2042 *Biochemistry* **2010,** *49,* 2042–2050
DOI: 10.1021/bi1000596

BIOCHEMISTRY
including biophysical chemistry & molecular biology
Article

Modulating the pH−Activity Profile of Cellulase by Substitution: Replacing the General Base Catalyst Aspartate with Cysteinesulfinate in Cellulase A from *Cellulomonas fimi*[†]

Darrell W. Cockburn, Chris Vandenende, and Anthony J. Clarke*

Department of Molecular and Cellular Biology, University of Guelph, Guelph, Ontario N1G 2W1, Canada

Received January 14, 2010; Revised Manuscript Received February 2, 2010

ABSTRACT: Cellulase A (CenA) from *Cellulomonas fimi* is an inverting glycoside hydrolase and a member of family 6 of the CAZy database classification system. We replaced its putative catalytic base aspartyl residues, Aps392 and Asp216, with cysteinesulfinate using a combination of site-directed mutagenesis and chemical modification to investigate the applicability of this approach for the modulation of enzymatic properties. The substituted cysteinyl residues were oxidized to cysteinesulfinic acid with hydrogen peroxide, and the resulting protein products were demonstrated to retain their native structure. Oxidation of the Asp392Cys mutant enzyme restored 52% of wild-type activity when assessed at pH 7.5, whereas Asp216Cys CenA remained inactive. This suggests that Asp216 is not the catalytic base and provides further support for Asp392 performing this role. Similar substitution of the catalytic acid residue Asp252 or the catalytic nucleophile of the retaining enzyme Cel5A from *Thermobifida fusca* failed to produce active enzymes. This indicates a potential utility of this approach for uniquely identifying catalytic base residues. The replacement of Asp392 with cysteinesulfinate induced an acidic shift in the pH profile of the enzyme such that this enzyme derivative was more active than wild-type CenA below pH 5.5. These data demonstrate the potential of combining site-directed mutagenesis with chemical modification as a viable approach for the modulation of cellulases, and potentially other glycoside hydrolases, at low pH.

Cellulose is the most abundant biopolymer on Earth, and as such, it has attracted considerable attention as a source of renewable biofuel. It is a linear polymer of β-(1→4)-linked anhydrous D-glucose residues which, depending upon the source, may extend to 15000 residues in length. Due to the nature of the β-linkage, the linear polymers form an extended ribbon structure with a 2-fold screw axis and each glucopyranose residue oriented 180° relative to its neighbors. As a result, the basic repeating unit of cellulose is not glucose but instead cellobiose. Intramolecular hydrogen bonding between adjacent residues confers rigidity, and intermolecular bonding between cellulose polymers leads to the crystallinity observed in fibers. This crystallinity provides rigidity to plant cell walls and makes them recalcitrant to biodegradation. Thus, the complete solubilization of crystalline cellulose requires the concerted and synergistic action of three classes of glycoside hydrolases, cellulase (EC 3.2.1.4, endoglucanase), cellobiohydrolase (EC 3.2.1.74), and β-glucosidase (EC 3.2.1.21) (reviewed in ref *1*). For each, examples of both retaining and inverting enzymes have been characterized.

Inverting glycoside hydrolases catalyze bond cleavage using a single-displacement mechanism involving the participation of two acidic amino acid residues positioned opposite each other across the active site cleft or tunnel (recently reviewed in ref *2*). In the enzyme's resting state, only one is deprotonated and it serves as a general base, removing a proton from water during its attack at the anomeric carbon of the glycosidic linkage (Figure 1). The second residue protonates the departing aglycone oxygen atom, thereby assisting its departure from the anomeric center. This reaction mechanism thus proceeds through a single oxocarbenium ion-like transition state in which the developing positive charge at the anomeric carbon is partially stabilized by donation of an electron from the ring oxygen of the glucose substrate. Retaining glycoside hydrolases utilize a double-displacement mechanism. In this mechanism, an acidic residue donates a proton to the leaving group as in the inverting mechanism, but the second catalytic residue functions as a nucleophile to directly attack the anomeric carbon of the glycosidic linkage, leading to the formation of a covalent bond between the two. The residue acting as the acid in the first stage of the mechanism then acts as a base, activating water for an attack on the anomeric carbon. The sugar is thus released with a net retention of its anomeric configuration.

In an earlier study with glucoamylase, a starch degrading inverting enzyme and a member of family 15 of the CAZy database classification system (http://www.cazy.org/), the carboxylate moiety of its general base catalyst was replaced with sulfinate to generate an enzyme with a higher level of activity compared to that of the wild type (*3*). Despite this success, the general applicability of this protein engineering approach has not been tested with another inverting glycoside hydrolase. Also, the effect of replacing the carboxylate of the catalytic base with sulfinate on the pH−activity profile of the engineered derivative merits further investigation. A slight acidic shift in the pH−activity profile was observed with the engineered glucoamylase despite the fact that the wild-type enzyme already has an acidic pH−activity optimum (*4*). Hence, the question of what effect the substitution of a sulfinate for a carboxylate would have on an

[†]This work was supported by an operating grant (RGPIN3215) to A.J.C. from the Natural Sciences and Engineering Research Council of Canada and an Ontario Graduate Scholarship to D.W.C.

*To whom correspondence should be addressed: E-mail: aclarke@uoguelph.ca. Telephone: (519) 824-4120. Fax: (519) 837-1802.

 Published on Web 02/05/2010

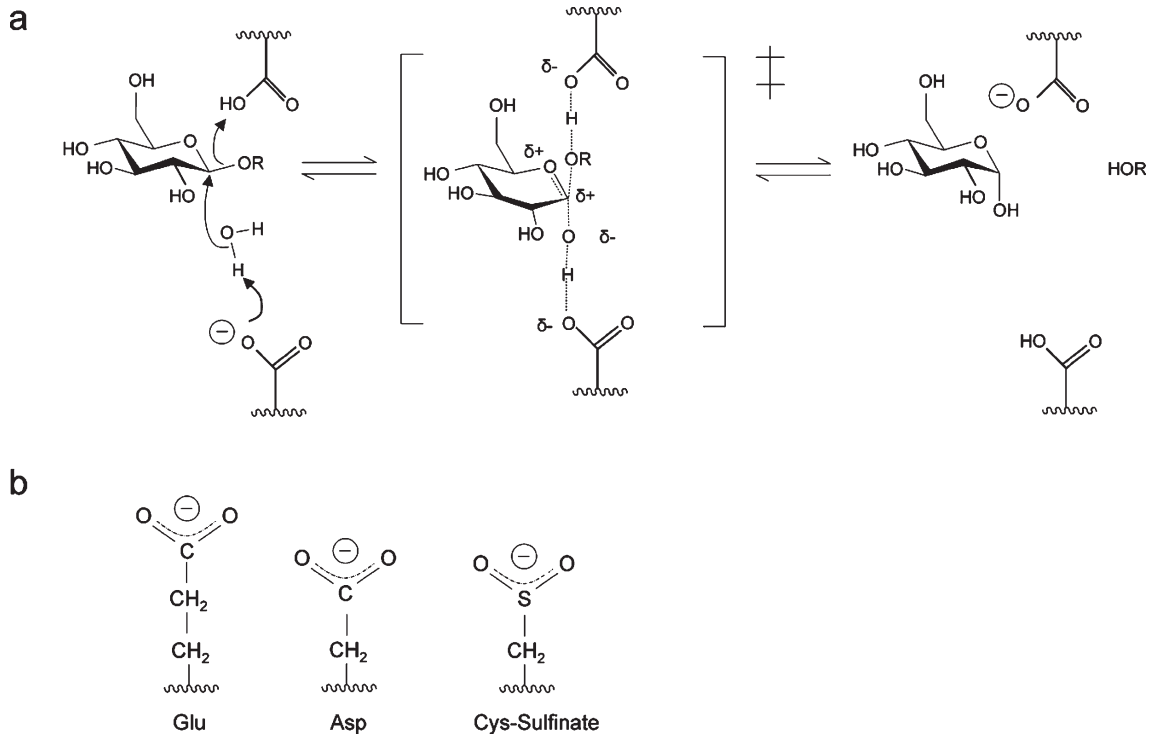

FIGURE 1: Proposed mechanism of action of inverting glycoside hydrolases (a) and structures of catalytic residues (b).

enzyme with a more neutral pH−activity optimum remains. To assess both of these issues, we identified cellulase A (CenA)[1] from *Cellulomonas fimi* as a model enzyme for investigation with the ultimate aim of engineering cellulolytic enzymes with enhanced properties for more efficient application. Also, Cel5A, a retaining enzyme from *Thermobifida fusca*, was used as a model retaining enzyme to determine if the catalytic nucleophile could similarly be replaced with cysteinesulfinate.

