Key Residues in Subsite F Play a Critical Role in the Activity of Pseudomonas fluorescens Subspecies cellulosa Xylanase A Against Xyooligosaccharides but Not Against Highly Polymeric Substrates such as Xylan*  

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In a previous study crystals of Pseudomonas fluorescens subspecies cellulosa xylanase A (XYLA) containing xylopentaose revealed that the terminal nonreducing end glycosidic bond of the oligosaccharide was adjacent to the catalytic residues of the enzyme, suggesting that the xylanase may have an exo-mode of action. However, a cluster of conserved residues in the substrate binding cleft indicated the presence of an additional subsite, designated subsite F. Analysis of the biochemical properties of Xyla revealed that the enzyme was a typical endo-β,1,4-xylanase, providing support for the existence of subsite F. The three-dimensional structure of four family 10 xylanases, including Xyla, revealed several highly conserved residues that are on the surface of the active site cleft. To investigate the role of some of these residues, appropriate mutations of Xyla were constructed, and the biochemical properties of the mutated enzymes were evaluated. N182A hydrolyzed xylotetraose to approximately equal molar quantities of xylotriose, xylobiose, and xylose, while native Xyla cleaved the substrate to primarily xylobiose. These data suggest that N182 is located at the C site of the enzyme. N126A and K47A were less active against xylan and aryl-β-glycosides than native Xyla. The potential roles of Asn-126 and Lys-47 in the function of the catalytic residues are discussed. E43A and N44A, which are located in the F subsite of Xyla, retained full activity against xylan but were significantly less active than the native enzyme against oligosaccharides smaller than xyloseptaose. These data suggest that the primary role of the F subsite of Xyla is to prevent small oligosaccharides from forming nonproductive enzyme-substrate complexes.

Xylan, the major hemicellulose in a range of plant cell wall material, comprises a backbone of linked β,1,4-xylose units which are substituted with acetyl groups and various sugars (1). The xylan backbone is hydrolyzed by endo-β,1,4-xylanases (xylanases (1)). The primary sequences of over 70 xylanases have now been determined (2). Hydrophobic cluster analysis of these enzymes has shown that they have evolved from two ancestral sequences (2, 3). Thus, xylanases are classified as either glycosyl hydrolase family 10 or glycosyl hydrolase family 11 enzymes (3, 4).

Analysis of the catalytic mechanism of xylanases suggests that they hydrolyze glycosidic bonds via a double displacement general acid-base mechanism (5). Elegant studies by Withers and colleagues (5), using a combination of suicide inhibitors, substrates with different leaving groups and site-directed mutagenesis, have identified the nucleophile and acid-base residues in a family 10 xylanase, designated Cex, from Cellulomonas fimi (6, 7). Cex displays significant activity against aryl-β-glucosides, soluble cellulose, and xylan (8–9). It is not clear, however, whether other family 10 xylanases display similar activity toward cellulose as Cex or whether the C. fimi enzyme is unusually active against the glucose polymer.

Recently, the three-dimensional structure of four family 10 xylanases have been solved by x-ray crystallography (10–13). The enzymes all consist of β-barrels containing deep active site grooves consistent with their endo-mode of action. However, when xylopentaose was soaked into crystals of an inactive mutant (E246C) of xylanase A (XYLA)1 from Pseudomonas fluorescens subsp. cellulosa, the glycosidic bond linking the terminal nonreducing xylose residue with the rest of the oligosaccharide was adjacent to the enzyme’s nucleophile and acid-base residues, suggesting that Xyla was an exo-acting xylanase (10). In contrast, the xylanase also contained residues Glu-43, Asn-44, and Lys-47, which were conserved in all family 10 xylanases, that could constitute a further xylose binding site, designated subsite F, that was not filled by xylopentaose. To establish whether Xyla is an endo-acting glycosidase and to evaluate the importance of conserved residues located at the putative F, E, and C sites of the enzyme, the biochemical properties of the enzyme were evaluated, and the effect, on enzyme activity, of creating E43A, N44A, M46A, K47A, N126A,

