Site-directed Mutagenesis of Essential Carboxylic Residues in Clostridium thermocellum Endoglucanase CelD*

(Received for publication, October 9, 1991)

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12 carboxyl residues of the Clostridium thermocellum endoglucanase CelD were mutated to alanine. The specific activity of five of the mutated proteins was 1% or less that of wild type. The Ca²⁺ binding isotherms of these five were similar to those of wild type CelD, consistent with the fact that none of the mutated residues is observed to be directly involved in Ca²⁺ binding in the three-dimensional structure of the protein (Juy, M., Anit, A. G., Alzuri, P. M., Poljak, R. J., Claeyssens, M., Béguin, P., and Aubert, J.-P., manuscript in preparation) and suggesting that the mutations did not result in gross alterations of the tertiary structure. Analysis of the physico-chemical and enzymatic properties of the five purified mutated proteins and consideration of their position in the three-dimensional structure suggest that carboxyl groups identified may play roles as a general acid catalyst and a source of negative charge in stabilizing a carbonium ion intermediate. Among mutated residues, Glu-555 appears as the most likely candidate for participating in the catalytic mechanism of endoglucanase CelD.

Cellulases, like other carboxydrases, are thought to act by general acid catalysis involving carboxylic residues. Although a great variety of cellulases has been characterized from many microorganisms, most of them can be ordered into six different structural paradigms (families A to F) according to hydrophobic cluster analysis (1).

Experimental data concerning the catalytic mechanism of cellulases are available for a small number of enzymes. These include the chemical modification and spectrophotometric analysis of essential tryptophanyl and carboxylic residues in Schizosaccharomyces pombe endoglucanase I (family A) (2–4), the site-directed mutagenesis of an essential Glu residue in Bacillus subtilis and Bacillus polymyxa endoglucanases (both belonging to family A) (5), the three-dimensional structure of the catalytic core and the identification of the active site of Trichoderma reesei cellobiohydrolase II (family B) (6, 7), and the mapping of a peptide containing an essential carboxylic residue of the catalytic site of T. reesei cellobiohydrolase I (family C) (8). The role of carboxylic residues of T. reesei cellobiohydrolase I has also been studied by site-directed mutagenesis (9).

*C. thermocellum endoglucanase CelD was chosen as a representative member for detailed structural and functional studies of family E cellulases. Family E includes, beside C. thermocellum CelD (10), a number of cellulases of bacterial, fungal, and plant origin, such as Butyrivibrio fibrisolvens cellobextrinase CelD (11), C. thermocellum endoglucanase CelE, Cellulomonas fimii endoglucanase CenB (12), Clostridium stercolorum Avicelase I (13), Persea americana endoglucanase (14), Dictyostelium discoideum endoglucanase (15), Cellulomonas fimii endoglucanase CenC (16), and Pseudomonas fluorescens var. cellulosa endoglucanase A (17). CelD is part of the high molecular weight cellulase complex, or cellulosome, of C. thermocellum. The corresponding gene, celD, has been overexpressed in Escherichia coli, and the enzyme is easily purified in large amounts from cytoplasmic inclusion bodies (19). Purified CelD forms crystals suitable for x-ray diffraction analysis (20) and the three-dimensional structure of the enzyme was solved recently. Furthermore, several biochemical features of CelD have been established. Calcium binding parameters and the effect of calcium on the Kₘ and thermostability of the enzyme have been determined (21). In addition, a histidyl residue has been identified within the active center (22).

In this paper, we tried to localize the carboxylic residue(s) (aspartate, glutamate) which are involved in the catalytic mechanism of CelD. 5 carboxylic residues whose mutation inactivated the enzyme were identified. The inactive mutated proteins were purified. Catalytic and physicochemical properties of wild type and mutated forms were analyzed and compared with structural data derived from x-ray diffraction analysis in order to determine which residues were most likely to participate directly in the catalytic mechanism of the enzyme.

MATERIALS AND METHODS

Strain

The host strain was E. coli TG1 (31) = K-12Δ(lac-proA),thi,supE,hsdD8(F'traD36,proAB*,lacFΔZΔM15).

Plasmid Constructions

In all plasmids encoding CelD, the coding sequence of celD was cloned at the HindIII site of the vector, fusing the 25th codon of celD in-frame with the first codons of the lacZ' gene (10). Plasmids

1 To confirm with accepted practice, the endoglucanases encoded by C. thermocellum cel genes and previously termed EGA, EGB, etc. have been renamed CelA, CelB, etc.