CenA is an extracellular cellulase produced by the Gram-positive, non-spore-forming facultative anaerobic soil bacterium *C. fimi* (*5*) which, in vitro, is active on carboxymethyl (CMC) and phosphoric acid-swollen cellulose. Typical of cellulases, CenA is weakly active on crystalline cellulose (*10*). It is a 46.7 kDa protein composed of 449 amino acids that form three distinct modules, a cleavable N-terminal leader sequence followed by a carbohydrate-binding module which is tethered through a Pro-Thr rich linker sequence ("Pro-Thr box") to the catalytic module. The catalytic module of 280 amino acid residues has been classified as a CAZy family 6 glycoside hydrolase. Whereas its three-dimensional structure has yet to be determined, those of four other related family 6 enzymes from *Humicola insolens* (PDB entry 1BVW), *Hypocrea jecorina* (formerly *Trichoderma reesei*) (PDB entry 1CB2), *T. fusca* (PDB entry 1TML), and *Mycobacterium tuberculosis* (PDB entry 1UOZ) have been determined. Nonetheless, a significant amount of mutational work and biochemical characterization has been conducted on CenA (*5*−*9*). These studies have identified unequivocally Asp252 as the catalytic acid (*9*), but its catalytic base may be either Asp216 or Asp392. Whereas site-specific replacement of Asp392 in CenA results in a dramatic decrease in activity (*9*), similar results were not achieved in studies with other related family 6 enzymes (*11*−*13*).

Herein, we describe the engineering of CenA to replace its putative catalytic base residues with cysteine followed by their oxidation to cysteinesulfinic acid which supports the identification of Asp392 as the catalytic base. In addition, we demonstrate the applicability of this approach generation of an enzyme derivative with enhanced activity at acidic pH.

EXPERIMENTAL PROCEDURES

Strains, Plasmids, and Reagents. The list of bacteria and plasmids used in this study, together with their respective sources, is given in Table 1 of the Supporting Information. *Escherichia coli* DH5α was used for all plasmid propagation, while *E. coli* BL21(DE3) pLysS was used for all gene expressions. Growth media were purchased from Fisher Scientific (Nepean, ON), and unless otherwise stated, all other chemical reagents were purchased from Sigma-Aldrich (Oakville, ON).

DNA Techniques. Isolation of all plasmids was performed using the QIAprep Spin Miniprep Kit from Qiagen (Mississauga, ON) according to the manufacturer's protocol. Genomic DNA was isolated using the DNeasy Blood & Tissue Kit from Qiagen, while primer synthesis and sequencing were performed by the Lab Services division of the University of Guelph (Guelph, ON).

The *cenA* gene was subcloned into the pET30a(+) vector. The gene was amplified by PCR from the pUCEC2 plasmid using the forward and reverse oligonucleotide primers, CenA-f and CenA-r, respectively (Table 2 of the Supporting Information). The PCR product was purified using the High Pure PCR Purification kit from Roche (Mississauga, ON) according to the manufacturer's instructions. An *Nde*I and *Hin*dIII (New England Biolabs, Pickering, ON) double digest was performed, and the PCR product was ligated into a similarly digested pET30a(+) plasmid. The ligation product was transformed into chemically competent cells of *E. coli* DH5α. Colonies were propagated and the plasmids

[1]Abbreviations: CD, circular dichroism; CenA, cellulase (endoglucanase) A from *C. fimi*; CHES, *N*-cyclohexyl-2-aminoethanesulfonic acid; Cm, chloramphenicol; CMC, carboxymethyl cellulose; DTNB, 5,5′-dithiobis(2-nitrobenzoate); DTT, dithiothreitol; IPTG, isopropyl β-D-thiogalactopyranoside; Km, kanamycin; LB, Luria-Bertini culture medium; Ni-NTA, nickel-nitrilotriacetic acid; PDB, Protein Data Bank.

isolated and subjected to analytical digestion with *Nde*I and *Hin*dIII. Plasmids containing inserts of the appropriate size (~1.4 kb) were sequenced to confirm their identity.

The *cel5A* gene from *T. fusca* YX was amplified from genomic DNA with primers Cel5A-f and Cel5A-r (Table 2 of the Supporting Information). The gene was inserted into the pET-30a(+) vector as described above for *cenA*.

Site-directed mutagenesis was performed using the QuikChange site-directed mutagenesis kit from Stratagene (La Jolla, CA) according to the manufacturer's protocol. Primers used for site-directed mutagenesis are listed in Table 2 of the Supporting Information. The PCR product was treated with *Dpn*I (Stratagene) and transformed into *E. coli* DH5α. Plasmids were isolated from the colonies, and the mutations were confirmed by sequencing.

Protein Production and Purification. A single colony of *E. coli* BL21(DE3) pLysS transformed with a plasmid harboring either wild-type *cenA*, *cel5A*, or one of their mutant forms was inoculated into 10 mL of Luria-Bertani (LB) broth (*cenA*) or Super Broth (*cel5A*), supplemented with 50 μg/mL kanamycin (Km) and 34 μg/mL chloramphenicol (Cm) and grown overnight at 37 °C. This was then diluted into 1 L of LB broth or Super Broth similarly supplemented and incubated at 37 °C. When the cells reached the midexponential phase of growth (OD_{600} of 0.6 for *cenA* or OD_{600} of 0.8 for *cel6A*), 1 mM IPTG (final concentration) was added and incubation was continued overnight at 15 °C for *cenA* or for 3 h at 37 °C for *cel5A*. The cells were harvested by centrifugation (8000*g* for 5 min) and frozen for at least 1 h. After thawing, the cells were resuspended in 45 mL of preparation buffer [50 mM sodium phosphate buffer (pH 8.0) and 300 mM NaCl] containing 10 mM imidazole, treated with 3 miniComplete EDTA free protease inhibitor tablets (Roche), 5 μg/mL DNase, 10 μg/mL RNase, and 100 μg/mL lysozyme, and incubated for 1 h at 4 °C before being sonicated on ice for 3 min (10 s on, 10 s off) using a Sonics Vibra Cell VCX130 ultrasonic processor. Any intact cells and insoluble cellular debris were removed by centrifugation (12000*g* for 15 min). To this clarified cell lysate was added 2 mL of fresh Ni-NTA agarose (Qiagen), and the mixture was incubated overnight at 4 °C. The resin slurry was then packed into a disposable column and washed with 250 mL of prepartion buffer containing 30 mM imidazole. The recombinant His-tagged cellulases were recovered in 12 mL of preparation buffer containing 250 mM imidazole which was added to the column and allowed to stand for 5 min prior to elution. The eluate was dialyzed overnight at 4 °C against 50 mM ammonium bicarbonate buffer (pH 9.0) (for CenA) or 50 mM sodium phosphate buffer (pH 7) (for Cel5A), with at least two changes. The partially purified proteins were subjected to anion-exchange chromatography on SourceQ (Amersham, Baie d'Urfe, QC). Protein fractions were applied in dialyisis buffer at a flow rate of 0.7 mL/min and then eluted with the application of a linear gradient from 0 to 1.0 M NaCl over 30 min. Collected fractions were analyzed via SDS−PAGE, with the most pure being pooled and dialyzed overnight at 4 °C against 100 mM ammonium bicarbonate buffer (pH 8.5) with at least two buffer changes. These purified protein preparations were lyophilized and stored at −20 °C until they were needed. In some experiments, the entire isolation and purification process was conducted in the presence of 5 mM dithiothreitol (DTT).