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1 The abbreviations used are: Xyla, P. fluorescens subsp. cellulosa xylanase A; DNP, 2,4-dinitrophenoxy; 2,4-DNPy, 2,4-dinitrophenyl-β-d-celllobiose; DNPy, 2,4-dinitrophenyl-β-d-xyloside; PNP, 4-nitrophenol; PNP, 4-nitrophenyl-β-d-celllobiose; PNP, 4-nitrophenyl-β-d-xyloside; XYLALSL, S. lividans xylanase A; HPLC, high performance liquid chromatography.
Mutagenesis of Pseudomonas Xylanase A

Activity of native XYLA and Cex against aryl β-glycosides and xylan

| Enzyme | Substrate | $k_{cat}a$ | $K_m b$ | $k_{cat}/K_m c$
|--------|-----------|-----------|---------|---------|
| XYLA   | PNPC      | 157       | 50      | 3.14    |
| XYLA   | 2,4DNPC   | 456       | 38      | 14.9    |
| XYLA   | PNPX      | 8.8       | 308     | 0.03    |
| XYLA   | 2,4DNPC   | 1,146     | 10      | 141.6   |
| XYLA   | Xylan     | 3,146     | 1.0     | 3,146   |
| Xylan  | PNPX      | 60,137    | 54,670  |         |
| Cex    | PNPC      | 67.2      | 0.53    | 1,278   |
| Cex    | 2,4DNPC   | 419       | 0.06    | 6,983   |

Pre-steady State Kinetics—Pre-steady state kinetics were performed using stopped flow apparatus (Applied Photophysics model 5x-17MV, used with a 10-mm light path). Briefly, 60 μl of enzyme solution was mixed with an equal volume of substrate at 25 °C, and the release of the chromophore was monitored from 1 ms to 45 s at 400 nm.

**Circular Dichroism (CD) and Fluorescence Spectroscopy**—CD spectra were recorded with a Jobin-Yvon CD6 spectropolarimeter. The spectra were obtained at a residue concentration of 5.0 to 5.5 nm in 10 mM Tris/HCl buffer, pH 8.0, at 25 °C using a 0.1-mm path quartz cuvette (Hellma. 121.000 Q8). Each spectrum was accumulated from 20 to 30 scans between 190 and 250 nm, at a scan rate of 60 nm/min. Fluorescence spectroscopy was performed using an SLM 8100 spectro-motor operating in ratio mode with 8-nm excitation and 4-nm emission bandwidths. Proteins were diluted in 10 mM Tris/HCl buffer, pH 8.0, to a final concentration of 50 μg/ml. Samples were excited at 280 nm, and the emission spectra of the proteins were recorded between 290 and 430 nm at 25 °C. Samples were corrected by subtraction from a buffer blank.

**RESULTS**

Comparison of the Biochemical Properties of XYLA and Cex—Previous studies have shown that xylanases belonging to glycosyl hydrolase family 10, in addition to hydrolyzing xylan, also cleaved aryl-β-celllobiosides. Indeed, Cex displays significant activity against PNPC, 2,4DNPC, soluble cellulose, and xylan (9, 25). To evaluate whether other family 10 xylanases also displayed significant activity against cellulose substrates, we analyzed the biochemical properties of XYLA. The data (not shown) showed that XYLA was >50,000 times less active against soluble cellulose compared with oat spelt xylan. The enzyme exhibited considerably higher activity against and elevated affinity for aryl-β-xyloligosides and xylosides, compared with the corresponding celllobiosides and glucosides, respectively. XYLA also exhibited far lower affinity for aryl-β-celllobiosides compared with Cex (Table I).

Family 10 xylanases cleave glycosidic bonds via a double displacement mechanism as depicted in Equation 1.

