Calcium Protects a Mesophilic Xylanase from Proteinase
Inactivation and Thermal Unfolding*

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Crystal structure analysis of Pseudomonas fluorescens subsp. cellulosa xylanase A (XYLA) indicated that the enzyme contained a single calcium binding site that did not exhibit structural features typical of the EF-hand motif. Isothermal titration calorimetry revealed that XYLAs binds calcium with a $K_d$ of $4.9 \times 10^8$ M$^{-1}$ and a stoichiometry consistent with one calcium binding site per molecule of enzyme. Occupancy of the calcium binding domain with its ligand protected XYLAs from proteinase and thermal inactivation and increased the melting temperature of the enzyme from 60.8 to 66.5 °C. However, the addition of calcium or EDTA did not influence the catalytic activity of the xylanase. Replacement of the calcium binding domain, which is located within loop 7 of XYLAs, with the corresponding short loop from Cex (a Cellulomonas fimii xylanase/exoglucanase), did not significantly alter the biochemical properties of the enzyme. These data suggest that the primary function of the calcium binding domain is to increase the stability of the enzyme against thermal unfolding and proteolytic attack. To understand further the nature of the calcium binding domain of XYLAs, four variants of the xylanase, D256A, N261A, D262A, and XYLA, in which Asp-256, Asn-261, and Asp-262 had all been changed to alanine, were constructed. These mutated enzymes did not show any significant binding to Ca$^{2+}$, indicating that Asp-256, Asn-261, and Asp-262 play a pivotal role in the affinity of XYLAs for the divalent cation. In the presence or absence of calcium, XYLA exhibited thermal stability similar to that of the native enzyme bound to Ca$^{2+}$ ions, although the variant was sensitive to proteinase inactivation. The role of the calcium binding domain in vivo and the possible mechanism by which the domain evolved are discussed.

Endo-β1,4-xylanases (xylanases; EC 3.2.1.8) catalyze the cleavage of internal β-1,4 glycosidic linkages in the backbone of xylans (1, 2). Using hydrophobic cluster analysis, the catalytic domains of glycosyl hydrolases have been classified into 57 different enzyme families (3). Members of each family are thought to have evolved from a common ancestral sequence. Xylanases belong to either Family 10 or 11 (4). Recently, hydrophobic cluster analysis has shown that several enzyme families have common folds, suggesting that they evolved from a single sequence (5). Support for the concept of a common evolutionary link among some glycosyl hydrolase families is provided by the conservation of the three-dimensional structure of enzymes from different families, particularly in the vicinity of the active site, and the observation that glycosidases belonging to a specific clan cleave glycosidic bonds by the same mechanism (5, 6).

In general, glycosyl hydrolases are unusually resistant to proteolytic attack and thermal inactivation (7). Although calcium ions often play an important role in conferring structural stability on proteins, only two cellulases, a hybrid Bacillus glucanase and CelD from Clostridium thermocellum, have been shown to bind to Ca$^{2+}$ and to both Ca$^{2+}$ and Zn$^{2+}$ ions, respectively (8, 9). High concentrations of calcium enhanced the thermostability of both enzymes and decreased the $K_m$ of CelD for 4-nitrophenyl-β-cellobioside (9, 10).

Recently, the three-dimensional structures of the catalytic domain of four Family 10 enzymes have been solved (11-14). They all consist of an (α/β)$_n$-fold barrel structure in which two conserved glutamates function as the catalytic nucleophile and acid/base catalytic residues, respectively. Xylanase A (XYLA) from Pseudomonas fluorescens subsp. cellulosa is a modular enzyme comprising an NH$_2$-terminal cellulose binding domain linked to a COOH-terminal catalytic domain. The catalytic domain is unique within Family 10 enzymes as it is the only xylanase described to date which contains a calcium binding site (located in loop 7) (11), and it is the only xylanase from either mesophilic or thermophilic microorganisms which has been shown to be sensitive to proteinases (15). The function(s) of the calcium binding domain in XYLAs and the structural basis for the enzyme’s sensitivity to proteinases remain to be elucidated.