Circular Dichroism Spectroscopy. Protein solutions had a concentration of 0.1 mg/mL in 25 mM sodium phosphate buffer (pH 7.0), and 200 μL samples were transferred to a 0.1 cm cuvette. CD spectra were recorded using a Jasco J-810 spectropolarimeter. Data were recorded in the far-UV range from 180 to 260 nm with a 1 nm bandwidth, a 100 nm/min scan rate, and a 1 s response time. The averages of three scans, subtracted from a buffer blank and converted to mean residue ellipticity, are presented for each sample. The scans were analyzed for secondary structure predictions using the K2D algorithm (*14*) at http://www.embl-heidelberg.de/~andrade/k2d .html.

Activity Assays. All activity assays with purified enzymes were performed using 0.4% CMC as the substrate, except where otherwise noted. The following buffers were used in the indicated pH ranges: 50 mM sodium acetate from pH 4.5 to 5.5, 50 mM sodium phosphate from pH 6.0 to 8.0, and 50 mM CHES from 8.5 to 9.0. Enzyme was added to 1 mL of substrate to a final concentration of 3.7 μg/mL for wild-type CenA, Asp392Cys CenA, and wild-type Cel5A enzymes, 7.4 μg/mL for Asp316Asn CenA, or 16.8 μg/mL for Asp392Ala CenA, Asp392Asn CenA, Asp392Ser CenA, Asp216Cys CenA, Glu355Gln Cel5A, and Glu355Cys Cel5A and incubated at 37 °C for the appropriate period of time. For the determination of the Michaelis constant (K_M), a range of substrate concentrations was used from 0.05 to 1.6% of the CMC at pH 5. The K_M was determined by nonlinear regression of the Michaelis−Menten plot using the enzyme kinetics module of SigmaPlot (SysStat Software Inc., San Jose, CA). Reducing sugar production was determined by the Nelson−Somogyi method (*15*, *16*) by reading the absorbance at 660 nm on a Beckman DU520 spectrophotometer. Standard curves of glucose were prepared to determine reducing sugar concentrations. One unit of activity is defined as the number of micromoles of glucose equivalents released per minute.

An overlay assay using Congo Red staining was adopted to qualitatively determine the activity of the different CenA enzymes in vivo. Overnight cultures of *E. coli* BL21(DE3) pLysS, with plasmids harboring either wild-type *cenA* or one of its mutants, were diluted 10^6−10^8-fold and plated on LB agar supplemented with 50 μg/mL Km, 34 μg/mL Cm, and 0.1 mM IPTG. The colonies were allowed to grow overnight before being overlaid with a mixture of 0.8% agar and 0.5% CMC in 100 mM sodium phosphate buffer (pH 6.5). Following incubation at 37 °C for 1.5 h, these overlays were stained with 1 mg/mL Congo Red in 250 mM sodium phosphate buffer (pH 8.0) for 30 min at room temperature. Destaining was then performed with 500 mM NaCl for 20 min at room temperature.

Free Thiol Determination. The protein thiol content was determined by titration with 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB) (*17*). Protein solutions had a concentration of approximately 12 μM, in 25 mM sodium phosphate buffer (pH 7.0), and DTNB was added to a final concentration of 250 μM. Following incubation for 1 h at room temperature, the absorbance at 412 nm was measured and corrected by subtraction of a protein-free buffer blank. The concentration of free thiol in the protein was obtained from a standard curve prepared using 10−40 μM cysteine.

Tryptic Digest and Mass Spectrometry Analysis. Protein samples had a concentration of approximately 0.5 mg/mL in 25 mM ammonium bicarbonate buffer (pH 8.0) and were incubated for 5 min at 90 °C. This heat denaturation was found to be necessary to achieve efficient digestion. The heat-denatured protein was allowed to cool, and proteomics grade trypsin was added to a final concentration of 4 μg/mL and incubated overnight at 37 °C. The samples were then desalted using a ZipTip (Millipore, Etobicoke, ON) and analyzed by matrix-assisted laser

desorption ionization time-of-flight (MALDI-TOF) mass spectrometry at the Biological Mass Spectrometry Facility of the University of Guelph.

Oxidation of CenA, Cel5A, and Mutants. Proteins were resuspended in 25 mM sodium phosphate buffer (pH 7.0) to a final concentration of ~12.5 μM. For optimization of oxidation conditions for the Asp392Cys mutant CenA, hydrogen peroxide was added to final concentrations ranging from 10 μM to 100 mM. Reactions were allowed to proceed for either 3 min, 24 h, 48 h, 72 h, or 96 h. The 3 min reactions were conducted at room temperature, while all others were performed at 4 °C. The reactions were halted by the addition of catalase to a final concentration of 20 μg/mL. Samples were then analyzed for thiol content and/or activity assays. The optimal condition was found to be 100 μM hydrogen peroxide for 72 h, and these conditions were used for all subsequent oxidations of the Asp392Cys mutant, as well as the Asp216Cys and Asp252Cys mutants and wild-type CenA enzymes. A similar attempt was made to optimize Glu355Cys Cel5A oxidation; however, activity was not restored under any of these conditions.

Other Analytical Techniques. Protein concentrations were determined using a bicinchoninic acid-based assay (Pierce, Rockford, IL). SDS−PAGE was performed by the method of Laemmli (*18*) using 12% acrylamide gels and Coomassie Brilliant Blue staining. Secondary structure predictions and three-dimensional modeling of CenA were performed using the Jpred algorithm (http://www.compbio.dundee.ac.uk/~www-jpred/) and Phyre (http://www.sbg.bio.ic.ac.uk/~phyre/), respectively.

RESULTS

Overproduction and Purification of CenA and Its Derivatives. The *cenA* gene was amplified by PCR using pUCEC2 as a template and subcloned into the pET30a(+) vector. Similarly, *cel5A* was amplified from genomic DNA and cloned into the pET30a(+) vector. The resulting constructs pACDC-003 and pACDC-074 were transformed separately into *E. coli* DH5α, and their nucleotide sequences were confirmed prior to further experimentation. The protein products, now possessing C-terminal hexahistidine tags, were overproduced in cells of freshly transformed *E. coli* BL21(DE3) pLysS following their induction with 1 mM IPTG at the midexponential phase of growth. Confirmation of recombinant protein production was obtained by SDS−PAGE and Western blot analysis using and anti-hexahistidine antibody (data not shown). Overproduced recombinant CenA and Cel5A were purified to above 98% homogeneity from cell lysates of *E. coli* transformants by a combination of affinity and anion-exchange chromatographies on Ni^{2+}-NTA agarose and Source Q, respectively. Care was taken to use fresh chromatography medium for each purification to prevent any potential contamination of wild-type and mutant enzymes with one another. Yields and levels of purification achieved were similar to those reported previously (*19*, *20*).