In the first step the glycosidic bond in the substrate is cleaved, and the enzyme is glycosylated ($k_2$) by one of the reaction products. In the second step water attacks the anomer carbon attached to the nucleophile, Glu-246, with general base-catalytic assistance from the deprotonated carboxylate of the acid-base residue, Glu-127, resulting in deglycosylation ($k_3$) of the enzyme. When Cex hydrolyzes either PNPC or 2,4DNPC, deglycosylation is the rate-limiting step (8). To evaluate whether the rate-limiting step in the hydrolysis of 2,4DNPC by XYLA, pre-steady state hydrolysis of this molecule by XYLA was analyzed. The data, presented in Fig. 1, showed that there was no pre-steady state burst of DNP release, suggesting that the rate-limiting step in the action of XYLA against this substrate is glycosylation.
The Endo-mode of Action of XYLA—To evaluate the mode of action of XYLA, the products generated by the action of the enzyme against highly polymeric substrates and oligosaccharides were analyzed. The data, presented in Fig. 2, revealed that XYLA displayed typical endo activity against xylan; during the initial stages of hydrolysis a mixture of oligosaccharides was generated. As the reaction continued, the oligosaccharides were progressively degraded yielding primarily xylose, xylobiose, and xylotriose when the reaction was terminated. Similarly, the enzyme also exhibited an endo-mode of activity against oligosaccharides (Fig. 3). For example xylohexaose was cleaved, initially, to mainly xylooligosaccharides and small amounts of xylobiose and xylotetraose; xylolentaose to xylobiose and xylotriose; whereas xylotetraose was hydrolyzed, initially, to xylobiose with some xylotriose and xylose. No significant quantities of xylose were generated during the initial stages of hydrolysis of oligosaccharides consisting of four or more xylose units, strongly suggesting that XYLA does not successively release significant quantities of xylose from the nonreducing end of xylolentaose or other oligosaccharides. The relative activities of XYLA against xylotriose, xylotetraose, xylolentaose, and xylohexaose were 1:93:1516:8380, respectively. When the enzyme was incubated with high concentrations of xylotetraose or xylolentaose, initially, oligosaccharides consisting of up to 11 xylose units were generated (Fig. 4) indicating that the enzyme displayed significant transglycosylating activity, consistent with its double displacement mechanism of catalysis. The products generated by XYLA from the oligosaccharides clearly show that XYLA is not an exo-acting enzyme, suggesting that within the crystal structure of XYLA only substrate-binding sites A to E are available to xylolentaose and that there must be at least two xylose binding sites on either side of nucleophile and acid-base residues. Indeed, the relatively high activity of the enzyme against xylohexaose, and the predominant production of xylotriose from this substrate, suggests that there is a minimum of six xylose binding sites.

Modification of Conserved Residues at the F Subsite of XYLA—As stated above, XYLA contains a sixth xylose binding pocket adjacent to site E, which is designated site F. Inspection of the F site revealed residues, Glu-43, Asn-44, and Lys-47, on the surface of the binding pocket, and Met-46 which is located close to the sixth xylose binding region. All four residues are conserved in the family 10 xylanases analyzed to date. To investigate the role of these amino acids in XYLA, site-directed mutagenesis was used to generate E43A, N44A, K47A, and M46A variants of the xylanase. The four mutated forms of XYLA were purified to homogeneity as judged by SDS-polyacrylamide gel electrophoresis (data not shown) and their biochemical properties analyzed. The data (Table II) showed that E43A, N44A, and M46A displayed very similar kinetic properties to native XYLA, when using xylan as the substrate, whereas K47A was significantly less active against the polymeric substrate. Although N44A also exhibited similar activity to native XYLA against all the aryl-β-glycosides, K47A was less active against PNPC, 2,4DNPC, and 2,4DNPX2 than native XYLA. E43A was less active against PNPC compared with the native enzyme; however, the mutant displayed similar activity to the unmodified xylanase toward the other aryl-β-glycosides analyzed. M46A was 4–8 times less active than native XYLA against all the aryl-β-glycosides. E43A, N44A, and K47A were approximately 50–100 times less active against xylotriose, xylotetraose, and xylolentaose, compared with native XYLA, when the wild type and mutant enzymes were matched for arylglycosides, whereas M46A was about 8 times less active than the wild type enzyme, against these substrates (Table III). The pattern of products released from these substrates by the mutants K47A and M46A was similar to wild type XYLA (data not shown), whereas the mode of action of N44A against xylotetraose and xylohexaose and E43A against xylohexaose was distinct from the native xylanase (Fig. 3). N44A generated predominantly xylose from xylotetraose, whereas native XYLA produced significantly more xylose and xylotriose from this substrate than the asparagine mutant. Against xylohexaose N44A produced equal molar quantities of xylose, xylotriose, and xylotetraose, whereas wild type XYLA released primarily xylotriose. E43A generated a higher proportion of xylotriose from xylohexaose, compared with native XYLA.