The objective of this report is to determine the role of the calcium binding domain in XYLAs. The data presented show that occupation of the calcium binding loop with its ligand protected the enzyme from thermal inactivation, thermal unfolding, and proteolytic attack. A mutant of XYLAs in which the key residues of the calcium binding domain were replaced by alanine exhibited thermal stability similar to that of XYLAs complexed with Ca$^{2+}$ ions; however, the xylanase variant was susceptible to cleavage by chymotrypsin. The role of the calcium

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1 The abbreviations used are: XYLAs, xylanase A; MUC, 4-methylumbelliferyl-β-cellobioside; PAGE, polyacrylamide gel electrophoresis; ITC, isothermal titration calorimetry; DSC, differential scanning calorimetry; MOPS, 4-morpholinepropanesulfonic acid; $T_m$, thermal denaturation temperature.
cium binding domain in vivo and the possible mechanism by which the domain evolved are discussed.

**EXPERIMENTAL PROCEDURES**

**Mutagenesis of xynA**— Mutated forms of xynA (encodes the catalytic domain of XYLA) were prepared as described by Charnock et al. (16). The primers used to generate XYLA pol, XYLA Cex, D256A, D262A, and N261A were: 5’-CGATTGTTGTAACGGGGCATTACCGGGATAGGGATTAT-3’, 5’-GTCAGCAGCGCCGACAACCGTGA-5’, 5’-CTGGAATCCGGCATAACCTG-3’, 5’-GGCATGGTAAAGGCTTACTGGAAT-3’, and 5’-TGTTGGTATGCGCAGTCGGAGATTACCG-3’, respectively. The nucleotides that introduced the appropriate mutations into xynA are in bold.

**Purification of Wild Type and Mutant Forms of XYLA**— Recombinant strains of *Escherichia coli* expressing XYLA or its derivatives were cultured as in (17). Periplasmic fractions of the cells were prepared in the presence of 25 mM EDTA to complex both free and XYLA-associated calcium. The EDTA was then removed by dialysis against 100 volumes of 10 mM Tris/HCl buffer, pH 8.0, at 4 °C. XYLA was then purified by anion-exchange chromatography (17). The purified enzymes were dialyzed against 3 × 1,000 volumes of 50 mM Tris/HCl buffer, pH 7.5, containing 10 μl/liter Chelex 100 (Bio-Rad) to remove any remaining CaCl2. Aliquots were prepared in the subsequent analysis of the purified enzymes were treated with Chelex 100 (50 μl/liter) following the manufacturer’s instructions.

**Enzyme and Protein Assays**— XYLA activity was determined using oat spelt xylan, 4-nitrophenyl-β-d-cellobioside, and 4-methylumbelliferyl-β-d-cellobioside (MUC) as described previously (16). Assays were performed in 50 mM Tris/HCl buffer, pH 7.5, at 37 °C in the presence of the indicated concentrations of CaCl2 or EDTA. The cleavage of xylanose and the identity of the products released were evaluated by high performance liquid chromatography analysis (18). Protein concentration was determined by A280 nm using a molar extinction coefficient of 58,100 M−1 cm−1 (19) and by the method of Smedk and Grossberg (20).

**Proteolysis Studies**— Proteins were incubated with a-chymotrypsin in the ratio of 1:10 (w/w) in the presence of either 1 mM CaCl2 or 1 mM EDTA. Aliquots were removed at each time point between 0 and 20 min, heated to 100 °C in the presence of 2% SDS, and then subjected to SDS-PAGE (21). The NH2-terminal sequence of the major proteolytic product generated by proteolysis at D262 was determined as described previously (17). To determine the extent of proteolytic cleavage, the products released were electrophoresed on a 15% SDS/PAGE gel, stained with Coomassie Blue, and visualized using a gel documentation system.

**Circular Dichroism (CD) Spectroscopy**— CD spectra were recorded at 20 °C in a Jobin-Yvon CD6 spectropolarimeter using quartz cells of 0.01-cm path length. Proteins (1 mg/ml) were dialyzed as described for the DSC experiments. Spectra were collected before and after samples had been incubated at 57 °C for 30 min. Each spectrum was accumulated from at least three scans between 190 and 250 nm and was corrected for residual protein concentration from the A280 value.