PCR-based site-directed mutagenesis was employed to replace the putative catalytic acid residue Asp252 and the hypothetical base residues, Asp216 and Asp392, with Cys in CenA. In addition, mutant genes encoding the Asp392Ala, Asp392Asn, Asp392Ser, and Asp392Cys/Asp252Cys derivatives of CenA were also generated, and all were overexpressed in their respective *E. coli* transformants. For Cel5A, the catalytic nucleophile Glu355 was replaced with Gln and Cys. Each mutant enzyme was purified by the same method employed for the wild-type enzyme with similar yields (data not shown).

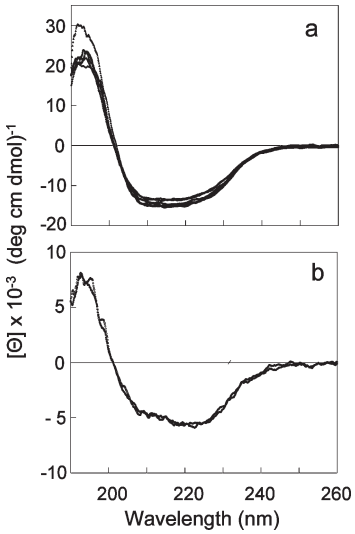

FIGURE 2: CD spectra of wild-type and mutant forms of CenA and Cel5A. Spectra of the various enzymes were recorded at a concentration of 0.1 mg/mL in 25 mM sodium phosphate buffer (pH 7.0), and the data are expressed as mean residual ellipticity: (a) wild-type (■), Asp392Cys (●), Asp252Cys (◆), Asp252Cys/Asp392Cys (▼), and Asp216Cys (▲) CenA and (b) wild-type (●) and Glu355Cys (○) Cel5A.

Characterization of Mutant Enzymes. The mutant enzymes generated were analyzed by CD spectroscopy (Figure 2) to ensure that there were no gross structural differences between them and the wild type. Measurements were conducted using the same temperature (37 °C) and buffer conditions [25 mM sodium phosphate (pH 7)] as for activity studies. The spectra obtained were essentially indistinguishable from one another, indicating that the mutant enzymes maintained an overall wild-type structure under the assay conditions employed. For each CenA mutant, the contents of α-helix, β-strand, and random coil were estimated to be 32−36, 19, and 45−49%, respectively, which is consistent with those predicted for CenA (29% helix, 20% β-strand, and 51% random coil) and also with the known structures of two other family 6 cellulases from *T. fusca* (PDB entry 1TML) and *M. tuberculosis* (PDB entry 1UOZ) (35−38% helix and 22% β-strand). It has been determined that the Asp216Ala and Asp392Ala replacements do not disrupt the overall structure of CenA (*9*), so those measurements were not repeated here. Also, only the oxidized versions of the Cys mutant proteins are presented as the unoxidized versions produced identical curves. For wild-type and Glu355Cys Cel5A, the contents of α-helix, β-strand, and random coil were estimated to be 24, 23, and 53%, respectively, which is consistent with that found in the crystal structure of the wild-type enzyme (PDB entry 2CKS) (26% helix, 25% β-strand, and 49% random coil).

We expected that any replacement of the catalytic residues would result in little, if any, detectable hydrolytic activity. This was indeed the case for both Glu355Gln Cel5A and Glu355Cys Cel5A (data not shown), as well as for Asp216Cys CenA, for Asp352Cys CenA, and for CenA with Asp392 replaced with either Ala, Asn, or Ser. With each, residual k_{cat} values were ≤0.24% of the wild-type value (Table 1). Surprisingly, however, purified Asp392Cys CenA and Asp216Asn CenA were found to possess approximately 17 and 4% of wild-type activity, respectively. To determine if the Asp392Cys replacement was active in vivo prior to its isolation, an overlay assay of transformed *E. coli* colonies was employed using 0.5% CMC as the substrate with

Table 1: Effect of Oxidation of CenA and Its Derivatives on Hydrolytic Activity

	before peroxide treatment		after peroxide treatment	
CenA	percent modified[a]	k_{cat} [s^{-1} (%)][b]	percent modified[a]	k_{cat} [s^{-1} (%)][b]
wild-type	0	5.92 (100)	0	5.81 (98.2)
Asp392Ala	0	0.0013 (0.02)	–	–
Asp392Asn	0	0.0059 (0.10)	–	–
Asp392Ser	0	0.0051 (0.09)	–	–
Asp392Cys	20	0.994 (16.8)	97.9	3.57 (60.2)
Asp252Cys	68.7	0.0073 (0.12)	92.8	0.014 (0.24)
Asp216Asn	0	0.227 (3.83)	–	–
Asp216Cys	13	0.0146 (0.24)	89.2	0.029 (0.48)

[a]As determined by DTNB titration of free sulfhydryl group(s) relative to protein concentration. [b]Assayed with 0.4% CMC in 50 mM sodium phosphate buffer (pH 7.0) at 37 °C and expressed as micromoles of glucose equivalents released per second per micromole of enzyme.

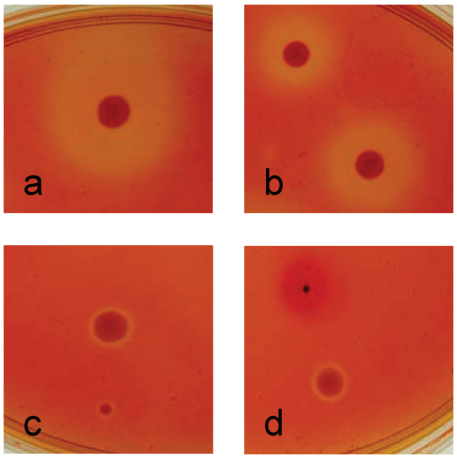

FIGURE 3: Colony assay of *E. coli* BL21(DE3) transformants expressing genes encoding different forms of CenA. Colonies of cells growing on LB agar were overlaid with 0.5% CMC and 0.8% agar in 100 mM sodium phosphate buffer (pH 6.5) for 1.5 h and then stained with Congo Red. Zones of clearing are indicative of cellulase activity: (a) wild-type CenA and (b) Asp392Cys, (c) Asp392Asn, and (d) Asp252Cys mutant enzymes.

Congo Red staining. As seen in Figure 3, a zone of clearing resulted around cells producing wild-type CenA or those with Asp392Cys CenA but not with the Asp392Asn (negative control) or Asp252Cys mutant. Whereas there was less clearing evident around the colonies of cells producing Asp392Cys CenA compared to those producing the wild-type enzyme, it was nonetheless clear that this mutant enzyme possessed residual activity prior to its isolation and purification.

It is highly unlikely that a free sulfhydryl group of Cys can functionally replace the carboxyl group of Asp as the catalytic base at the active center of CenA. Hence, we investigated the possibility that spontaneous oxidation of Cys392, and the other Asp → Cys replacements, occurred to yield cysteinesulfinic acid in a fraction of the protein during its production. To this end, isolated and purified enzymes were subjected to titration with DTNB to quantify the amount of free thiol present. Wild-type CenA contains six Cys residues that form three cystines (*6*), so it should be unreactive to the sulfhydryl reagent if folded properly; the Asp → Cys replacements should exhibit a single free Cys. The results of this titration, presented in Table 1, do indeed show that the control wild-type CenA and the Asp392Asn, Asp392Ala, and Asp392Ser mutant enzymes have no reactivity with DTNB under the conditions employed, indicating the formation of the three cystines. As expected, each of the Asp → Cys mutant enzymes did react with the DTNB, but not stoichiometrically. The apparent level of Cys modification in these mutant enzymes ranged from 13 to 68.7%. With Asp392Cys CenA, approximately 20% of the protein molecules have their free cysteine somehow modified, a level of modification roughly equal to the level of catalytic activity detected. The inclusion of up to 5 mM DTT throughout the isolation and purification process did lower the level of modification to approximately 16%, but it did not preclude it. Interestingly, however, while neither enzyme was catalytically active, the Asp216Cys and Asp252Cys mutant enzymes were nonetheless also modified with only 0.87 and 0.3 molar fractions of the proteins having a free sulfhydryl group, respectively.