2 Navarro, A., Chebrou, M.-C., Béguin, P., and Aubert, J.-P., (1992) Res. Microbiol., in press.

3 M. Juy, P. Alzari, and R. J. Poljak, personal communication.

* This work was supported by Grant EN3B-0062-F from the Commission of the European Communities and by research funds from the University of Paris 7. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

† Supported by a fellowship from the French Ministry of Research and Technology.

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pCT650, pCT651, and pCT6520 to pCT6532 carried a 1642-bp HindIII-HincII fragment, which encodes a truncated, but functional polypeptide corresponding to the active domain of the enzyme (21).

Reconstitution of a Cassette System for Introducing Mutations—The original celD gene did not contain restriction sites allowing to mutate small fragments whose nucleotide sequence could be easily verified and which could be easily reinserted into the gene. Consequently, five restriction sites not affecting the sequence of the encoded polypeptide were inserted by site-directed mutagenesis. The HindIII-HincII fragment of celD, encoding the catalytic domain of CelD, was cloned into the pTZ19U phagemid (23) to yield pCT650 (Fig. 1). pCT651 was obtained from pCT650 by introducing EcoRI, BamHI, PstI, KpnI, and XhoI sites (Fig. 2). To introduce a stop codon close to the 3' end of the truncated gene and to delete restriction sites of the multiple cloning site of the vector, the HindIII-HincII fragment from pCT651 was cloned into pUCS19 to yield pCT652. The pUCS19 plasmid was previously constructed by cloning the oligonucleotide 5'-AATTAGTTAACT-3' at the EcoRI site of pUC19, introducing an HpaI site immediately followed by a stop codon. From pCT652, small fragments of the celD gene (from 104 to 422 bp of length) were cloned into M13mp18 or M13mp19 to mutate each of the chosen carboxylic residues. 12 carboxylic residues were mutated to alanine. Wild type fragments of pCT652 were exchanged for homologous fragments carrying a mutation to yield pCT6521 to pCT6532.

Reconstitution of Plasmids Isogenic to pCT603—Mutated plasmids pCT6541 to pCT6545 were constructed to insert mutations carried by pCT6521, pCT6522, pCT6526, pCT6530, and pCT6532, respectively, into a background isogenic to the original overproducing clone carrying pCT603 (19). The clones were obtained by three-partner liguations between the HindIII-cut pUC8 vector, the mutated HindIII-HincII fragment from pCT6521, pCT6522, pCT6526, pCT6530, and pCT6532, respectively, and the HindII-HindIII fragment carrying the

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**Fig. 1.** Plasmid constructions. ○, celD coding sequence; ■, duplicated, 3'-terminal segment of celD; C. thermocellum DNA adjacent to the celD coding sequence. The name of each plasmid is underlined. The vector used for each construct is indicated on the left of each plasmid. The orientation of lacZ', fused to the 25th codon of celD (10), is indicated by a horizontal arrow. In pUCS19 and pCT652, the sequence of the oligonucleotide originally inserted at the EcoRI site of pUC19 is in boldface type. The stop codon in frame with the coding sequence of celD in pCT652 and in pCT6520 to pCT6532 is boxed. The star indicates a carboxylic residue mutation. See text for details.

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The abbreviations used are: bp, base pair(s); p-NPC, p-nitrophenyl-β-D-cellobioside; MOPS, 3-(N-morpholino)propanesulfonic acid; EGTA, [ethylenebis(oxyethylenenitrilo)]tetraacetic acid.
![Catalytic Domain](image)

**Fig. 2. Position of five new restriction sites within the celD gene.** The five new restriction sites are in boldface type, three-terminal segment of celD. The sizes of the celD gene and the HindIII-HincII fragment encoding the catalytic domain are 1847 and 1642 nucleotides, respectively.

The 3' end of the celD gene. The latter fragment was isolated from the pCT611 plasmid, whose HindIII site at the 5' end of celD had been deleted. The same procedure was used to obtain pCT6540, carrying the five restriction sites in the celD gene but encoding wild type CelD.

**Site-directed Mutagenesis**

Mutagenic oligonucleotides (16- to 23-mers) were synthesized using a MilliGen/Biosearch DNA synthesizer. All mutants were obtained using either the Muta-Gen Phagemid or the Muta-Gen M13 in vitro mutagenesis kits provided by Bio-Rad. Both kits are based on the counterselection of the uracil-substituted template strand obtained after growth in a dut, ung strain (24). The whole 3'-deleted celD gene carrying the five new restriction sites and all fragments carrying a mutation inactivating the enzyme were sequenced throughout to ascertain the absence of any secondary mutation.

