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Structural and Functional Analysis of the Metal-binding Sites of Clostridium thermocellum Endoglucanase CelD*

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Clostridium thermocellum synthesizes a multienzymatic cellulose complex with a molecular mass of 2–4 MDa, termed cellulosome (1, 2). Endoglucanase CelD is a component of the cellulosome, which can be easily purified in large amounts from inclusion bodies produced in recombinant Escherichia coli (3). CelD belongs to the family E of cellulases (4, 5). The three-catalytic domains that are closely associated: a small amino-terminal a-helical (CelD246A), B (CelD361A), or C (CelD523A) were compared with wild type CelD. The Ca2+-binding isotherm of wild type CelD was compatible with two high affinity sites (Km = 2 × 10^6 M^-1) and one low affinity site (Km < 10^5 M^-1). The Ca2+-binding isotherms of the mutated proteins showed that sites A and B were the high affinity sites and that site C was the low affinity site. Atomic absorption spectrometry confirmed the presence of a tightly bound Zn2+ atom per CelD molecule. The inactivation rate of CelD at 75 °C was decreased 1.9-fold upon increasing the Ca2+ concentration from 2 × 10^-6 to 10^-3 M. The Km of CelD was decreased 1.8-fold upon increasing the Ca2+ concentration from 5 × 10^-6 to 10^-4 M. Over similar ranges of concentration, Ca2+ did not affect the thermostability nor the kinetic properties of CelD361A. These findings suggest that Ca2+ binding to site C stabilizes the active conformation of CelD in agreement with the close vicinity of site C to the catalytic center.

Crystallographic analysis indicated that Clostridium thermocellum endoglucanase CelD contained three Ca2+-binding sites, termed A, B, and C, and one Zn2+-binding site. The protein contributed five, six, and three of the coordinating oxygen atoms present at sites A, B, and C, respectively. Proteins altered by mutation at site A (CelD246A), B (CelD361A), or C (CelD523A) were compared with wild type CelD. The Ca2+-binding isotherm of wild type CelD was compatible with two high affinity sites (Km = 2 × 10^6 M^-1) and one low affinity site (Km < 10^5 M^-1). The Ca2+-binding isotherms of the mutated proteins showed that sites A and B were the high affinity sites and that site C was the low affinity site. Atomic absorption spectrometry confirmed the presence of a tightly bound Zn2+ atom per CelD molecule. The inactivation rate of CelD at 75 °C was decreased 1.9-fold upon increasing the Ca2+ concentration from 2 × 10^-6 to 10^-3 M. The Km of CelD was decreased 1.8-fold upon increasing the Ca2+ concentration from 5 × 10^-6 to 10^-4 M. Over similar ranges of concentration, Ca2+ did not affect the thermostability nor the kinetic properties of CelD361A. These findings suggest that Ca2+ binding to site C stabilizes the active conformation of CelD in agreement with the close vicinity of site C to the catalytic center.

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MATERIALS AND METHODS

Crystallographic Analysis—Two isomorphous crystal forms of CelD were grown using ammonium sulfate (i.e. no added calcium) or 300 mM calcium chloride as precipitants. Structure determination and independent refinement of the two forms at 2.3 Å resolution have been described elsewhere (6). The present models comprise residues 36–574 and include three calcium ions, one zinc ion, and 221 (ammonium sulfate) or 204 (calcium chloride) water molecules. The final agreement factors between observed and calculated structure factor amplitudes in the resolution range 6–2.3 Å were 17.0% for 33,211 observed reflections with F > 5 σ(F) (ammonium sulfate) and 17.4% for 29,797 observed reflections (calcium chloride). Root mean squares deviations of bond lengths and angles from ideality were 0.007 Å and 1.6°, respectively, in both crystal structures.

Bacterial Strains and Plasmids—Plasmids pCT6523, pCT6525, and pCT6527, encoding the catalytic domain of CelD and carrying the D246A, D361A, and D523A mutations, respectively, were previously obtained (10). Each of the mutations was inserted into a plasmid whose sequence included the 3′-end of celD, as previously described (10). The resulting plasmids, carrying the D246A, D361A, and D523A mutations, were termed pCT6543, pCT6545, and pCT6547, respectively. The plasmid pCT6540, encoding the wild type enzyme, has been described (10). The pCT6540 and pCT6547 plasmids were harbored by E. coli TG1 (12) = R-K12, Δ(lac-proAB), thi, supE, hsdD5 (F′ traD36, proAB+, lacF−, lacZAM15). pCT6543 and pCT6545 were harbored by E. coli JM101 (13) = K-12, Δ(lac-proAB), thi, supE, hsdD5 (F′ traD36, proAB+, lacF−, lacZAM15). Purification of Wild Type and Mutant Forms of CelD—E. coli cells harboring the appropriate plasmids were grown to stationary phase at 37 °C in Luria Bertani broth (14) containing 100 µg/ml ticarcillin. Wild type and mutant forms of CelD were purified from inclusion bodies as previously described (10). Low and high M′, forms of CelD361A (CelD-A′)