To distinguish the nature of the Cys modification that had occurred, tryptic peptide mapping coupled with mass spectrometric analysis was performed on Asp392Cys CenA. From the mixture of resulting peptides, two with apparent masses of 2902 and 2934 (Δ32) were detected which would correspond to the unmodified Cys-containing peptide Leu387−Arg414 (expected mass of 2903.25) and the same peptide with the addition of two oxygen atoms and lacking one hydrogen (expected mass of 2934.23). Similar analysis of the wild-type enzyme did not provide analogous peptides with theses masses but rather a single peptide with a mass of 2914 corresponding to that containing Asp (expected mass of 2915.20). As would be expected, no peptide with a mass of 2946 (i.e., 32 mass units higher) was found in this digest, indicating that the modification is unique to the Cys-containing version of the peptide. Taken together, these data suggest that ~20% of the Asp329Cys CenA is produced in a modified form, and that this modification is most likely oxidation of the introduced Cys to cysteinesulfinic acid.

Complete Oxidation of Asp → Cys Mutant CenAs. Initial attempts to oxidize the free sulfhydryl of Asp → Cys mutant enzymes to sulfinic acid while minimizing its overoxidation to the sulfonic acid or causing oxidative damage to the protein involved the protocol developed by Fierobe et al. for the oxidation of glucoamylase (*3*). Thus, treatment of Asp392Cys CenA with the combination of KI and Br did result in the complete loss of free Cys, as monitored by DTNB titration (data not shown). However, similar treatment of the wild-type enzyme resulted in a loss of catalytic activity, presumably as a result of general oxidative damage. Despite repeated attempts using various concentrations of the two reagents for different periods of time, this damage could not be avoided. Consequently, the KI/Br procedure was abandoned and replaced with a protocol based on the use of hydrogen peroxide as the oxidant.

A series of oxidations were performed using hydrogen peroxide concentrations ranging from 10 μM to 100 mM, representing 0.8−8000-fold molar excesses over protein, and for incubation times of up to 96 h. Whereas treatment of each of the Asp → Cys enzymes with 100 mM hydrogen peroxide for 3 min led to >94% oxidation of the free sulfhydryl group, these conditions also resulted in the loss of activity with the wild-type enzyme. Complete oxidation (>97%) of each could also be achieved with 100 μM peroxide (an 8-fold molar excess under the conditions employed), but these reactions involving relatively low oxidant concentrations required considerably longer reaction times of up to 72 h. However, these latter conditions did not affect the activity of wild-type CenA despite the extended incubation.

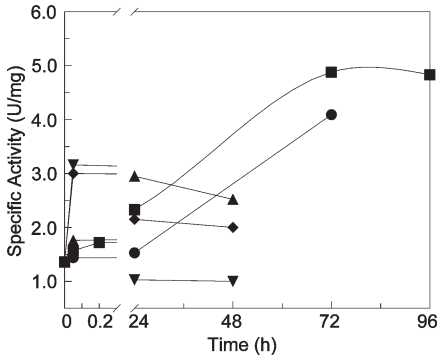

FIGURE 4: Optimization of oxidation conditions for Asp392Cys CenA with hydrogen peroxide. Enzyme (12.5 μM) in 25 mM sodium phosphate buffer (pH 7.0) was treated with hydrogen peroxide at final concentrations of (■) 10 μM, (●) 0.1 mM, (▲) 1 mM, (◆) 10 mM, or (▼) 100 mM. Following incubation for the indicated times, reactions were halted with the addition of catalase and then mixtures assayed for specific activity using 0.4% CMC in 50 mM sodium phosphate buffer (pH 7.0) as the substrate.

Optimization of the Restoration of Hydrolytic Activity for Asp392Cys Enzymes. Characterization of *E. coli* transformants overproducing Asp392Cys mutant CenAs and the isolated enzymes (described above) suggested that oxidation of the free sulfhydryl to sulfinic acid leads to the restoration of catalytic activity, as observed previously with glucoamylase (*3*). Thus, oxidation conditions were optimized to yield the highest possible recovery of hydrolytic activity. As seen in Figure 4, oxidation of Asp392Cys CenA with higher concentrations of peroxide led to a rapid increase in its specific activity against CMC as the substrate to levels of approximately 40% of the wild-type level. This, however, was followed by its loss upon continued incubation. Under such conditions, it is possible that overoxidation of the free sulfhydryl group to its sulfonic acid state and/or oxidative damage elsewhere in the protein occurred. While requiring extended incubation, the use of lower concentrations of peroxide proved to be more effective. Thus, incubation of Asp392Cys CenA with 100 μM peroxide for 72 h proved to be optimal and led to the restoration of approximately 60% of the specific activity of the wild-type enzyme (Figure 4 and Table 1). These conditions did not, however, produce significant recovery of activity with the Asp252Cys mutant or any other CenA mutant; no restoration of hydrolytic activity was detected with these forms of CenA under any conditions employed. In addition, no conditions could be found that would lead to the recovery of activity in Glu355Cys Cel5A, despite achieving up to 80% oxidation (data not shown).

Dependence of CenA Activity on pH. The dependence of hydrolytic activity against CMC on pH was determined for both Asp216Asn CenA and the oxidized form of the Asp392Cys enzyme and compared to that of the wild type. As is typical of a carbohydrase with two acidic catalytic residues, the semilog plot of CenA activity as a function of pH was bell-shaped, with maximal activity centered between pH 7 and 7.5 (Figure 5). Interestingly, the plot for the oxidized form of Asp392Cys CenA indicated both a broadening and an acidic shift in the pH−activity optimum. Moreover, oxidized Asp392Cys CenA was more active at pH < 5.5 compared to the wild-type enzyme at the same pH. Thus, at pH 4.5, the mutant derivative of CenA exhibited almost 300% of the activity associated with the wild-type enzyme at this pH (compare 0.312 s^{-1} vs 1.02 s^{-1}). Whereas the activity of Asp216Asn CenA was relatively low, its pH−activity optimum

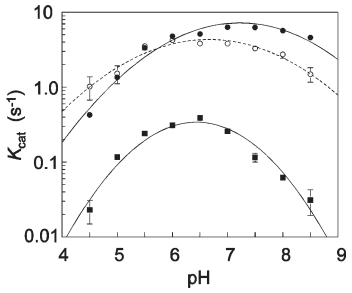

FIGURE 5: Dependence of activity on pH for CenA and mutant enzymes. The activities (relative to their respective maximal specific activity) of (●) wild-type, (○) oxidized Asp392Cys CenA, and (■) Asp216Asn CenA were determined using 0.4% CMC in 50 mM sodium acetate (pH 4.5−5.5), 50 mM sodium phosphate (pH 6.0−8.0), and 50 mM CHES (pH 8.5−9.0).

also decreased from >7 to 6.5. However, more striking is the dramatic decrease in activity seen above pH 6.5, suggesting the pK_a of the catalytic acid is being disrupted in this mutant enzyme.

Determination of the Michaelis Constant (K_M). To gain a better understanding of the nature of the improved specific activity seen at lower pH, the K_M value was determined for oxidized Asp392Cys at pH 5 and compared to the wild-type value. The K_M increases from 0.43 ± 0.05% CMC in the wild type to 0.64 ± 0.16% CMC in the Asp392Cys mutant. The slightly higher K_M seen in the mutant suggests that the improved activity is not due to any improvement in binding, but rather an improvement in the base catalysis at lower pH.