Modification of Asn-182—Previous studies by Moreau et al. (26) showed that Asn-173 in Streptomyces lividans xylanase A (XYLASL) played an important role in binding xylose units at the B site. The equivalent residue in Pseudomonas XYLA, Asn-182, appeared to be located at the boundary of the enzyme’s B and C xylose binding sites. To investigate the importance of this residue at the active site of XYLA, N182R and N182A were created and the biochemical properties of the mutants evaluated. The data, displayed in Tables II and III, showed that neither the N182A nor N182R mutation had a significant effect on the activity of the enzyme against xylotriose or xylan, although there was a modest reduction in the rate at which the mutants hydrolyzed xylotetraose, xylotriose, and xylohexaose. The initial products generated by the mutants against xylan and the oligosaccharides were similar to native XYLA except for xylotetraose; in contrast to the native xylanase, which generated primarily xylose, N182A produced equal molar amounts of xylose, xylotriose, and xylotetraose. The initial products generated by the mutants against xylan and the oligosaccharides were similar to native XYLA except for xylotetraose; in contrast to the native xylanase, which generated primarily xylose, N182A produced equal molar amounts of xylose, xylotriose, and xylotetraose (Fig. 3). These data suggest that Asn-182 does play an important role in substrate binding in XYLA; however, it is also apparent that the conserved asparagine residue does not play an equivalent role in all family 10 xylanases.

Modification of Asn-126 and Glu-127—N126A and E127G mutants of XYLA were constructed and purified to apparent homogeneity. Both mutants were considerably less active than wild type XYLA against all substrates evaluated. Although the $K_m$ values of E127G against both PNPC and 2,4DNPC were very low, only against 2,4DNPC was the $K_m$ of N126A significantly lower than native XYLA (Tables I and II). Biophysical Properties of N126A and K47A—As both N126A and K47A displayed significantly lower activity against polymeric substrates, compared to XYLA, circular dichroism (CD) and fluorescence spectroscopy were used to probe the extent to which the mutations had altered the three-dimensional structure of the enzymes. CD spectra of both enzymes were very
similar to wild type XYLA (data not shown), suggesting that the two amino acid replacements had not significantly altered the secondary structure of the two proteins. Fluorescence spectroscopy of N126A and K47A showed a shift in the wavelength of maximum emission intensity from 328 nm in the native enzyme to 331 and 326 nm, respectively (Fig. 5).

Pre-steady State Kinetics of N126A—
The reduction in $K_m$ of N126A against 2,4DNPC suggests that the rate-limiting step in the hydrolysis of this substrate by the mutant enzyme is de-glycosylation. To evaluate this possibility, the pre-steady state kinetics of this reaction were analyzed using stopped-flow apparatus. The data, presented in Fig. 1, show a rapid burst of 2,4DNP release from the substrate 2,4DNPC which fitted Equation 2:

$$[P_2] = A \cdot t \cdot B \cdot (1 - e^{-kt})$$  \hspace{1cm} (Eq. 2)