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and CelD(oxa) (CelD-B*) were separated on a Mono-Q anion exchange column using a fast performance liquid chromatography system (Pharmacia Biotech Inc.). Up to 4 mg of purified protein was loaded on a Mono-Q HR6/5 anion exchange column (1 ml) equilibrated with 20 mM Tris-HCl, pH 7.7, at a rate of 1 ml/min. Elution was performed at 0.7 ml/min using a linear gradient from 100 to 250 mM NaCl in the same buffer. The low M_r and high M_r peaks were eluted at 150 and 180 mM NaCl, respectively, and concentrated by ultrafiltration using a YM10 Amicon membrane. All samples were dialyzed against 40 mM Tris-HCl, pH 7.7.

Protein Electrophoresis—SDS-PAGE was performed according to Laemmli (11). Samples were boiled for 5 min in 2% SDS, 10% glycerol, 5% β-mercaptoethanol, 62.5 mM Tris-HCl, pH 6.8. Non-denaturing PAGE was performed using the same procedure, omitting SDS and β-mercaptoethanol and the heat treatment of the samples.

Zinc Assay—The zinc content of wild type CelD was assayed by flame atomic absorption spectroscopy at 213.9 nm using a Varian AA-1275 spectrophotometer (Varian Techtron, Springdale, Australia), with a single element hollow-cathode lamp for zinc (16).

Ca²⁺-binding Assay—Binding of ⁴⁰Ca to purified proteins was assayed by monitoring the release of ⁴⁰Ca from Chelex-100 (Bio-Rad) previously equilibrated with various concentrations of ⁴⁰Ca (11).

Enzyme and Protein Assays—All reagents used in assays performed in the presence of controlled concentrations of Ca²⁺ were kept in disposable plasticware (Sterilin) and were handled with disposable plastic pipettes or pipette tips. Divalent metals were removed from 50 mM Na-MOPS buffer, pH 6.3, and from 20 mM p-NPC, dissolved in the same buffer, by shaking with 10% (w/v) Chelex-100. The resin was removed by centrifuging at 1,000 × g for 2 min. Ca²⁺ was removed from CelD by shaking in the presence of 10% Chelex-100 followed by decantation. Alternatively, the enzyme was diluted in Chelex-treated buffer so that the contribution of protein-bound Ca²⁺ in the assay medium was less than 5 × 10⁻⁹ M, assuming 3 mol of Ca²⁺ bound/mol of CelD. No difference was observed between the results obtained with either procedure, even when no Ca²⁺ was added (data not shown).

Enzyme activity was assayed at 60 °C in 50 mM Na-MOPS buffer, pH 6.3, containing CaCl₂, EGTA, or ZnCl₂ as indicated for each experiment and 0.5–20 mM p-NPC as substrate. The reaction was stopped after less than 5% of the substrate had been hydrolyzed by adding ½ vol 1 M Na₂CO₃. One unit of activity is defined as the amount of enzyme liberating 1 μmol of p-nitrophenol (ε = 1.61 × 10⁴ cm⁻¹ M⁻¹) per min. Protein concentration was measured using the Coomassie Blue reagent supplied by Bio-Rad (17), with bovine serum albumin as a standard.

Thermostability—Proteins were either treated with Chelex-100 or diluted so that their contribution to the concentration of Ca²⁺ in the inactivation reaction was less than 1.5 × 10⁻⁴ M. No difference was observed between the results obtained with either procedure, even when no Ca²⁺ was added (data not shown).

Proteins were incubated at 75 °C as a concentration of 3–5 × 10⁻⁸ M in 50 mM MOPS buffer, pH 6.3, containing CaCl₂, EGTA, or ZnCl₂ as indicated for each experiment. Temperature control was ascertained by checking the temperature inside of a plastic vial similar to those in which the inactivation reaction was performed. Samples were withdrawn at several time intervals and chilled on ice, and ZnCl₂ and CaCl₂ were added to a final concentration of 1 mM (2 mM CaCl₂ in the case of samples containing 1 mM EGTA). Residual activity was assayed as described above, using 0.9 mM p-NPC.

