the substrate was intact and no inhibitors were present was provided by the addition of wild type EABase directly to the assays, leading to rapid turnover. Since the CD spectra of these mutants show that they are folded, the lack of activity is likely due to their role in deprotonation and/or positioning of the water molecule for attack at the anomeric center; therefore, one or both residues may function as the base (*36*). The k_{cat} value for the E354A mutant was only 10 times lower than that for the wild type enzyme. Because this mutant retains considerable activity, this residue is unlikely to serve as a base but may serve as an acid residue. The K_M of E354A is reduced by a factor of 2 compared to the wild type, which is of marginal significance but may be a result of more favorable interactions of the aromatic leaving group with a less polar residue at position 354. The k_{cat} and K_M values for the D429A and E467A mutants are similar to those of the wild type EABase, indicating that they are not important in catalysis. No rescue was seen upon addition of formate or azide anions to any of the mutants. However, this lack of rescue is consistent with studies on other inverting β-glycosidases, in which acid−base residues have been mutated (*36*).

Unactivated Natural Substrate. Analysis of the hydrolysis of a natural, nonactivated substrate by each mutant was carried out first by monitoring the hydrolysis of A^+PGM by TLC (Figure 1, Supporting Information). The wild type EABase efficiently hydrolyses the substrate with release of A-trisaccharide. However, even when incubated with enzyme concentrations up to 1000 times higher than those used for the wild type

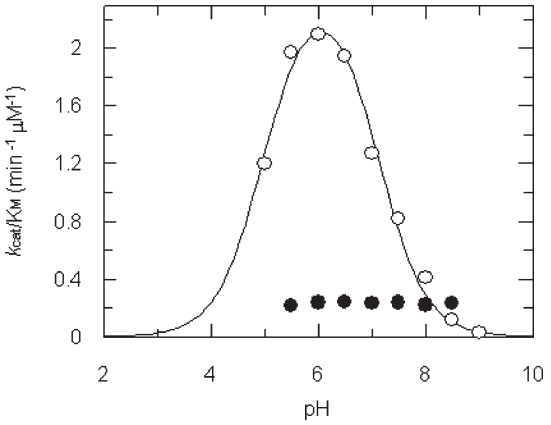

FIGURE 6: pH Dependence of k_{cat}/K_M for the hydrolysis of DNP-A-trisaccharide by wild type EABase (○) and its E354A mutant (●).

EABase, no hydrolysis whatsoever of A^+PGM by D453A or E506A is observed. This is completely consistent with observations with the activated substrate. By comparison, a small amount (≈20%) of substrate hydrolysis by E354A was observed after 16 h, but only when the mutant enzyme was used at concentrations 20 times higher than that in the wild type EABase experiment. This allows an approximate estimation of a 1000-fold rate reduction for nonactivated substrates as a consequence of the removal of E354.

Kinetic parameters for the hydrolysis of A-pentasaccharide were determined spectrophotometrically using a stopped reducing sugar assay. As can be seen in Table 2, kinetic parameters of of $K_M = 384 \pm 42\ \mu M$ and a $k_{cat} = 4.1 \pm 0.2\ min^{-1}$ were obtained with the wild type EABase. Interestingly, this K_M value is 6-fold higher than that obtained with DNP-A-trisaccharide implying that the dinitrophenyl moiety binds more tightly than the natural sugar at this site. Furthermore, the k_{cat} is significantly lower than that for the aryl glycoside. The rates of hydrolysis of A-pentasaccharide by E354A were far lower, an approximate k_{cat} value of $(3.7 \pm 0.1) \times 10^{-3}\ min^{-1}$ being obtained at saturating (3 mM) substrate concentrations. Thus, replacement of Glu354 with Ala results in an 1100-fold rate reduction for nonactivated substrates. As is elaborated below, such kinetic behavior with an activated and unactivated substrate is completely consistent with a role for E354 as the acid catalyst.

pH Profiles. Values of k_{cat}/K_M for the hydrolysis of DNP-A-trisaccharide by wild type EABase and the E354A mutant were determined as a function of pH within the stability range for each enzyme: 5.0−9.0 for wild type and 5.5−8.5 for E354A. These values are plotted in Figure 6. The pH profile of the wild type enzyme is a typical bell-shaped curve with optimal activity at pH 6.0. The pK_a values for the general base and general acid ionizable groups in the free enzyme, obtained from the plot of k_{cat}/K_M versus pH, are pK_{a1} = 5.0 and pK_{a2} = 7.1. However, the value of pK_{a1} should be taken only as an estimate since the data obtainable for this limb of the profile were limited due to the instability of the enzyme at lower pH values. The pH profile of the E354A mutant was significantly different from that of the wild type enzyme, with the k_{cat}/K_M values remaining constant over the whole pH range at which the mutant was stable. The profile could not be obtained at sufficiently low pH values to report on the acidic limb of the pH dependence. However, the absence of a basic limb in the profile of the mutant is completely consistent with the notion that the residue mutated, E354, is indeed the acid catalytic residue.