**Sequence Analysis**— Sequences between the highly conserved motif ENMK at the NH2 terminus and KXXY at the COOH terminus of the catalytic domains of 23 of the 28 Family 10 xylanases (the five sequences not compared did not contain these highly conserved motifs) were aligned using the CLUSTAL W version 1.6 multiple alignment program (27). Initially, this alignment comprised 410 amino acid positions but was later reduced to 394 on removal of the positions corresponding to extended loop 7. Parsimony analyses were carried out using the PROTPARS routine in the PHYLIP 3.57c Phylogeny inference package (http://evolution.genetics.washington.edu). To evaluate the robustness of the inferred trees, 1,000 bootstrap resamplings of the data were made using SEQBOOT prior to parsimony analysis. A consensus tree was produced using CONSENSE. Both the SEQBOOT and CONSENSE routines are included as part of the PHYLIP 3.57c package.

**RESULTS**

**XYLA Binds to Calcium**— Crystallographic analysis of XYLA in the presence of 200 mM CaCl2 showed that the crystals contained generated Ca2+. The topology of the putative calcium binding domain of the enzyme is shown in Fig. 1. To confirm that XYLA binds to calcium, the purified enzyme was subjected to nondenaturing PAGE, blotted onto nitrocellulose membrane, and then probed with 45Ca. The data presented in Fig. 2A showed that the xylanase existed in two forms, presumably the monomer and dimer, which migrated differently on nondenaturing PAGE and confirmed that XYLA bound to calcium (Fig. 2B). The divalent ion did not appear to influence the oligomeric state of the enzyme (Fig. 2C). To determine the affinity of the xylanase for calcium and the stoichiometry of binding, the enzyme–ligand interaction was monitored using ITC. Fig. 3A shows a typical calorimetric titration of XYLA with Ca2+ at 25 °C. After correction for appropriate heats of dilution of the data were analyzed using Microcal ORIGIN software. The normalized results are shown in Fig. 3B and give the association constant (Kd) of 4.9 × 104 M−1, a stoichiometry (n) of 1, and an
The enthalpy of binding ($\Delta H^\circ$) of $\approx 4.615$ kJ mol$^{-1}$.

**Residues of XYLA Which Bind to Calcium**—The geometry of the putative calcium binding domain of XYLA suggests that Asp-256 (O$_{d1}$ and O$_{d2}$), Asn-261 (O$_{d1}$), Asp-262 (O$_{d1}$), and the carbonyl oxygens of Asn-253 and Asn-258 coordinate with the Ca$^{2+}$ ion (Fig. 1). The domain does not appear to exhibit a classical EF-hand motif, which is characterized by three or four glutamate or aspartate residues coordinating with calcium within a helix-loop-helix structure. To establish the importance of Asp-256, Asn-261, and Asp-262 in ligand binding, site-directed mutagenesis was used to construct three variants of XYLA containing D256A, N261A, and D262A mutations, respectively. A fourth mutant, designated XYLA$^{999}$, in which all three residues were replaced by alanine, was also generated.

The different migration patterns of the native and mutant forms of the xylanase, observed when the enzymes were subjected to nondenaturing gel electrophoresis (Fig. 2A), could reflect differences in either the overall charge and/or the size of the XYLA derivatives. Both ITC and $^{45}$Ca autoradiography of XYLA variants blotted onto nitrocellulose membranes (Fig. 2B) showed that XYLA$^{999}$ and each of the mutants containing single amino acid substitutions did not bind calcium. These data suggest that all three amino acids play an important role in calcium binding and support the view that the calcium binding domain of XYLA is located in the extended loop prior to $\alpha$-helix 7.

**The Influence of the Calcium Binding Domain on the Catalytic Properties of XYLA**—XYLA and XYLA$^{999}$ were purified to apparent homogeneity (Fig. 2D), and the activity of the two enzymes in the presence of calcium or EDTA was determined. The data (Table I) showed that the catalytic properties of the two forms of XYLA were very similar and were not influenced by either EDTA or calcium. The products released from xylohexaose, by XYLA (in the presence of 1 mM Ca$^{2+}$ or 1 mM EDTA) and XYLA$^{999}$, over time, were indistinguishable (data not shown). These data indicate that the calcium binding domain of XYLA does not play an important role in the catalytic activity of the enzyme.