There was little dependence of the steady-state rate ($A$) on substrate concentration in the range 0.5–5 mM with the apparent $K_m$ (0.08 ± 0.01 mM) in agreement with the separately determined value given in Table II. The extrapolated value of the burst amplitude ($B$), expressed as a fraction of the total protein concentration, was 0.77 ± 0.04, consistent either with $k_2/k_3 = 7.1$ or with 77% of the protein being catalytically active if $k_2 >>>>> k_3$. Analysis of the ratio $B/A$ as a function of substrate concentration gave an estimate of $k_2(k_2 + k_3)/k_3$ of 0.071 ± 0.004 s\(^{-1}\), in good agreement with the value of 0.06 ± 0.015 s\(^{-1}\) obtained from the plot of the rate constant against substrate concentration. The latter plot was linear, consistent with a $K_a$ 5 mM, and the slope gave a value of $k_{cat}/K_m$ of 500 M\(^{-1}\)s\(^{-1}\).

With PNPC, progress curves were linear, with steady-state

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**Fig. 2. HPLC analysis of xylan hydrolysis by XYLA.** A, XYLA (3.68 μg/ml) was incubated with 2 mg/ml oat spelt xylan in PC buffer at 37 °C. At regular intervals aliquots were removed, after 0 (1), 5 (2), 10 (3) and 30 min (4), boiled for 5 min, and subjected to HPLC analysis. The position at which xylose (A), xylitolose (B), xylotriose (C), xylotetraose (D), xylpentose (E), and xylohexaose (F) were eluted from the HPLC column are indicated. B is a graphical presentation of the products generated from xylan hydrolysis by XYLA. The quantity of xylose ([ ], xylitolose (○), xylotriose (△), xylotetraose (▼), xylpentose (●), and xylohexaose (■) produced during the course of the reaction is indicated.
rate similar to that for 2,4DNPC. The linear progress curve for the wild type enzyme with 2,4DNPC suggests that \( k_2 \) is rate-limiting. A rapid burst, complete within the dead time of mixing, cannot be ruled out, but a \( K_m \) value lower than 40 mM (Table I) would then be expected.

**DISCUSSION**

The primary objectives of this study were (i) to evaluate the biochemical properties of XYLA to establish whether there are substantial differences in the biochemical properties of glycosyl hydrolase family 10 enzymes; and (ii) to investigate the role of highly conserved residues in the xylose binding sites C, E, and F of XYLA.

**Biochemical Properties of XYLA**—Data presented in this paper clearly show that XYLA is an endo-acting xylanase. The relative activity of XYLA against the oligosaccharides indicates that the enzyme exhibits a dramatic increase in affinity for xylotetraose, compared with xylotriose, suggesting that binding of the substrate to two sites either side of the glycosidic bond cleaved plays an important role in enzyme action. In addition, the enzyme was more active against xylohexaose than xylopentaose indicating that XYLA contains at least six xylose
binding sites. It can be confirmed, therefore, that occupation of subsites A to E by xyloentaose in the crystal, and not B–F, is a result of contacts between XYLA molecules in the crystals making site F far less accessible to the substrate. The data also indicate that binding of the substrate to site F is essential for efficient hydrolysis by XYLA of xyooligosaccharides and that site E must bind weakly to these substrates. This interpretation of our data is supported by previous studies (27) which showed that subsites adjacent to the site of bond cleavage in xylanases exhibit weak affinity for xylose units. It has been proposed that these sites distort the chair conformation of the xylose sugars and thus assist in the formation of oxocarbonium ion-like transition states prior to the generation of the glycosyl enzyme intermediate, rather than binding tightly to the substrate (28).

Data presented in this report showed that although the $k_{cat}$ values for *Pseudomonas* XYLA against both PNPC and 2,4DNPC were similar to Cex, the $K_m$ of XYLA against these substrates were 2 and 3 orders of magnitude higher for the respective substrates, compared with the *Cellulomonas* enzyme. The rate-limiting step of the cleavage of the two aryl-β-glycosides by XYLA and Cex was glycosylation and deglycosylation, respectively. These data clearly show that there are significant differences in the capacity of cellobiose to bind to the E and F sites of the two xylanases. However, given that the $k_{cat}$ for aryl-β-cellobioside hydrolysis is similar for Cex and XYLA, it is surprising that in contrast to Cex, the *Pseudomonas* enzyme displays virtually no activity against polymeric cellulolic substrates even at very high substrate concentrations. It is possible that differences in the two enzymes’ activities against cellulose is reflected in the capacity of sites A–D of XYLA and Cex to accommodate glucose molecules. It remains to be established whether Cex or XYLA represents the best paradigm for family 10 xylanases. However, the observation that XYLASL has a $K_m$ for PNPC intermediate between XYLA and Cex (29) suggests that family 10 enzymes will exhibit a range of different activities for the aryl β-glycosides.