DISCUSSION

In contrast to what had been proposed by Rigden, EABase follows an inverting mechanism as observed by ^{1}H NMR spectroscopy. Mutation of D467 and D429 had little or no effect on the kinetic parameters for EABase, indicating that these residues do not play important roles in catalysis. This is also consistent with the fact that D467 is not entirely conserved and that the sequence around D429 is not conserved, which is once again in conflict with Rigden's predictions. By contrast, mutation of each of the three residues in highly conserved regions severely impacts the catalytic activity of EABase. Residues D453 and E506 are particularly important for catalysis as no catalytic activity was observed with the alanine mutants, even with the highly activated substrate, DNP-β-A-trisaccharide. Upon the basis of precedent with the inverting β-glycosidase CenA from *C. fimi*, this would suggest that one or both of these residues functions as the base catalyst (*36*). Indeed, it is quite common to find two residues acting in concert as catalytic acids/bases in glycosidases (*37*, *38*). Alternatively, one of the carboxylic acids may play a particularly important role in coordinating substrate hydroxyls, possibly bridging across an adjacent pair as is seen in GH13 enzymes (*37*). The kinetic data strongly support a role for E354 as the acid catalyst since the activity of the E354A mutant is 1100-fold lower than that of the wild type enzyme when assayed with a natural, nonactivated substrate, which absolutely needs acid catalytic assistance for bond cleavage. By contrast, its activity is only 10-fold (k_{cat}) or even 5-fold (k_{cat}/K_M) lower than that of the wild type enzyme when assayed with an activated substrate that requires little to no acid catalytic assistance (DNP-β-A-trisaccharide). This is behavior that is characteristic of a mutant in which the acid catalyst has been removed. This conclusion is considerably strengthened by the finding that the activity of the E354A mutant is essentially independent of pH in the range over which the activity of the wild type enzyme decreased severely with pH: the acid catalyst had been removed.

Unfortunately the observation that EABase is an inverting glycosidase considerably complicates efforts to generate a glycosynthase for the modification of surfaces of blood cells. Unlike retaining glycosidases, inverting glycosidases do not possess true transglycosylation activity since there is no intermediate to partition; thus, only the inefficient thermodynamic approach can be employed with wild type enzymes. Furthermore, only limited success has been attained in generating glycosynthases in inverting glycosidases, generally by the mutation of the general base residue, making this a somewhat less promising route. However, through some combination of saturation mutagenesis of conserved residues, it may prove possible to engineer such an activity in the future.

ACKNOWLEDGMENT

We thank Maria Ezhova for her technical assistance in running the 600 MHz spectrometer used for the determination of the stereochemical outcome.

SUPPORTING INFORMATION AVAILABLE

Illustration of the differences in the rate of hydrolysis of a natural substrate, A^+PGM by wild type EABase, and mutants. This material is available free of charge via the Internet at http://pubs.acs.org