Computations—Kinetic constants (including the 95% confidence interval) for the rate of inactivation were computed from linear regressions of log (residual activity) versus time, using the Instat Mac® program (version 2.0, GraphPad Software). K_m and k_cat values were calculated by non-linear regression using the KaleidaGraph® program (version 2.1, Abelbeck Software).

RESULTS

Crystallographic Analysis of Ca²⁺-binding Sites in CelD—The three-dimensional structure of CelD revealed four metal-binding sites occupied by atoms heavier than water in the crystal. A first internal site is located immediately behind a protein loop involved in substrate binding and catalysis (Zn sphere in Fig. 1). The tetrahedral coordination by two Cys and two His side chains and the displacement by Hg suggests that this site is occupied by a Zn⁺⁺ ion (6). The three other metal binding sites are located close to the molecular surface in different regions of the protein (spheres A, B, and C in Fig. 1).

From the coordination geometry, these three positions could be identified as Ca²⁺-binding sites.

The coordination of the Ca²⁺ ion bound at site A appears as a slightly distorted octahedral arrangement with a water molecule at one of the vertices (Fig. 2A). Protein groups donate the five other oxygen ligands: two main chain carbonyls at positions 236 and 241, and the side chains of residues Asn-239, Asp-243, and Asp-246. The loop forming this site protrudes into the solvent and appears to be stabilized by calcium.

Seven oxygen atoms chelate the Ca²⁺ ion at site B. In this case, the coordination polyhedron appears as a distorted pentagonal bipyramid with Asp-362 and a main chain carbonyl at position 401 on the vertices, or alternatively as a distorted octahedral arrangement with one bidentate ligand, Asp-361 (Fig. 2B). In addition to the aspartate residues, protein oxygens involved in Ca²⁺ binding include the side chain of Thr-356 and the main chain carboxyl groups at positions 358 and 401. As shown in Fig. 2B, this site appears to have a structural role in linking together two different regions of the protein.

The protein loop forming site C is completely exposed to the solvent, with three out of the six oxygen ligands donated by water molecules (Fig. 2C). Main chain carbonyls at positions 520 and 525 and the carboxylate group of Asp-523 complete the calcium coordination polyhedron. Unlike sites A and B, the protein loop forming binding site C is partially involved in intermolecular interactions in the crystal. The side chain of Arg-314 from a neighbor molecule is stacked against Trp-526, and the carbonyl group at position 524 forms an intermolecular hydrogen bond with the guanido group of Arg-416 (data not shown).
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each other in loop conformation as well as in the side chains and the number of water molecules involved in the coordination polyhedra.

Overall, only small structural differences were observed for the structure of CelD at 0 and 300 mM calcium. The coordination geometry of the three sites was essentially the same within experimental error (Table I). Only the temperature factors of the calcium atoms bound at sites A and C were different in the two crystal forms (the temperature factors for the three calcium atoms were 27, 25, and 32 Å², respectively, at 300 mM CaCl₂, and 43, 28, and 47 Å² at 0 mM CaCl₂), suggesting partial calcium occupancy of sites A and C in ammonium sulfate-grown crystals.

The D246A, D361A, and D523A mutations were chosen to inactivate Ca²⁺-binding sites A, B, and C, respectively. The corresponding proteins will be termed CelD-A*, CelD-B*, and CelD-C*, respectively.

Separation of High and Low M₉ Forms of CelD-A* and CelD-B*-SDS-PAGE analysis indicated that the wild type and the three mutant proteins were mainly composed of 65-kDa CelD, with 68- and 63-kDa CelD being present as minor species in some of the preparations (Fig. 3A). Previous work has shown that proteolysis accounts for some heterogeneity of the COOH terminus of CelD. However, cleavage does not affect the catalytic domain of the protein, and the 68-, 65-, and 63-kDa species were shown to share very similar catalytic properties (9, 11, 19).

In non-denaturing electrophoresis (Fig. 3B), CelD-C* displayed the same mobility as wild type CelD, which is a monomeric protein (3). However, CelD-A* and CelD-B* could be separated into a form with a mobility similar to that of the wild type monomer and a slower migrating, higher Me₉ form, presumably resulting from self-association. The two forms could be separated by ion exchange chromatography on a Mono-Q column (Fig. 3B) or by gel filtration on a TSK G2000 column (data not shown) but tended to reequilibrate over a period of a few days. This explains the partial contamination of one form by the other seen in Fig. 3B.