**Preparation of Crude Extracts**

Recombinant *E. coli* strains expressing mutated celD genes were grown overnight with aeration at 37 °C in 50 ml of Luria-Bertani medium (25) containing 100 μg/ml carbenicillin. Cells were pelleted at 5,000 × g for 10 min, suspended in 10 ml of PC buffer (50 mM K3HPO4, 12.5 mM citric acid, pH 6.3), and disrupted by sonication. The extract was cleared by centrifugation at 12,000 × gmax for 10 min.

**Enzyme Assay**

In crude extracts, the activity toward p-NPC was measured in PC buffer as described previously (21). For Kₐ and kₐ determinations, purified proteins were incubated at 60 °C in a 50 mM sodium MOPS buffer, pH 6.3, containing 1-9 mM p-NPC plus either 10 mM EGTA or 1 mM CaCl₂. The hydrolysis of p-NPC was followed continuously in a Unikon 930 spectrophotometer (Kontron) fitted with a thermostat. Activities were calculated using experimentally determined ε coefficients for p-nitrophenol dissolved in the assay buffers. One unit of activity is defined as the amount of enzyme liberating 1 μmol of p-nitrophenol per min.

**Assay of CelD Protein**

Protein concentration was determined using the Coomassie Blue G-250 binding assay (26) with bovine serum albumin as a standard.

The concentration of CelD present in the crude extracts of strains expressing mutated genes was estimated from Western blots. 4 μg of total protein from each crude extract was loaded on a SDS-polyacrylamide gel. After electrophoresis, proteins were transferred to nitrocellulose (27), and the blot was probed with anti-CelD antiserum as detected in a weighed sample from an empty area of the blot.

**Calcium-binding Assay**

Calcium-binding of purified mutated proteins was assayed as described for wild type CelD (21). The release of 45Ca from Chelex-100 previously saturated with 45Ca was measured in the presence of purified mutated proteins.

**Calcium-binding Assay**

Calcium-binding assay was performed using crude extracts of mutated clones containing pCT6521 to pCT6532, which code a truncated polypeptide containing the catalytic domain of CelD.

**Specific Activity of Mutated Proteins**

The activity present in crude extracts was related to the amount of CelD protein detected by immunoblotting. The percentage of residual activity of each mutated CelD protein was determined relative to the activity of wild type CelD expressed from pCT6520.

### Table I

**Specific activity of mutated proteins**

| Clones   | Mutation          | Relative specific activity |
|----------|-------------------|----------------------------|
| pCT6520  | None (wild type)  | 100                        |
| pCT6521  | Asp-198 → Ala     | 0.08                       |
| pCT6522  | Asp-201 → Ala     | 0.2                        |
| pCT6523  | Asp-246 → Ala     | 72                         |
| pCT6524  | Asp-317 → Ala     | 98                         |
| pCT6525  | Asp-361 → Ala     | 86                         |
| pCT6526  | Asp-517 → Ala     | 0.8                        |
| pCT6527  | Asp-522 → Ala     | 2.24                       |
| pCT6528  | Glu-527 → Ala     | 127                        |
| pCT6529  | Asp-543 → Ala     | 91                         |
| pCT6530  | Asp-546 → Ala     | 1                          |
| pCT6531  | Asp-549 → Ala     | 118                        |
| pCT6532  | Glu-555 → Ala     | 0.3                        |

### Table II

**Calcium-binding parameters of wild type and mutated CelD**

| Mutation          | Kₐ (10⁻⁶ M⁻¹) | Number of Ca⁺⁺ binding sites |
|--------------------|---------------|-----------------------------|
| None (wild type)   | 3.0           | 2.2                         |
| Asp-198 → Ala      | 2.3           | 2.1                         |
| Asp-201 → Ala      | 2.3           | 2.4                         |
| Asp-517 → Ala      | 3.4           | 2.1                         |
| Asp-546 → Ala      | 4.6           | 1.5                         |
| Glu-555 → Ala      | 2.8           | 2.3                         |

**RESULTS**

**Choice of Mutagenized Residues**—Among the 64 carboxylic residues present in the catalytic domain of CelD, 12 were chosen for site-directed mutagenesis according to the following criteria: (i) residues Asp-198, Asp-201, Asp-246, Asp-317, Asp-361, Asp-546, and Glu-555 were conserved in *C. thermocellum* CelF and *P. fluorescens var. cellulosa* endoglucanase A; (ii) residues Asp-517, Asp-523, Glu-527, Asp-543, and Asp-549 were located in a region where chemical modification studies had indicated the presence of a carboxylic residue participating in the catalytic center of CelD.