REFERENCES

1. Petit, L., Gibert, M., and Popoff, M. R. (1999) Clostridium perfringens: toxinotype and genotype. *Trends Microbiol. 7*, 104–110.
2. Barth, H., Aktories, K., Popoff, M. R., and Stiles, B. G. (2004) Binary bacterial toxins: biochemistry, biology, and applications of common *Clostridium* and *Bacillus* proteins, Microbiol. Mol. Biol. Rev. 68, 373−402; table of contents.
3. Minutti, C. Z., Immergluck, L. C., and Schmidt, M. L. (1999) Spontaneous gas gangrene due to *Clostridium perfringens*. *Clin. Infect. Dis. 28*, 159–160.
4. Awad, M. M., Bryant, A. E., Stevens, D. L., and Rood, J. I. (1995) Virulence studies on chromosomal alpha-toxin and theta-toxin mutants constructed by allelic exchange provide genetic evidence for the essential role of alpha-toxin in *Clostridium perfringens*-mediated gas gangrene. *Mol. Microbiol. 15*, 191–202.
5. Alape-Giron, A., Flores-Diaz, M., Guillouard, I., Naylor, C. E., Titball, R. W., Rucavado, A., Lomonte, B., Basak, A. K., Gutierrez, J. M., Cole, S. T., and Thelestam, M. (2000) Identification of residues critical for toxicity in *Clostridium perfringens* phospholipase C, the key toxin in gas gangrene. *Eur. J. Biochem./FEBS 267*, 5191–5197.
6. Cassidy, J. T., Jourdian, G. W., and Roseman, S. (1965) The sialic acids. VI. Purification and properties of sialidase from *Clostridium perfringens*. *J. Biol. Chem. 240*, 3501–3506.
7. Sabban, E., Laster, Y., and Loyter, A. (1972) Resolution of the hemolytic and the hydrolytic activities of phospholipase-C preparation from *Clostridium perfringens*. *Eur. J. Biochem./FEBS 28*, 373–380.
8. Yamakawa, Y., and Ohsaka, A. (1977) Purification and some properties of phospholipase C (alpha-toxin) of *Clostridium perfringens*. *J. Biochem. 81*, 115–126.
9. Anderson, K. M., Ashida, H., Maskos, K., Dell, A., Li, S. C., and Li, Y. T. (2005) A clostridial endo-beta-galactosidase that cleaves both blood group A and B glycotopes: the first member of a new glycoside hydrolase family, GH98. *J. Biol. Chem. 280*, 7720–7728.
10. Cantarel, B. L., Coutinho, P. M., Rancurel, C., Bernard, T., Lombard, V., and Henrissat, B. (2009) The Carbohydrate-Active EnZymes database (CAZy): an expert resource for glycogenomics. *Nucleic Acids Res. 37*, D233–238.
11. Lowe, J. (1995) Biochemistry and Biosynthesis of ABH and Lewis Antigens: Characterization of Blood Group-Specific Glycosyltransferases, in Blood Cell Biochemistry: Molecular Basis of Human Blood Group Antigens (Cartron, J.-P. R., and Rouger, P., Eds) pp 75−109, Plenum Press, Paris, France.
12. Oriol, R. (1995) ABO, Hh, Lewis, and Secretion: Serology, Genetics, and Tissue Distribution, in Blood Cell Biochemistry: Molecular Basis of Human Blood Group Antigens (Cartron, J.-P. R., and Rouger, P., Eds), pp 37−67, Plenum Press, Paris, France.
13. Holgersson, J., Breimer, M. E., and Samuelsson, B. E. (1992) Basic biochemistry of cell surface carbohydrates and aspects of the tissue distribution of histo-blood group ABH and related glycosphingolipids. *Apmis 27*, 18–27.
14. Huppes, W., Paulonis, J., Dijk, H., Van Rooijen, N., and Van Bekkum, D. W. (1993) The role of natural antibodies and ABO (H) blood groups in transplantation of human lymphoid cells into mice. *Eur. J. Immunol. 23*, 26–32.
15. Murphy, S. (1988) ABO blood groups and platelet transfusion. *Transfusion 28*, 401–402.
16. Gordon, R. D., Iwatsuki, S., Esquivel, C. O., Todo, S., Makowka, L., Tzakis, A., Marsh, J. W., and Starzl, T. E. (1987) Experience with primary liver transplantation across ABO blood groups. *Transplant. Proc. 19*, 4575–4579.
17. Hendriks, G. F., Wenting, G. J., Mochtar, B., Bos, E., Simoons, M. L., Balk, A. H., Laird-Meeter, K., Essed, C. E., and Weimar, W. (1989) The influence of ABO blood groups on the incidence of cardiac allograft rejection in males. *Transplant. Proc. 21*, 803.
18. White, D. J., Gore, S. M., Barroso, E., and Calne, R. Y. (1987) The significance of ABO blood groups in liver transplant patients. *Transplant. Proc. 19*, 4571–4574.
19. Goldstein, J., Siviglia, G., Hurst, R., Lenny, L., and Reich, L. (1982) Group B erythrocytes enzymatically converted to group O survive normally in A, B, and O individuals. *Science* (*New York*) *215*, 168–170.