**The Influence of the Calcium Binding Domain on the Bioophysical Properties of XYLA**—To investigate whether the calcium binding domain played an important role in the thermostability of the enzyme, the activity of native and mutant forms of XYLA was determined at different temperatures. In the presence of calcium, XYLA exhibited increased thermostability compared with the enzyme incubated with EDTA (Fig. 4). The
shows the calorimetric titrations of XYLA (135 μM) with 25 × 10⁻¹ μl injections of 8 mM CaCl₂ in Buffer A (50 mM Tris/HCl buffer, pH 7.5, containing 100 mM NaCl) at 25 °C (1); 13 × 10⁻¹ μl injections of Buffer A into protein solution (protein dilution) (2); 13 × 10⁻¹ μl injections of 8 mM CaCl₂ into Buffer A (calcium dilution) (3). Panel B displays the integrated injection heats from panel A (1), corrected for control dilution heats (panel A (2) and (3)). The solid line is the best fit curve that was used to derive parameters n, Kₘ, and ΔH°.

FIG. 3. ITC analysis of binding of calcium to XYLA. Panel A shows the calorimetric titrations of XYLA (135 μM) with 25 × 10⁻¹ μl injections of 8 mM CaCl₂ in Buffer A (50 mM Tris/HCl buffer, pH 7.5, containing 100 mM NaCl) at 25 °C (1); 13 × 10⁻¹ μl injections of Buffer A into protein solution (protein dilution) (2); 13 × 10⁻¹ μl injections of 8 mM CaCl₂ into Buffer A (calcium dilution) (3). Panel B displays the integrated injection heats from panel A (1), corrected for control dilution heats (panel A (2) and (3)). The solid line is the best fit curve that was used to derive parameters n, Kₘ, and ΔH°.

TABLE I

Activity of native and mutant forms of XYLA in the presence and absence of calcium

| Enzyme | Ca²⁺/EDTA | PNPC | Xylan |
|--------|-----------|------|-------|
|        | Kᵣ | kᵣ | Kᵣ | kᵣ |
| XYLA   | 1 mM Ca²⁺ | 40  | 50  | 0.7 | 21,000 |
| XYLA   | 1 mM EDTA | 40  | 63  | 1.0 | 22,000 |
| XYLA   | 1 mM Ca²⁺ | 50  | 100 | 1.1 | 28,000 |
| XYLA   | 1 mM EDTA | 50  | 100 | 1.4 | 34,000 |
| XYLA/Cex | 1 mM Ca²⁺ | 40  | 63  | 1.1 | 17,000 |
| XYLA/Cex | 1 mM EDTA | 40  | 63  | 0.9 | 17,000 |

*a* PNPC, 4-nitrophenyl-β-cellobioside.

first order rate constant (Kᵢₐ₅) for thermal inactivation of XYLA in the presence of 1 mM EDTA and 1 mM calcium at 57 °C was 5.1 s⁻¹ and 0.24 s⁻¹, respectively (Table II). The protection afforded by calcium exhibited saturation kinetics (Fig. 5) with the divalent ion having its maximal effect at a concentration of approximately 1 mM and above. These data clearly show that the association of the calcium binding domain with its ligand stabilizes the enzyme against thermal inactivation.

To establish whether the substitution of the key residues in the calcium binding domain of XYLA influenced the rate of thermal inactivation, the Kᵢₐ₅ of D256A, N261A, D262A, and D262A, and XYLA” at 57 °C was determined. The data, presented in Table II, showed that although XYLA” in the presence and absence of calcium, had Kᵢₐ₅ values similar to those of to Ca²⁺-XYLA, the other three variants were more prone to thermal inactivation, both in the presence and absence of calcium. These data indicate that Asp-256, Asn-261, and Asp-262 interact with each other to destabilize loop 7, resulting in an increase in the enzyme’s susceptibility to thermal inactivation. These deleterious interactions can be prevented by either the binding of calcium to loop 7 or the replacement of all three amino acids by alanine.