**Importance of F Site Amino Acids Glu-43, Asn-44, and Lys-47**—The location of the highly conserved F site residues Glu-43, Asn-44, and Lys-47 are depicted in Fig. 6. To investigate the role of these residues, they were substituted with alanine, and the biochemical properties of the resultant mutants were analyzed. Against the aryl-β-cellobiosides N44A displayed the same activity as the native enzyme. This suggests that, although highly conserved, Asn-44 does not play a pivotal role in binding cellobiose in the active site of the enzyme. This is in contrast to the findings of White *et al.* (30) who suggested that the equivalent residue in Cex forms a H bond with the C-3-OH of the distal glucose of the covalently bound 2-deoxy-2-fluoro cellobiose-enzyme intermediate and thus plays an important role in the active site binding of aryl-β-cellobiosides. The different role of the conserved asparagine could reflect the way cellobiose fits into the active site of Cex and XYLA. It is clear that the two enzymes display very different affinities for cellulolic substrates, and it is possible that in XYLA the C-3-OH of the distal glucose of cellobiose is too distant from Asn-44 to form a H bond.

Although E43A displayed similar activity toward 2,4DNPC as native XYLA, the mutant was significantly less active against PNPC than the unmodified enzyme. This could reflect differences between the leaving groups of the two substrates; 2,4DNP has a $pK_a$ of 3.96 (8), and thus at the pH of the assay (7.2) 2,4DNP will function as a good leaving group in the absence of protonation. In contrast PNP has a $pK_a$ of 7.18 (8), and thus will only function as a good leaving group if the glycosidic oxygen between PNP and cellobiose in the substrate

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**Fig. 3—continued**
PNP is protonated. Once the aryl group has been cleaved the cellbiose-enzyme intermediate generated will be cleaved by a water molecule with general base-catalytic assistance from deprotonated Glu-127. Three possible mechanisms by which E43A influences the protonation of the glycosidic oxygen are as follows. (i) The mutation causes a subtle change in the position of the acid-base residue within Xyla such that the amino acid is not sufficiently close to the glycosidic oxygen to effect protonation. (ii) The mutation influences the environment of Glu-127 such that the residue is not fully protonated. (iii) The E43A modification affects the way cellbiose sits in the active site such that the glycosidic oxygen is not close enough to Glu-127 to be protonated. If mechanisms i or ii are correct, then modification of the position or protonation state of Glu-127 should reduce the rate of the glycosylation step of all substrates, such as xylan, which contain poor leaving groups, and significantly decrease the rate of deglycosylation for all the substrates evaluated. However, the observation that E43A retains full activity against 2,4DNPC and xylan argues against mechanisms i and ii. Support for mechanism iii is provided by White et al. (30) who showed that when 2-deoxy-2-fluoro-β-cellobiose was covalently linked to the nucleophile of Cex, the Glu-43 equivalent formed a H bond with C-2-OH of the distal saccharide unit. These data suggest that in Xyla Glu-43 forms a H bond with the same OH group of cellbiose. We suggest that disruption of this bond in E43A could change the position of PNP within the active site, such that the glycosidic oxygen is no longer in close proximity to Glu-127. In contrast, in the glycosylated enzyme the covalent linkage between Glu-246 and C1 of cellbiose positions the anomeric carbon in close proximity to the water molecule that is deprotonated by Glu-127. Thus, the $k_{\text{cat}}$ for E43A against 2,4DNPC is similar to native Xyla, as protonation of the leaving group is not essential for deglycosylation to occur. It is unclear, however, precisely what role Glu-43 plays in the hydrolysis of xylan, as removal of this residue does not affect the activity of the enzyme against the polysaccharide. It is possible that xylan forms such strong interactions with other residues within the active site cleft that removal of the C-2-O/E43 interaction does not alter the position of the substrate within the enzyme. This hypothesis is in agreement with Moreau et al. (29), who also demonstrated that a mutation within XYLASL, D124E, had a much greater effect on the affinity of the enzyme for PNP compared with xylan.