20. Liu, Q. P., Sulzenbacher, G., Yuan, H., Bennett, E. P., Pietz, G., Saunders, K., Spence, J., Nudelman, E., Levery, S. B., White, T., Neveu, J. M., Lane, W. S., Bourne, Y., Olsson, M. L., Henrissat, B., and Clausen, H. (2007) Bacterial glycosidases for the production of universal red blood cells. *Nat. Biotechnol. 25*, 454–464.
21. Shaikh, F. A., and Withers, S. G. (2008) Teaching old enzymes new tricks: engineering and evolution of glycosidases and glycosyl transferases for improved glycoside synthesis. *Biochem. Cell Biol. 86*, 169–177.
22. Williams, S. J., and Withers, S. G. (2002) Glycosynthases: Mutant glycosidases for glycoside synthesis. *Aust. J. Chem. 55*, 3–12.
23. Williams, S. J., and Withers, S. G. (2000) Glycosyl fluorides in enzymatic reactions. *Carbohydr. Res. 327*, 27–46.
24. Mackenzie, L. F., Wang, Q., Warren, R. A. J., and Withers, S. G. (1998) Glycosynthases: mutant glycosidases for oligosaccharide synthesis. *J. Am. Chem. Soc. 120*, 5583–5584.
25. Withers, S. G. MacKenzie, L. F., and Wang, Q. (1998) Methods and compositions for synthesis of oligosaccharides, and the products formed thereby, US Patent 5716812.
26. Honda, Y., and Kitaoka, M. (2006) The first glycosynthase derived from an inverting glycoside hydrolase. *J. Biol. Chem. 281*, 1426–1431.
27. Wada, J., Honda, Y., Nagae, M., Kato, R., Wakatsuki, S., Katayama, T., Taniguchi, H., Kumagai, H., Kitaoka, M., and Yamamoto, K. (2008) 1,2-alpha-l-Fucosynthase: a glycosynthase derived from an inverting alpha-glycosidase with an unusual reaction mechanism. *FEBS Lett. 582*, 3739–3743.
28. Rigden, D. J. (2005) Analysis of glycoside hydrolase family 98: catalytic machinery, mechanism and a novel putative carbohydrate binding module. *FEBS Lett. 579*, 5466–5472.
29. Ballardie, F., Capon, B., Sutherland, J. D. G., Cocker, D., and Sinnott, M. L. (1973) A simple general synthesis of 2,4-dinitrophenyl glycopyranosides. *J. Chem. Soc., Perkin Trans. 1*, 2418–2419.
30. van Boom, J. H., Koeners, A. J., deKok, and Romers, C. (1980) The use of the 2,4-dinitrophenyl group in sugar chemistry reexamined. *J. R Neth. Chem. Soc. 99*, 355–362.
31. Drouillard, S., Driguez, H., and Samain, E. (2006) Large-scale synthesis of H-antigen oligosaccharides by expressing *Helicobacter pylori* alpha1,2-fucosyltransferase in metabolically engineered *Escherichia coli* cells. *Angew. Chem., Int. Ed. 45*, 1778–1780.
32. Withers, S. G., Dombroski, D., Berven, L. A., Kilburn, D. G., Miller, R. C. Jr., Warren, R. A., and Gilkes, N. R. (1986) Direct ^{1}H n.m.r. determination of the stereochemical course of hydrolyses catalysed by glucanase components of the cellulase complex. *Biochem. Biophys. Res. Commun. 139*, 487–494.
33. Shah, P. S., Bizik, F., Dukor, R. K., and Qasba, P. K. (2000) Active site studies of bovine alpha-1−3-galactosyltransferase and its secondary structure prediction. *Biochim. Biophys. Acta 1480*, 222–234.
34. Gastinel, L. N., Bignon, C., Misra, A. K., Hindsgaul, O., Shaper, J. H., and Joziasse, D. H. (2001) Bovine alpha1,3-galactosyltransferase catalytic domain structure and its relationship with ABO histo-blood group and glycosphingolipid glycosyltransferases. *EMBO J. 20*, 638–649.
35. Koeners, H. J., Dekok, A. J., Romers, C., and Vanboom, J. H. (1980) The use of the 2,4-dinitrophenyl group in sugar chemistry re-examined. *J. R Neth. Chem. Soc. 99*, 355–362.
36. Damude, H. G., Withers, S. G., Kilburn, D. G., Miller, R. C. Jr., and Warren, R. A. (1995) Site-directed mutation of the putative catalytic residues of endoglucanase CenA from *Cellulomonas fimi*. *Biochemistry 34*, 2220–2224.
37. Rydberg, E. H., Li, C., Maurus, R., Overall, C. M., Brayer, G. D., and Withers, S. G. (2002) Mechanistic analyses of catalysis in human pancreatic alpha-amylase: detailed kinetic and structural studies of mutants of three conserved carboxylic acids. *Biochemistry 41*, 4492–4502.
38. Joshi, M. D., Sidhu, G., Pot, I., Brayer, G. D., Withers, S. G., and McIntosh, L. P. (2000) Hydrogen bonding and catalysis: a novel explanation for how a single amino acid substitution can change the pH optimum of a glycosidase. *J. Mol. Biol. 299*, 255–279.