Although calcium stabilizes XYLA from thermal inactivation, it is not clear whether this involves a local effect that influences the structure of the active site or whether the divalent ion stabilizes the complete structure of the enzyme. To investigate the effect of calcium on the tertiary structure of XYLA, CD spectroscopy and DSC were employed. CD spectra of XYLA, treated for 30 min at 57 °C in the absence of calcium, indicated that the enzyme had very little secondary structure, suggesting that the protein was essentially unfolded (Fig. 6). In contrast, the CD spectra of XYLA-Ca²⁺, and XYLA’” (±CaCl₂), with and without pretreatment for 30 min at 57 °C, were very similar, suggesting that the higher temperature did not cause a significant unfolding of the two proteins. The stability of XYLA and its derivatives toward thermal unfolding was also measured using DSC. The thermal denaturation temperature (Tᵡ) of these proteins are shown in Table III and Fig. 7. The data showed that removal of calcium from XYLA resulted in an 6 °C drop in the Tᵡ (Fig. 7A). In the absence and presence of the divalent cation XYLA’” had a Tᵡ similar to that of XYLA-
Ca\(^{2+}\) (Fig. 7B). The \(T_m\) values of D256A, N261A, and D262A were significantly lower than XYLA-Ca\(^{2+}\) or XYLA\(^{-}\) and were unaffected by the presence or absence of calcium. These data indicate that the binding of calcium to XYLA causes a gross stabilization of the protein which results in a decrease in the thermal inactivation of the enzyme. Similarly, replacing the three amino acids that bind to the metal ion with alanine stabilizes the three-dimensional structure of the xylanase against thermal unfolding in the absence of calcium.

Proteolysis of XYLA—To establish whether calcium stabilizes the enzyme against proteinases, the susceptibility of XYLA to proteolytic inactivation in the presence of excess calcium or EDTA was assessed. The data, presented in Fig. 8, showed that the divalent ion protected the xylanase from chymotrypsin attack. The protection afforded by calcium exhibited saturation kinetics (Fig. 8); at concentrations \(\approx 1\) mM Ca\(^{2+}\) XYLA was completely resistant to proteolytic attack. Disruption of the calcium binding domain by replacing Asp-256, Asn-261, Asp-262, or all three residues with alanine increased the enzyme’s susceptibility to proteinase inactivation in the presence of calcium (Table II). To investigate whether chymotrypsin cleaved a specific peptide bond within the calcium binding domain, XYLA was incubated with chymotrypsin in the presence and absence of Ca\(^{2+}\) and subjected to SDS-PAGE. The data, presented in Fig. 9, show that the divalent ion protected the xylanase from chymotrypsin attack. The protection afforded by calcium exhibited saturation kinetics (Fig. 8); at concentrations \(\approx 1\) mM Ca\(^{2+}\) XYLA was completely resistant to proteolytic attack.

Disruption of the calcium binding domain by replacing Asp-256, Asn-261, Asp-262, or all three residues with alanine increased the enzyme’s susceptibility to proteinase inactivation in the presence of calcium (Table II). To investigate whether chymotrypsin cleaved a specific peptide bond within the calcium binding domain, XYLA was incubated with chymotrypsin in the presence and absence of Ca\(^{2+}\) and subjected to SDS-PAGE. The data, presented in Fig. 9, show that the major transient proteolysis product had a molecular mass of approximately 29 kDa (29,918 Da as determined by electrospray ionization mass spectrometry) and an NH\(_2\)-terminal sequence that matched XYLA.

It would appear, therefore, that cleavage of XYLA within the calcium binding loop between residues Asp-262 and Tyr-263 (would generate an NH\(_2\)-terminal peptide of 29,910 Da) gave rise to the 29-kDa polypeptide.

Does a Loop Swap between XYLA and Cex Stabilize the Xylanase?—Replacement of loop 7, between residues 252 and 273, with the corresponding smaller loop from the Cellulomonas fimi xylanase, Cex (28), generated a truncated enzyme, designated XYLA/Cex, which had a \(M_r\) of 37,000. XYLA/Cex was purified to homogeneity (Fig. 2D), and the biochemical and
The data, presented in Table I, showed that XYLA/Cex exhibited catalytic properties that were virtually indistinguishable from XYLA. In contrast, XYLA/Cex was more sensitive to both thermal and proteinase inactivation (Table II) than native XYLA bound to calcium.