**Specific Activity of Mutated CelD**—A preliminary characterization of the mutations was carried out using crude extracts of mutated clones containing pCT6521 to pCT6532 plasmids, which encode a truncated polypeptide containing the catalytic domain of CelD.

Two types of mutants were obtained (Table I). Seven clones retained at least 70% of the wild type activity. A 2-fold increase in specific activity was even observed for the Asp-198 → Ala mutation, but was not further investigated. The other five clones, pCT6521, pCT6522, pCT6526, pCT6530, and pCT6532 carrying the Asp-198 → Ala, Asp-201 → Ala, Asp-517 → Ala, Asp-546 → Ala, and Glu-555 → Ala mutations, respectively, had at most 1% of the wild type activity. Similar results were obtained when activity assays were performed at 30 °C, indicating that the phenotype of the inactive

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4 P. Tomme and M. Claeyssens, personal communication.
mutants was not due to thermal inactivation.

**Purification of Mutated Forms of CelD**—Purification of CelD forms with strongly decreased activity was greatly facilitated by constructing plasmids pCT641 to 6545, in which the Asp-198 → Ala, Asp-201 → Ala, Asp-517 → Ala, Asp-546 → Ala, and Glu-555 → Ala mutations, respectively, were carried in a background isogenic to pCT603. As observed for wild type CelD, mutated proteins carrying the noncatalytic COOH-terminal domain formed cytoplasmic inclusion bodies.

**TABLE III**

Catalytic parameters of wild type and mutated CelD and influence of calcium on activity

| Mutated residue | $k_{cat}$ (EGTA) | $K_m$ (EGTA) | $k_{cat}$ (CaCl$_2$) | $K_m$ (CaCl$_2$) |
|-----------------|-----------------|-------------|---------------------|-----------------|
| None (wild type)| 11.0 $\times 10^{-3}$ | 13.9 $\times 10^{-3}$ | 11.3 $\times 10^{-3}$ | 2.7 $\times 10^{-3}$ |
| Asp-198         | NM$^a$          | NM$^b$      | NM$^a$              | NM$^b$          |
| Asp-201         | 3.5 $\times 10^{-2}$ | 4.2 $\times 10^{-2}$ | 29                 | 16.9            |
| Asp-517         | 3.0 $\times 10^{-2}$ | 4.2 $\times 10^{-2}$ | 9.2                | 1.6             |
| Glu-555         | 2.2 $\times 10^{-3}$ | 3.4 $\times 10^{-3}$ | 9.2                | 1.6             |

$^a$ NM, not measurable. Activity in the presence of 9 mM p-NPC was less than 0.003% of the wild type under the same assay conditions.

$^b$ Activity in the presence of 9 mM p-NPC was less than 0.001% of the wild type under the same assay conditions.

$^c$ Activity in the presence of 9 mM p-NPC was less than 0.0001% of the wild type under the same assay conditions.

$^d$ Activity in the presence of 9 mM p-NPC was less than 0.000% of the wild type under the same assay conditions.

(21, 29), from which the mutated proteins could be purified as described by Joliff et al. (19) for wild type CelD.

**Enzymatic Properties of Mutated Forms of CelD**—Wild-type CelD is known to have a strong affinity for calcium, with two calcium binding sites per molecule and a binding constant of about $2 \times 10^6$ M$^{-1}$. Calcium enhances the activity of CelD by increasing the affinity for the substrate by about 4-fold and increases the thermostability of the protein (21).

As shown in Table II, wild type and mutated forms of CelD displayed very similar calcium binding properties, with affinity constants comprised between 2.3 and $4.6 \times 10^6$ M$^{-1}$, and 1.5–2.4 sites per molecule.

Catalytic parameters of mutated proteins were determined in the presence of 1 mM CaCl$_2$ or 10 mM EGTA (Table III). Both in the presence and absence of calcium, activity was below reliable assay level for CelD Asp-198 → Ala. In the absence of calcium, the activity of CelD Asp-201 → Ala was also not measurable. In other cases, inactivation of the mutated CelD proteins was mostly due to a decrease in $k_{cat}$, ranging between 30- and 5000-fold. However, a significant increase in $K_m$ could be observed for CelD Asp-546 → Ala and, in the presence of calcium, for CelD Asp-201 → Ala and CelD Asp-517 → Ala. By contrast, the Glu-555 → Ala mutation did not increase the $K_m$, whereas the $k_{cat}$ was decreased by about 5000-fold in the absence of calcium, and by about 4000-fold in the presence of calcium.