DISCUSSION

This study has demonstrated that the ability to replace the carboxylate moiety of the general base catalyst of an inverting carbohydrase with sulfinate to generate a functional enzyme derivative is not restricted to the isolated case of family 15 glucoamylase. Moreover, a kinetic analysis of this engineered enzyme revealed an acidic shift in its pH−activity profile. An added feature of this study was the finding that hydrolytic activity could be restored to Asp392Cys CenA but not the Asp216Cys enzyme by peroxide oxidation, thus supporting the identification of Asp392 as the general base catalyst in CenA.

Compared to the situation with glucoamylase, the restoration of hydrolytic activity to Asp392Cys CenA by oxidation of the free sulfhydryl group to sulfinate is only modest when assessed at neutral pH. However, at acidic pH, the oxidized CenA derivative activity level greatly exceeded wild-type activity levels, reaching 300% of wild-type activity at pH 4.5. While these activities at low pH are small in absolute terms, they do point the way toward a potential strategy for increasing the activity of enzymes which already possess high activity at low pH.

This increase in activity at lower pH was expected given the lower pK_a of the side chain functional group of cysteinesulfinic acid compared to that of Asp and Glu residues (compare 1.5 with 3.8−4.1, respectively) (*21*). Such a lower pK_a of the catalytic base would permit it to remain in its deprotonated state further into the acidic region. This deprotonation is required because hydrolysis of most natural glycoside substrates by inverting glycosidases requires both acid and base catalysis. In the single-displacement mechanism catalyzed by the inverting enzymes, the general base catalytic residue assists by removing a proton from water, thus making it more nucleophilic for its direct attack on the anomeric center of the glucoside substrate (Figure 1). In concert, the acid catalyst facilitates bond cleavage by donating a

proton to the glycosidic oxygen as cleavage of the linkage occurs, thereby stabilizing the leaving group.

The acid and base catalysts of inverting enzymes are two carboxylic acid residues (Asp or Glu) that are located, on average, 10.5 Å apart on opposite sides of an active site (*2*). This gap is sufficient to permit a water molecule to position between the general base catalyst and the anomeric center of the bound substrate. Whereas the acid catalyst has to be positioned relatively precisely to donate its proton to the departing aglycone oxygen atom, the earlier study with glucoamylase suggested that the inverting mechanism is relatively "flexible" in terms of positioning the general base catalyst, at least for that enzyme. With glucoamylase, the Glu400 base catalyst was replaced with cysteinesulfinate and thus the side chain would be shorter by a methylene group (Figure 1b), resulting in an increase in the size of the gap between the two catalytic residues of approximately 1.5 Å, assuming no structural alterations in the enzyme backbone occurred. Presumably, the hydroxide ion generated from the active site water by the new catalytic base is free and able to migrate the extra distance for nucleophilic attack of the anomeric center. With CenA, however, replacement of the catalytic base Asp392 with cysteinesulfinate would result in very little change in the spatial separation. Hence, despite the successful restoration of hydrolytic activity upon its oxidation, the question of why a similar enhancement to >100% wild-type levels at neutral pH as achieved with glucoamylase was not possible with Asp392Cys CenA remains. Both enzymes are inverting glycoside hydrolases acting on homopolymers of glucose, but that is the extent of their similarities. There is no significant sequence similarity between them, nor do they share similar three-dimensional folds. They act on substrates of opposite anomeric configuration, and wild-type CenA has a neutral pH optimum while glucoamylase an acidic one. However, the most significant difference between these two enzymes with respect to the engineering of a sulfinate functional group at their active centers is likely the difference in their respective amino acids that serve as the general base catalyst. As noted above, an aspartic acid/aspartate pair constitutes the catalytic acid and base for CenA, and all family 6 glycoside hydrolases. Glucoamylase, on the other hand, is somewhat unusual in that it possesses a glutamic acid/glutamate pair. Indeed, of the 115 glycoside hydrolase families currently listed in the CAZy database, only three involve inverting enzymes that use Glu as their catalytic base. Thus, with the oxidized Glu400Cys glucoamylase, the resulting catalytic side chain would be shorter by a methylene group compared to the wild-type form, and it is possible that the larger gap between the two catalytic groups accommodated any subtle conformational adjustments that needed to be made. Such conformational flexibility may not have been afforded CenA as the resulting similar gap between the catalytic residues would limit any required movements to accommodate the engineered stronger acid. Alternatively, but not mutually exclusively, it is conceivable that any changes to the hydrogen bonding network around the catalytic bases that were made in the respective enzymes were better accommodated in glucoamylase. In this regard, the three-dimensional structure of *C. fimi* CenA remains unknown, but the structures of four other family 6 enzymes have been determined. With each of these, the respective constellations of catalytic residues are observed to interact with each other and bound ligands. For the closely related cellulase E2 from *T. fusca*, the equivalent residue Asp265 is hydrogen bonded to Lys259 and Tyr191 and also forms a salt bridge with Arg221 which is proposed to aid in maintaining the Asp residue in a deprotonated, and thus catalytically functional, state (*18*). Such interactions in CenA could be affected by the replacement of a carboxylic acid with the stronger sulfinic acid.

Whereas Asp216 is totally invariant among the family 6 glycoside hydrolases, its role in the catalytic mechanism remained uncertain. Replacement of Asp216 in CenA with Ala was shown previously to lead to only modest decreases in catalytic efficiency compared to the effect of replacing Asp392, suggesting that it is not catalytically important (*9*). Analysis of the known structure of *H. jecorina* (formerly *Trichoderma reesei*) CBHII (PDB entry 1CB2) reveals that the corresponding Asp175 is positioned near the acid catalyst Asp221 where it is proposed to help increase the latter's pK_a (*22*). On the other hand, its role in Cel6A from *T. fusca* (PDB entry 1TML) is considered to be more substantial (*12*), perhaps even playing the role of the catalytic base (*23*). While not conclusive, the results of this study support the role of Asp392, and not Asp216, as the catalytic base in CenA. The failure of cysteinesulfinate to functionally replace Asp216 in CenA suggests that both its positioning and chemical properties are critical. As discussed above, it would appear that the general base catalyst of an inverting enzyme is less dependent on such factors. On the other hand, these physicochemical properties would, for example, greatly affect the precision of the hydrogen bonding network and influence the pK_a of the catalytic acid, the role originally proposed for the residues equivalent to Asp216 in *T. fusca* Cel6A (*12*). The dramatic decrease in activity seen in the Asp216Asn mutant at pH >6.5 (Figure 5) provides strong evidence that the role of this residue in CenA is to increase the pK_a of the catalytic acid, allowing it to remain protonated at higher pH. While this seems to eliminate Asp216 as a candidate, a definitive identification of the catalytic base will require further experimentation, possibly involving the azide rescue technique (*24*), as recently applied to *T. fusca* β-1,3-glucanase (*25*).

The finding that Asp252 could not be replaced with cysteinesulfinic acid to yield an enzyme active against CMC, a substrate for which an acid catalyst is required, is consistent with its putative role as the catalytic acid (*9*). As such, it needs to be precisely positioned and in the protonated state for catalysis. Both conditions are achieved through interactions with surrounding residues, and the decreased pK_a of the introduced cysteinesulfinic acid would appear to have a catastrophic effect. Similarly, the catalytic nucleophile of Cel5A, Glu355, cannot be replaced with cysteinesulfinate. This speaks to the unique ability among catalytic residues in glycoside hydrolases of a catalytic base to tolerate this substitution. Indeed, we have shown that replacement of a catalytic acid, the nucleophile of a retaining enzyme, and a residue critical to modulating the pK_a of catalytic residues each retain only minimal activity after such replacements. Even substrate binding appears to be negatively impacted by the presence of this moiety, judging by the increase in K_M seen in the Asp392Cys mutant. It would seem that the base position is the only one with sufficient flexibility to allow this replacement. Thus cysteinesulfinate replacement should prove to be a valuable tool for the determination of catalytic base residues.