**DISCUSSION**

The data presented in this paper showed that the $K_a$ of XYLA for calcium is $4.9 \times 10^4$ M$^{-1}$, which represents a relatively weak affinity compared with EF-hand calcium binding domains that bind their ligands with $K_a$ values of $10^5$–$10^9$ M$^{-1}$ (29). The low affinity of XYLA for the divalent ion could reflect the environment of *P. fluorescens* subsp. *cellulosae*. The bacterium was isolated from soil samples of neutral pH in which Ca$^{2+}$ would be at a concentration $>1$ mM, sufficient to saturate XYLA (30). Inspection of the location of the calcium binding domain in the xylanase suggested that its structure may affect the catalytic properties of the enzyme as Tyr-255, which apparently forms part of subsite B, is positioned on the Ca$^{2+}$ binding loop (11). However, the activity of the enzyme was not significantly influenced by either the disruption of the metal binding domain, through amino acid substitution or EDTA treatment, or saturation of the domain with its appropriate ligand. This suggests that either Tyr-255 does not play a pivotal role in xylene binding at site B, or the change in conformation of loop 7 through calcium binding does not influence the ability of the tyrosine residue to participate in substrate binding. It is also apparent that the conformational changes associated with calcium binding to loop 7, which influenced the biophysical properties of the enzyme, did not affect the structure of the extended substrate binding cleft.

The binding of the calcium binding loop to its ligand caused a substantial effect on the biophysical properties of XYLA. The enzyme was less sensitive to thermal inactivation, which reflected a general increase in the stability of the protein, as evidenced by a considerable increase in the $T_m$ of XYLA in the presence of the divalent cation. These data are in agreement with several previous studies demonstrating that calcium stabilizes the structure of many proteins (9, 10). Substituting Asp-256 or Asn-261 for alanine resulted in a substantial destabilization of the enzyme, whereas the D262A mutation resulted in a modest reduction in the thermal stability of the enzyme. It could be argued that these data suggest that Asp-262 is the key...
The microbial origin and the SWISS-PROT accession numbers of the enzymes compared were as follows: XYNA PENCH (Penicillium chrysogenum; P29417), XYNA ASPAK (Aspergillus awamori; P33559), GUNF FUSOX (Pusarium oxysporum; P46239), XYNA STRELI (Streptomyces lividans; P26514), XYNA CLOTM, XYN CLOTM, and XYNA CLOTM (Clostridium thermocellum; P38535, P51584, and P10478, respectively), GUX CELFI (Cellulomonas fim; P07986), XYNA PSEFL (Pseudomonas fluorescens subsp. cellulosa; P14788), XYN1 BACST and XYN2 BACST (Bacillus stearothermophilus; P49943 and P49703, respectively), XYNA BACOV (Bacteroides ovatus; P49942), XYNA PRERU (Prevotella ruminicola; P48789), XYNA RUMFL (Ruminococcus flavefaciens; P29126), XYNA BUTFI and XYNB BUTFI (Butyrivibrio fibrisolvens; P23551 and P26223, respectively), XYNA CALSA, GUNB CALSA, and XYNA CLOSA (Caldocellum saccharolyticum; P23556, P10474, and P23557, respectively), XYNA THER8 (Thermophilic bacterium RT8.B4; P40944), XYNA THESA (Thermoaerobacter saccharolyticum; P36917), and XYNA BAC55 (Bacillus sp. strain C-125; P07528).