For all proteins with detectable activity, the addition of calcium stimulated the rate of p-NPC hydrolysis. In the case...
FIG. 4. Sedimentation of wild type and mutated CelD proteins on sucrose gradients. 500 µg of inactive mutated CelD plus 50 µg of wild type CelD were layered onto 5 ml of 5–20% sucrose gradients containing 50 mM Tris–HCl, pH 7.7, plus 1 mM CaCl₂ and centrifuged at 300,000 × g for 21 h 30 min at 15 °C in a SW50-Ti rotor (Beckman). 200-µl fractions were collected from the bottom of the gradient. ◇, activity (arbitrary units); □, A₂₅₀.

FIG. 5. Amino acid sequence alignment of highly conserved regions within cellulases of family E. In the three regions of homology, numbers indicate the position of the first amino acid displayed. For each protein, the number starts at the initial Met residue. Shaded areas indicate amino acids strictly conserved in at least five of the enzymes. Residues whose mutagenesis led to inactivation of CelD are indicated by a negative sign. I, C. thermocellum endoglucanase CelD (10); II, B. fibrisolvens cellobiohydrolase Cel1 (11); III, C. thermocellum endoglucanase CelF; IV, C. fimi endoglucanase CenB (12); V, C. stercorarium Avicelase I (13); VI, P. americana endoglucanase (14); VII, D. discoideum endoglucanase (15); VIII, C. fimi endoglucanase CenC (16); IX, P. fluorescens var. cellulosa endoglucanase A (17).

of the Asp-517 → Ala and Asp-546 → Ala mutations, the effect was mostly due to an increase in kₐ, whereas for the wild type and for the Glu-555 → Ala mutation, calcium mostly decreased the kₐ, with little effect on kₐ.

Given the low activity of the mutated proteins, it was impractical to check whether calcium enhanced their half-life at high temperature. However, like wild type CelD, the five mutated proteins began to precipitate after 20–30 min at 60 °C when incubated at a concentration of 100 µg/ml in the presence of 10 mM EGTA. Aggregation was prevented in all cases when the proteins were incubated in the presence of 1 mM CaCl₂.

Ultrogel AcA-44 Gel Chromatography and Sedimentation Analysis—The behavior of wild type and mutated proteins was compared in gel filtration experiments using LKB Ultrogel AcA-44. A tracer amount of wild type enzyme was loaded together with each mutated protein, and the elution of either species was followed by monitoring the A₂₅₀ or the activity, respectively. Fig. 3 shows that all mutated proteins migrated faster than the wild type enzyme except CelD Glu-555 → Ala, which comigrated with the wild type. However, the migration of wild type and mutated forms of CelD corresponded to molecular masses of between 36 and 41 kDa, as estimated from the migration of standard molecular weight markers (bovine serum albumin, ovalbumin, chymotrypsinogen, and ribonuclease). Since the molecular mass of CelD had previously been estimated at 65 kDa by sedimentation equilibrium (19), the anomalously slow migration of the proteins was most likely due to some interaction with a component of the gel matrix, presumably agarose, which amounts to 4% of Ultrogel AcA-44. Since differences in migration could not unambiguously be ascribed to changes in affinity for the gel matrix or to changes in Stokes' radius, the sedimentation of mutated proteins was analyzed using sucrose gradients (Fig. 4). Cosedi-
mentation with wild type CelD was observed with all proteins. Thus, except for the Glu-555 → Ala mutation, all mutations affected the interaction of CelD with the gel matrix of AcA-44.

**DISCUSSION**

Among the 12 mutations studied, five inactivated CelD: Asp-198 → Ala, Asp-201 → Ala, Asp-517 → Ala, Asp-546 → Ala, and Glu-555 → Ala. The 5 residues are located within three segments highly conserved in cellulases of type E (1) (Fig. 5). Asp-198, Asp-201, Asp-546, and Glu-555 are strictly conserved in all enzymes, whereas Asp-517 is replaced by His in most cases, by Leu in B. fibrisolvens cellodextrinase CelD, and by Ser in D. discoideum endoglucanase.