Presence of Zn²⁺—Atomic absorption spectroscopy showed the presence of 1.0 ± 0.2 mol of Zn²⁺/mol of wild type CelD. No change in Zn²⁺ content was detected when the enzyme was incubated for 15 min at room temperature or at 60 °C in the presence of 10% (w/v) Chelex-100, but incubation with Chelex at 75 °C for 9 min resulted in total loss of detectable enzymatic activity.

The coordination polyhedra of the three Ca²⁺-binding sites. The course of the polypeptide chain is indicated by a smooth tracing. Liganded groups (side chain residues, main chain carbonyls, water molecules) are indicated explicitly. Oxygen and nitrogen atoms are in gray. Ca²⁺ is drawn as a larger sphere inside of the coordination polyhedron. A, site A; B, site B; C, site C. Diagrams were drawn with MOLSCRIPT (21).

Sites B and C are close to either end of the substrate-binding groove and are expected to have some influence on the catalytic activity of CelD. On the opposite site of the α-barrel, the Ca²⁺ ion bound at site A stabilizes a helix-connecting loop with no obvious role in enzymatic activity. As a general rule, the conformation of the loops forming the three Ca²⁺-binding sites does not follow the EF-hand pattern observed in many Ca²⁺-binding proteins (18). Moreover, they differ significantly from each other in loop conformation as well as in the side chains and the number of water molecules involved in the coordination polyhedra.

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Addition of 1 mM EGTA had little effect on the inactivation rate of each purified protein were analyzed by SDS-PAGE. 

The presence of one Zn$^{2+}$ ion/mol of CelD, predicted from the crystallographic analysis of the protein, was confirmed by biochemical analysis. Zn$^{2+}$ binding appeared quite stable at room temperature and at 60 °C, and dissociation of Zn$^{2+}$ at 75 °C was accompanied by rapid denaturation of the enzyme. By contrast, Ca$^{2+}$ could be dissociated from CelD without denaturing the protein.

Previous interpretation of Ca$^{2+}$ binding data had led to the conclusion that CelD contained two high affinity Ca$^{2+}$-binding sites (11). Points extending beyond two sites/molecule in the Scatchard plots were not considered in the analysis. However, crystallographic analysis revealed the presence of three putative Ca$^{2+}$-binding sites in CelD (6). The presence of three functional Ca$^{2+}$-binding sites was confirmed by the analysis of CelD$^{C*}$, whose mutation affects site C. The Ca$^{2+}$-binding isotherm of CelD-C$^{*}$ displayed two high affinity sites similar to those of the wild type, but, in contrast to the wild type, binding did not exceed 2.1 mol of Ca$^{2+}$ bound/mol of protein. This suggests that in the wild type, points extending between 2 and 3 mol of Ca$^{2+}$ bound/mol of protein were due to the presence of site C, which behaved like a low affinity site. High affinity Ca$^{2+}$ binding to sites A and B was confirmed by analysis of CelD-A$^{*}$ and CelD-B$^{*}$. The Ca$^{2+}$-binding isotherms of both proteins showed that each mutation abolished high affinity binding to one site. The relative affinities of sites A, B, and C were consistent with the fact that in sites A and B, the protein contributes five and six, respectively, of the coordinating oxygens but only three of the coordinating oxygens of site C.

Mutagenesis of site A or B seemed to abolish binding to site C, as if site C could form only when both sites A and B are occupied. Why this should be the case is not obvious from structural analysis.

Investigation of the kinetic parameters of CelD indicated that the change in $k_{\text{cat}}$ of the enzyme as a function of the Ca$^{2+}$ concentration was strongest between 5 × 10^{-6} and 10^{-4} M. This range is most likely accounted for by the increased occupancy of the low affinity site C rather than the high affinity sites A and B. The fact that the kinetic parameters of CelD-C$^{*}$ were not affected by Ca$^{2+}$ confirms this interpretation.

The stabilization of wild type CelD occurred at concentrations that were an order of magnitude higher than those required to affect catalytic parameters. This may be explained by the fact that changes in catalytic properties induced by Ca$^{2+}$ dissociation are reversible, whereas thermal denaturation is not. The Ca$^{2+}$ concentrations at which stabilization was observed were consistent with a requirement for occupancy of site C rather than site A and B. Accordingly, inactivation of site C abolished Ca$^{2+}$-induced stabilization of CelD.
The lowest Ca$^{2+}$ concentration was calculated from the contribution of Ca$^{2+}$ initially bound to the enzyme added to the assay. Except for the EGTA-treated samples, all samples contained 1 mM ZnCl$_2$ in addition to the Ca$^{2+}$ concentrations indicated.