The central unanswered questions are why do only 5 Family 10 xylanases contain an extended loop 7, and how did the calcium binding domain in Xyla evolve? Loop 7 may confer specific catalytic properties on the enzyme. However, the observation that the biochemical characteristics of Xyla are not altered when the extended loop is replaced with the corresponding shorter loop from Cex argues against this view. It is possible that the ancestral protein that gave rise to Family 10 xylanases contained an extended loop 7. Although inspection of the extended loop in Xyla shows that the extended structure of loop 7, either against thermal unfolding or proteinase attack. As Ca\(^{2+}\) only protects Xyla from thermal inactivation at temperatures above 55 °C, and the natural habitat of Pseudomonas is normally at a temperature lower than 30 °C, it is unlikely that the enhanced thermal stability afforded by calcium plays an important role in the survival of Xyla. We propose that the major function of the calcium binding domain of the xylanase is to protect the enzyme from proteinase attack. This view is supported by the unusual stability of other xylanases, from both mesophilic and thermophilic microorganisms, to proteolytic attack (7), suggesting that this property exerts a strong selection pressure on the evolution of xylanases.

To date only 5 of the 28 known Family 10 xylanases contain an extended loop 7. Although inspection of the extended loop in Xyla, Prevotella ruminicola Xyla (Xyna PRERU), Bacteroides ovatus Xyla (Xyna BACOV), and Bacillus stearothermophilus XYN1 and XYN2 (XYN1 BACST and XYN2 BACST, respectively) did not reveal motifs, such as the EF-hand, which are characteristic of calcium binding domains, it is apparent that loop 7 in Xyla did bind to the divalent metal ion. As XYN1 and 2 BACST function at elevated temperatures, and Xyna PRERU and Xyna BACOV in environments that contain high levels of proteinase activity, it is likely that loop 7 in these xylanases is stabilized, possibly through interactions with metal ions or other sequences within the respective proteins. The central unanswered questions are why do only 5 Family 10 xylanases contain an extended loop 7, and how did the calcium binding domain in Xyla evolve? Loop 7 may confer specific catalytic properties on the enzyme. However, the observation that the biochemical characteristics of Xyla are not altered when the extended loop is replaced with the corresponding shorter loop from Cex argues against this view. It is possible that the ancestral protein that gave rise to Family 10 xylanases contained an extended loop 7 that destabilized the protein, and through natural selection deletions within the loop have resulted in the evolution of stable xylanases. Mutations in loop 7 of the ancestral sequence, which gave rise to Xyla, generated a calcium binding domain that stabilized the loop, hence there was no requirement for the loop to be reduced in size in the Pseudomonas enzyme. An alternative possibility is that the extended loop 7, in 5 of the 28 Family 10 xylanases, is the result of a DNA insertion into a DNA sequence that gave rise to the genes encoding the 5 enzymes. In xynA the inserted DNA subsequently acquired mutations such that it encoded a cal-
cium binding domain in XYLA. To explore this hypothesis further, the primary structure of Family 10 xylanases were subject to phylogenetic analysis using parsimony methods. Initially, all sequence positions (410) were included in the analysis, which suggested a relationship between the xylanases containing the extended loop 7 (data not shown). To remove any bias due to the inclusion of extended loop 7 in the alignments, the corresponding sequence positions were removed and the analysis repeated (Fig. 10). It is interesting that the relationship between 4 of the 5 xylanases is maintained, indicating that the respective genes have evolved from a common ancestral sequence that contained a DNA insertion in the region encoding loop 7. However, this conclusion must be viewed with some caution as it is apparent that there has been considerable horizontal gene transfer between Family 10 xylanase genes (evidenced by the fact that very similar sequences occur in taxonomically diverse groups of organisms), and the bootstrap scores suggest that some clades are not particularly stable. Based on the relationship between the *Pseudomonas* xylanase (XYNA PSEFL) and the clade containing the actinomycete (GUX CELFI and XYNA STRLI) and fungal (XYNA PENCH, GUNF FUSOX, and XYNA PENCH) xylanases, one might hypothesize that the latter had evolved, following loss of the extended loop 7, from XYNA PSEFL. However, additional sequence information from this region of the tree is needed to validate this hypothesis.

To conclude, data presented in this report clearly showed that the extended loop 7 of *Pseudomonas* XYLA was stabilized by a calcium binding loop. Whether the corresponding extended loops of XYNA PRERU, XYNA BACOV, XYN1 BACST, and XYN2 BACST are also stabilized by binding to divalent metal ions, remains to be elucidated.

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