K47A was 18, 51 and 190 times less active against 2,4DNPC, xylan, and PNP, respectively. These data suggest that Lys-47 plays an important role in positioning the substrate into the active site. The retention, in K47A, of $K_m$ values that are similar to native Xyla, against the three substrates, suggests that the mutation is influencing the glycosylation step; if deglycosylation was reduced then the hydrolyzed substrate would accumulate at the active site, causing an apparent increase in the affinity of the enzyme for the substrate. As the enzyme was less active against substrates with moderate (PNP) and good (2,4DNPC) leaving groups, the mutation is probably affecting the proximity of the anionic carbon of the sugar at the E site to the nucleophile. This view is supported by the study of White et al. (30) who showed that Lys-47 in Cex formed H bonds with both the ring O and C-3-0H of the distal and proximal glucose molecules of 2-deoxy-2-fluoro-β-cellobiose, respectively, in the glycosyl-enzyme complex. Removal of these H bonds could significantly alter the position of the substrate at the active site such that the nucleophile, Glu-246, is not in close proximity with the anionic carbon of the proximal sugar at the E site. However, it is also possible that K47A is having an indirect effect by altering the environment of aromatic residues at the active site of the enzyme. Data in this report showed that the K47A mutation caused a subtle change in the fluorescence spectrum of the enzyme; the 2-nm decrease in $\lambda_{\text{max}}$ with excitation at 280 nm suggests that one or more tryptophan residues are located in a slightly more hydrophobic environment. A potential candidate is Trp-83, which is only 3.4-Å from Lys-47 (Fig. 6), and thus the removal of the charged nitrogen in the Lys-47 side chain could enhance the hydrophobic environment of Trp-83. This change could alter either the binding of the substrate at the active site or possibly the ionization state of the nucleophile.

**Effect of F Site Mutants on Xylan and Xylooligosaccharide Hydrolysis**—The observation that E43A, N44A, and K47A were less active against xylooligosaccharides compared with xylan could reflect a reduction in the capacity of the F site, in the three mutants, to bind substrate. The oligosaccharides are thus more prone to forming dead-end complexes by binding randomly to subsites A–D along the cleft. These complexes will block the formation of productive complexes in which the substrate spans sites E and D. In contrast, the highly polymeric structure of xylan ensures that when the polysaccharide binds to sites A–D, adjacent xylose residues fill site E ensuring the formation of an active complex. This interpretation of our data is supported by two reports (26, 27) which demonstrated that in both a family 10 and 11 xylanase, xylose binding sites adjacent to the site of bond cleavage did not bind to the substrate, hence the importance of site F in positioning small substrates into sites D and E of the enzyme.

**Importance of Asn-126**—Modification of Asn-126 to alanine caused a significant decrease in the catalytic activity of Xyla against all the substrates tested and, against 2,4DNPC, resulted in a large decrease in the $K_m$. Pre-steady state kinetics
showed that the rate-limiting step of hydrolysis of 2,4DNPC by N126A was deglycosylation, whereas in the native enzyme glycosylation was the limiting step in the cleavage of 2,4DNPC. These data suggest that N126A is affecting both the efficient protonation of the substrate by Glu-127 and the capacity of this residue to mediate subsequent general base catalysis. Clearly, this is having a greater effect on the glycosylation step of PNPC cleavage, as PNP constitutes a moderate leaving group.