The fact that Ca$^{2+}$ binding to site C enhanced the substrate binding affinity and stabilized the conformation of the catalytic site is consistent with the close vicinity of the two sites. The loop containing the Ca$^{2+}$-coordinating residues Ser-520, Asp-523, and Ile-525 is connected to the substrate-binding residues His-516 and Arg-518. His-516 and Arg-518 formed hydrogen bonds with hydroxyl groups of the inhibitor o-iodobenzyl-I$_3$-D-celllobioside in the crystal structure of the enzyme-inhibitor complex (8). In addition, chemical modification and mutagenesis studies identified His-516 as an important residue of the catalytic center (20).

The self-association of monomeric CelD-A* and CelD-B* into a high $M_r$, presumably dimeric form was not correlated with the occupancy of Ca$^{2+}$-binding sites. For both proteins, addition of Ca$^{2+}$ or EGTA during non-denaturing electrophoresis failed to alter the proportion of the two forms (data not shown). Both forms displayed very similar Ca$^{2+}$-binding isotherms. Self-association did not seem to influence thermostability nor kinetic parameters (data not shown). However, the compound effects of site A and B mutations on site C precluded a straightforward analysis of the influence of Ca$^{2+}$ on the stability and kinetic properties of the mutant enzymes.

Unlike catalytic residues, none of the residues involved in Ca$^{2+}$ binding is strictly conserved among all catalytic domains of family E cellulases. At present, it is difficult to predict from sequence analysis which of the other members of family E may be stabilized in a similar manner by Ca$^{2+}$. It would be of interest to know whether the presence of functional Ca$^{2+}$-binding sites is correlated with the thermostability of the enzymes.

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REFERENCES
1. Lamed, R., Setter, E., and Bayer, E. A. (1983) J. Bacteriol. 156, 828–836
2. Lamed, R., Setter, E., Kenig, R., and Bayer, E. A. (1983) Biotechnol. Bioeng. Symp. 13, 163–181.
3. Joliff, G., Béguin, P., Juy, M., Millet, J., Ryter, A., Poljak, R., and Aubert, J.-P. (1986) Bio/Technology 4, 896–900.
4. Henrissat, B., Claeyssens, M., Tomme, P., Lemele, L., and Mornon, J.-P. (1989) Gene (Amst.) 81, 83–95.
5. Gilkes, N. R., Henrissat, B., Kilburn, D. G., Miller, R. C., Jr., and Warren, R. A. J. (1991) Microbiol. Rev. 55, 363–315.
6. Juy, M., Amit, A. G., Alturi, P. M., Poljak, R. J., Claeyssens, M., Béguin, P., and Aubert, J.-P. (1990) Nature 347, 89–91.
7. Henrissat, B. (1993) Gene (Amst.) 125, 199–204.
8. Tokatlidis, K., Salamitou, S., Béguin, P., Dhurjati, P., and Aubert, J.-P. (1991) FEBS Lett. 284, 183–188.
9. Tokatlidis, K., Dhurjati, P., and Béguin, P. (1993) Protein Eng. 6, 947–952.
10. Chauvaux, S., Béguin, P., and Aubert, J.-P. (1992) J. Biol. Chem. 267, 4472–4478.
11. Chauvaux, S., Béguin, P., Aubert, J.-P., Bhat, K. M., Gow, L. A., Wood, T. M., and Baird, A. (1990) Biochem. J. 265, 261–265.
12. Gibson, T. J. (1984) Studies on the Epstein-Barr Virus Genome. Ph.D. thesis, University of Cambridge, Cambridge.
13. Yanisch-Perron, C., Vieira, J., and Messing, J. (1985) Gene (Amst.) 33, 103–119.
14. Maniatis, T., Fritsch, E. F., and Sambrook, J. (1982) Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.
15. Laemmli, U. K. (1970) Nature 227, 680–685.
16. Moret, S. and Henkin, R. I. (1971) Clin. Chem. 17, 369–373.
17. Bradford, M. (1976) Anal. Biochem. 72, 248–254.
18. Kretsinger, R. (1980) CRC Crit. Rev. Biochem. 8, 119–174.
19. Tokatlidis, K., Dhurjati, P., Millet, J., Béguin, P., and Aubert, J.-P. (1991) FEBS Lett. 282, 205–208.
20. Tomme, P., Chauvaux, S., Béguin, P., Millet, J., Aubert, J.-P., and Claeyssens, M. (1991) J. Biol. Chem. 266, 10313–10318.
21. Kraulis, P. J. (1991) J. Appl. Crystallogr. 24, 946–950.