While this paper was being written, the three-dimensional structure of CelD was solved by x-ray crystallography. The most likely site for catalysis consists of a cleft at the surface of the protein which binds the cocrystallized inhibitor α-iodobenzyl-β-D-thioglucoside. The cleft is formed by three loops connecting six of the 12 α-helices which constitute the core of the enzyme. Asp-198 and Asp-201 are located on the first loop, which spans residues 152 to 207, whereas Asp-517, Asp-546, and Glu-555 are located on the third loop, which spans residues 413 to 558. The second loop, spanning residues 259 to 296, does not contain conserved carboxylic residues. The loops are expected to have a more flexible structure than the α-helix core, which is highly compact. Accordingly, mutations affecting the loops may result in local structural alterations, but should not induce major changes in the folding of the protein. This may explain why none of the studied mutations affected the sedimentation coefficient of the protein significantly.

The effects of the Glu-555 → Ala mutation were closest to those expected for a mutation affecting a catalytic residue. The $k_{cat}$ of the enzyme was reduced by at least 4000-fold, but other properties, such as $K_m$, affinity for Ca$^{2+}$, effect of Ca$^{2+}$ on enzymatic properties, and chromatographic behavior on Ultrogel AcA-44 were quite similar to those of wild type CelD. The properties of Glu-555 are in agreement with results indicating that a carboxylic residue essential for the activity of CelD lies between Leu-507 and Val-572 and with the structure of the enzyme, in which Glu-555 points toward the inside of the catalytic cleft. The effects of the Glu-555 → Ala mutation on the $k_{cat}$ and $K_m$ of CelD were similar to those observed with the Glu-179 → Gin mutation of Aspergillus awamori glucoamylase. In the latter case, the $k_{cat}$ was reduced almost 2000-fold, with no decrease of the $K_m$, and Glu-179 was proposed as the general catalytic acid of the enzyme (30). A similar role may therefore be suggested for Glu-555 of CelD. The Asp-198 → Ala and Asp-201 → Ala mutations also resulted in very strong inactivation of CelD. They caused a small, but significant decrease in the elution volume of CelD on Ultrogel AcA-44. Assuming that the effect is due to a decrease in affinity for the carbohydrate component of the matrix, this would suggest that the mutations affect substrate binding. Indeed, the $K_m$ in the presence of Ca$^{2+}$ of the enzyme carrying the Asp-201 → Ala mutation was increased 13-fold as compared to the wild type. However, this was accompanied by a 1000-fold decrease in $k_{cat}$, and, although activities were too low to determine activity parameters, very low $k_{cat}$ values can also be assumed for Asp-198 → Ala CelD and for Asp-201 → Ala CelD assayed in the absence of Ca$^{2+}$. At present, structural data suggest that either carboxylic residue might be a candidate to stabilize the positively charged transition state of the substrate. The residual activity observed with the mutations affecting Asp-517 and Asp-546 was between one and three orders of magnitude higher than with Asp-198, Asp-201, and Glu-555 mutations. This is in agreement with the position of the side chains of Asp-517 and Asp-546, which are not favorably located to participate directly in the hydrolysis reaction. Conformational alterations may explain the effects observed with the mutations affecting these residues, in particular the changes in $K_m$, and chromatographic behavior.

Like wild type CelD, all mutated inactive proteins tested displayed Ca$^{2+}$ binding, Ca$^{2+}$-enhanced stability at 69 °C, and, as far as could be assayed, Ca$^{2+}$-enhanced activity, although the effect of Ca$^{2+}$ on activity parameters depended on the mutation. This observation is in agreement with structural data showing that complexation of Ca$^{2+}$ involves none of the mutated residues, although Ca$^{2+}$ does appear to stabilize a conformation of CelD with an increased affinity for the substrate. It will be of interest to investigate whether other cellulases of family E also show Ca$^{2+}$-binding and Ca$^{2+}$-sensitive properties.

Conservation of Ca$^{2+}$-binding properties further confirms that the mutations did not result in gross structural alterations of the protein. In addition, our results are in good agreement with structural data derived from x-ray diffraction analysis. They support the general hypothesis that enzymatic hydrolysis of carbohydrates involves a proton donor (Glu-555) and a negatively charged residue (Asp-198 or Asp-201) stabilizing the positively charged intermediate of the reaction.

**ACKNOWLEDGMENTS**—We wish to thank M. Jay and P. Alzari for discussing features of the three-dimensional structure of CelD prior to publication, M. Claeyssens and P. Tomme for communicating their results on chemical modification of carboxylate residues prior to publication, M. Goldberg for useful discussions, and M.-K. Daniel for skilful technical help.

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