An interesting finding of this study was that oxidation of the respective Cys mutant proteins was taking place during their production in vivo. However, while not encountered previously with glucoamylase (*3*), a similar situation was found with other enzymes in which oxidation of cysteine to cysteinesulfinic acid was used to alter the acidic properties of a residue in an enzyme (*26*−*28*). With lactose permease, Voss et al. (*26*) did not investigate the oxidation state of their Cys mutant enzyme

prior to its oxidation, but they did observe an unexpected high level of residual activity (~10% of the wild-type level) prior to its treatment. In the other two studies involving Δ5−3-ketosteroid isomerase (*27*) and phosphoribulokinase (*28*), the mutant enzymes with Cys replacements were found to be oxidized to levels as high as 30% prior to any treatment. As found also in this study, the addition of high concentrations of a reducing agent (DTT) was found to be ineffective in preventing the spontaneous oxidation of Δ5−3-ketosteroid isomerase. The difference in the susceptibility of these Cys replacements to spontaneous oxidation is likely due to their respective local environments. Indeed, Blackinton et al. (*29*) previously demonstrated that replacing the residues around a Cys residue in the DJ-1 protein has profound consequences on its susceptibility to oxidation. In an extreme example, a single-amino acid substitution was able to shift the protein from being produced in a completely unoxidized form to being 50% oxidized prior to any treatment. Thus, it would appear that environments around the Cys replacements in CenA, and especially that of Asp- or Cys252, are conducive to oxidation. Presumably, this would not be the case for the active site of glucoamylase, and it would explain why it required more than 96 h for complete oxidation of the Glu400Cys enzyme (*3*).

Another major difference between the production of the Glu → Cys glucoamylase and the other enzymes, including CenA, was the expression system used. With the latter studies, *E. coli* was used, but the production of the mutant glucoamylase involved the fungal expression host *Pichia pastoris*. Whereas both eukaryotes and prokaryotes maintain reducing environments in their cytoplasms (*30*), the compartmentalization of oxidative phosphorylation (aerobic respiration) in eukaryotes within the mitochondria provides physical containment of any reactive oxygen species that are created. Prokaryotes, on the other hand, do not have this separation, and while they do have systems to manage any reactive oxygen species, it is possible that they are less efficient during overproduction of recombinant proteins. Also, the signal sequence of CenA is recognized by *E. coli* (*31*), resulting in the transport of a significant proportion of the expressed protein to the periplasm where a strict reducing environment is not maintained.

This study has demonstrated the possibility of combining genetic engineering with chemical modification to generate designer enzymes for industrial and/or biotechnological applications, such as the production of cellulosic ethanol. Currently, the limiting factor to the economic viability of ethanol production from cellulosic materials is the efficient release of its component glucose molecules for subsequent fermentation (*32*). One favored approach is to pretreat the cellulosic material (e.g., straw) with steam and strong acid to release the cellulose from other plant cell wall polymers (primarily xylan and lignin) and its subsequent neutralization before digestion with a cocktail of cellulolytic enzymes. Clearly, generating enzymes that are more active and tolerant under acidic conditions, in addition to being more thermostable and produced in high yields, would help to reduce the costs associated with the pretreatment process. Whereas considerable advances have been made with respect to overexpression and/or production and thermostability of cellulolytic enzymes (*33*−*38*), manipulation of their pH−activity profiles remains an issue. Some success has been realized in shifting pH−activity profiles in the alkaline direction (*39*−*41*), but little has been reported on engineering acidic shifts. The results of this study suggest that the engineering of cysteinesulfinate as the general base catalyst for inverting enzymes provides a viable approach for further experimentation.

ACKNOWLEDGMENT

We thank Dr. Tony Warren (University of British Columbia, Vancouver, BC) for the generous gift of plasmid pUCEC2.

SUPPORTING INFORMATION AVAILABLE

Tables listing the bacterial strains and plasmids used in this study and the sequences of the oligonucleotides used for site-directed mutagenesis of *cenA* and *cel5A*. This material is available free of charge via the Internet at http://pubs.acs.org.

REFERENCES

1. Clarke, A. J. (1997) Biodegradation of Cellulose. Enzymology and biotechnology, CRC Press, Boca Raton, FL.
2. Zechel, D. L., and Withers, S. G. (2000) Glycosidase mechanisms: Anatomy of a finely tuned catalyst. *Acc. Chem. Res. 33*, 11–18.
3. Fierobe, H. P., Mirgorodskaya, E., McGuire, K. A., Roepstorff, P., Svensson, B., and Clarke, A. J. (1998) Restoration of catalytic activity beyond wild-type level in glucoamylase from *Aspergillus awamori* by oxidation of the Glu400 → Cys catalytic-base mutant to cysteinesulfinic acid. *Biochemistry 37*, 3743–3752.
4. Fierobe, H. P., Clarke, A. J., Tull, D., and Svensson, B. (1998) Enzymatic properties of the cysteinesulfinic acid derivative of the catalytic-base mutant Glu400 → Cys of glucoamylase from *Aspergillus awamori*. *Biochemistry 37*, 3753–3759.
5. Wong, W. K., Gerhard, B., Guo, Z. M., Kilburn, D. G., Warren, A. J., and Miller, R. C., Jr. (1986) Characterization and structure of an endoglucanase gene cenA of *Cellulomonas fimi*. *Gene 44*, 315–324.
6. Gilkes, N. R., Claeyssens, M., Aebersold, R., Henrissat, B., Meinke, A., Morrison, H. D., Kilburn, D. G., Warren, R. A., and Miller, R. C., Jr. (1991) Structural and functional relationships in two families of β-1,4-glycanases. *Eur. J. Biochem. 202*, 367–377.
7. Damude, H. G., Ferro, V., Withers, S. G., and Warren, R. A. (1996) Substrate specificity of endoglucanase A from *Cellulomonas fimi*: Fundamental differences between endoglucanases and exoglucanases from family 6. *Biochem. J. 315* (Part 2), 467–472.
8. Shen, H., Schmuck, M., Pilz, I., Gilkes, N. R., Kilburn, D. G., Miller, R. C., Jr., and Warren, R. A. (1991) Deletion of the linker connecting the catalytic and cellulose-binding domains of endoglucanase A (CenA) of *Cellulomonas fimi* alters its conformation and catalytic activity. *J. Biol. Chem. 266*, 11335–11340.
9. 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.
10. Meinke, A., Gilkes, N. R., Kwan, E., Kilburn, D. G., Warren, R. A., and Miller, R. C., Jr. (1994) Cellobiohydrolase A (CbhA) from the cellulolytic bacterium *Cellulomonas fimi* is a β-1,4-exocellobiohydrolase analogous to *Trichoderma reesei* CBH II. *Mol. Microbiol. 12*, 413–422.
11. Koivula, A., Ruohonen, L., Wohlfahrt, G., Reinikainen, T., Teeri, T. T., Piens, K., Claeyssens, M., Weber, M., Vasella, A., Becker, D., Sinnott, M. L., Zou, J. Y., Kleywegt, G. J., Szardenings, M., Stahlberg, J., and Jones, T. A. (2002) The active site of cellobiohydrolase Cel6A from *Trichoderma reesei*: The roles of aspartic acids D221 and D175. *J. Am. Chem. Soc. 124*, 10015–10024.
12. Wolfgang, D. E., and Wilson, D. B. (1999) Mechanistic studies of active site mutants of *Thermomonospora* fusca endocellulase E2. *Biochemistry 38*, 9746–9751.
13. Vuong, T. V., and Wilson, D. B. (2009) The absence of an identifiable single catalytic base residue in *Thermobifida fusca* exocellulase Cel6B. *FEBS J. 276*, 3837–3845.
14. Andrade, M. A., Chacón, P., Merelo, J. J., and Morán, F. (1993) Evaluation of secondary structure of proteins from UV circular dichroism using an unsupervised learning neural network. *Protein Eng. 6*, 383–390.
15. Somogyi, M. (1952) Notes on sugar determination. *J. Biol. Chem. 195*, 19–23.
16. Nelson, N. (1944) A photometric adaptation of the Somogyi method for the determination of glucose. *J. Biol. Chem. 153*, 375–380.
17. Ellman, G., and Lysko, H. (1979) A precise method for the determination of whole blood and plasma sulfhydryl groups. *Anal. Biochem. 93*, 98–102.

18. Laemmli, U. K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature 227*, 680–685.
19. Gilkes, N. R., Warren, R. A., Miller, R. C., Jr., and Kilburn, D. G. (1988) Precise excision of the cellulose binding domains from two *Cellulomonas fimi* cellulases by a homologous protease and the effect on catalysis. *J. Biol. Chem. 263*, 10401–10407.
20. Irwin, D. C., Spezio, M., Walker, L. P., and Wilson, D. B. (1993) Activity studies of eight purified cellulases: Specificity, synergism and binding domain effects. *Biotechnol. Bioeng. 42*, 1002–1013.
21. Palmieri, F., Stipani, I., and Iacobazzi, V. (1979) The transport of L-cysteinesulfinate in rat liver mitochondria. *Biochim. Biophys. Acta 555*, 531–546.
22. Spezio, M., Wilson, D. B., and Karplus, P. A. (1993) Crystal structure of the catalytic domain of a thermophilic endocellulase. *Biochemistry 32*, 9906–9916.
23. André, G., Kanchanawong, P., Palma, R., Cho, H., Deng, X., Irwin, D., Himmel, M. E., Wilson, D. B., and Brady, J. W. (2003) Computational and experimental studies of the catalytic mechanism of *Thermobifida fusca* cellulase Cel6A (E2). *Protein Eng. 16*, 125–134.
24. Viladot, J. L., de Ramon, E., Durany, O., and Planas, A. (1998) Probing the mechanism of *Bacillus* 1,3−1,4-β-D-glucan 4-glucanohydrolases by chemical rescue of inactive mutants at catalytically essential residues. *Biochemistry 37*, 11332–11342.
25. McGrath, C. E., Vuong, T. V., and Wilson, D. B. (2009) Site-directed mutagenesis to probe catalysis by a *Thermobifida fusca* β-1,3-glucanase (Lam81A). *Protein Eng., Des. Sel. 22*, 375–382.
26. Voss, J., Sun, J., Venkatesan, P., and Kaback, H. R. (1998) Sulfhydryl oxidation of mutants with cysteine in place of acidic residues in the lactose permease. *Biochemistry 37*, 8191–8196.
27. Holman, C. M., and Benisek, W. F. (1994) Extent of proton transfer in the transition states of the reaction catalyzed by the Δ5−3-ketosteroid isomerase of *Comamonas* (*Pseudomonas*) *testosteroni*: Site-specific replacement of the active site base, aspartate 38, by the weaker base alanine-3-sulfinate. *Biochemistry 33*, 2672–2681.
28. Runquist, J. A., and Miziorko, H. M. (2002) Anionic substitutes for catalytic aspartic acids in phosphoribulokinase. *Arch. Biochem. Biophys. 405*, 178–184.
29. Blackinton, J., Lakshminarasimhan, M., Thomas, K. J., Ahmad, R., Greggio, E., Raza, A. S., Cookson, M. R., and Wilson, M. A. (2009) Formation of a stabilized cysteine sulfinic acid is critical for the mitochondrial function of the parkinsonism protein DJ-1. *J. Biol. Chem. 284*, 6476–6485.
30. Stewart, E. J., Aslund, F., and Beckwith, J. (1998) Disulfide bond formation in the *Escherichia coli* cytoplasm: An in vivo role reversal for the thioredoxins. *EMBO J. 17*, 5543–5550.
31. Guo, Z., Arfman, N., Gilkes, N. R., Kilburn, D. G., Warren, R. A. J., and Miller, R. C., Jr. (1987) Leakage of *Cellulomonas fimi* cellulases from *Escherichia coli*. *FEMS Microbiol. Lett. 49*, 279–283.
32. Wilson, D. B. (2009) Cellulases and biofuels. *Curr. Opin. Biotechnol. 20*, 295–299.
33. Karhunen, T., Mäntylä, A., Nevalainen, K. M., and Suominen, P. L. (1993) High frequency one-step gene replacement in *Trichoderma reesei*. I. Endoglucanase I overproduction. *Mol. Gen. Genet. 241*, 515–522.
34. Rahman, Z., Shida, Y., Furukawa, T., Suzuki, Y., Okada, H., Ogasawara, W., and Morikawa, Y. (2009) Evaluation and characterization of *Trichoderma reesei* cellulase and xylanase promoters. *Appl. Microbiol. Biotechnol. 82*, 899–908.
35. Paloheimo, M., Mäntylä, A., Kallio, J., Puranen, T., and Suominen, P. (2007) Increased production of xylanase by expression of a truncated version of the *xyn11A* gene from *Nonomuraea flexuosa* in *Trichoderma reesei*. *Appl. Environ. Microbiol. 73*, 3215–3224.
36. Miettinen-Oinonen, A., Paloheimo, M., Lantto, R., and Suominen, P. (2005) Enhanced production of cellobiohydrolases in *Trichoderma reesei* and evaluation of the new preparations in biofinishing of cotton. *J. Biotechnol. 116*, 305–317.
37. Miettinen-Oinonen, A., and Suominen, P. (2002) Enhanced production of *Trichoderma reesei* endoglucanases and use of the new cellulase preparations in producing the stonewashed effect on denim fabric. *Appl. Environ. Microbiol. 68*, 3956–3964.
38. Heinzelman, P., Snow, C. D., Wu, I., Nguyen, C., Villalobos, A., Govindarajan, S., Minshull, J., and Arnold, F. H. (2009) A family of thermostable fungal cellulases created by structure-guided recombination. *Proc. Natl. Acad. Sci. U.S.A. 106*, 5610–5615.
39. Becker, D., Braet, C., Brumer, H. E., Claeyssens, M., Divne, C., Fagerström, B. R., Harris, M., Jones, T. A., Kleywegt, G. J., Koivula, A., Mahdi, S., Piens, K., Sinnott, M. L., Stahlberg, J., Teeri, T. T., Underwood, M., and Wohlfahrt, G. (2001) Engineering of a glycosidase family 7 cellobiohydrolase to more alkaline pH optimum: The pH behaviour of *Trichoderma reesei* Cel7A and its E223S/A224H/L225V/T226A/D262G mutant. *Biochem. J. 356*, 19–30.
40. Wang, T., Liu, X., Yu, Q., Zhang, X., Qu, Y., Gao, P., and Wang, T. (2005) Directed evolution for engineering pH profile of endoglucanase III from *Trichoderma reesei*. *Biomol. Eng. 22*, 89–94.
41. Qin, Y., Wei, X., Song, X., and Qu, Y. (2008) Engineering endoglucanase II from *Trichoderma reesei* to improve the catalytic efficiency at a higher pH optimum. *J. Biotechnol. 135*, 190–195.