**Table II**

Activity of mutant forms of XYLA against aryl β-glycosides and xylan

| Enzyme | Substrate | $k_{cat}$ | $K_m$ | $k_{cat}/K_m$ |
|--------|-----------|----------|-------|--------------|
| E43A   | PNPC      | 4        | 61    | 0.066        |
| E43A   | 2,4DNPC   | 498      | 44    | 11.3         |
| E43A   | XYLXAN    | 40,718   | 0.9   | 45,242       |
| N44A   | PNPC      | 188      | 57    | 3.3          |
| N44A   | 2,4DNPC   | 1,063    | 24    | 44.3         |
| N44A   | XYLXAN    | 29,996   | 0.7   | 42,851       |
| M46A   | PNPC      | 29.2     | 81    | 0.36         |
| M46A   | 2,4DNPC   | 163.4    | 43    | 3.8          |
| M46A   | XYLXAN    | 61,311   | 0.7   | 87,587       |
| K47A   | PNPC      | 1.1      | 100   | 0.011        |
| K47A   | 2,4DNPC   | 48.2     | 40    | 1.21         |
| K47A   | XYLXAN    | 1,188    | 0.8   | 1,485        |
| N126A  | PNPC      | 2.6      | 32    | 0.0828       |
| N126A  | 2,4DNPC   | 7.78     | 0.19  | 40.9         |
| N126A  | XYLXAN    | 715      | 0.3   | 2,383        |
| E127G  | PNPC      | 0.48     | 0.66  | 0.727        |
| E127G  | 2,4DNPC   | 0.872    | 0.013 | 67.1         |
| N44A   | PNPC      | 1995     | 51    | 3.9          |
| N44A   | XYLXAN    | 76,738   | 0.5   | 153,576      |
| N44A   | PNPC      | 215      | 55    | 3.91         |
| N44A   | XYLXAN    | 64,780   | 1.0   | 64,780       |

$a k_{cat}$ values are in mol of product/mol of enzyme/min.

$b K_m$ values are in mM for the aryl-xylooligosaccharides β-glycosides and mg/ml for xylan.

**Table III**

Hydrolysis of xylooligosaccharides by native and mutant forms of XYLA

| Enzyme | $X_3$ | $X_4$ | $X_5$ | $X_6$ |
|--------|-------|-------|-------|-------|
| Native | 1.2 $\times$ 10$^{-4}$ | 1.1 $\times$ 10$^{-2}$ | 1.8 $\times$ 10$^{-1}$ | 1$^b$ |
| E43A   | 1.5 $\times$ 10$^{-6}$ | 1.0 $\times$ 10$^{-4}$ | 1.7 $\times$ 10$^{-3}$ | 4.0 $\times$ 10$^{-2}$ |
| N44A   | 2.9 $\times$ 10$^{-6}$ | 1.9 $\times$ 10$^{-4}$ | 3.4 $\times$ 10$^{-3}$ | 3.1 $\times$ 10$^{-2}$ |
| M46A   | 1.5 $\times$ 10$^{-5}$ | 8.3 $\times$ 10$^{-4}$ | 1.5 $\times$ 10$^{-2}$ | ND $^c$ |
| K47A   | 1.9 $\times$ 10$^{-6}$ | 1.0 $\times$ 10$^{-4}$ | 1.7 $\times$ 10$^{-3}$ | ND $^c$ |
| N182A  | 1.1 $\times$ 10$^{-6}$ | 4 $\times$ 10$^{-3}$ | 6 $\times$ 10$^{-2}$ | 3 $\times$ 10$^{-3}$ |

$a$ The substrates used were xylotriose ($X_3$), xylotetraose ($X_4$), xylopentaose ($X_5$), and xylohexaose ($X_6$).

$b$ The activities of the enzymes were relative to the hydrolysis of xylohexaose by native XYLA. The units of enzyme used were matched against xylan such that they all hydrolyzed xylan at the same rate.

$c$ ND, not determined.
whereas protonation of 2,4DNP is not required for Xyla to cleave 2,4DNP; hence, the decrease in the rate at which this substrate is deglycosylated results in a similar decrease in \( k_{\text{cat}} \) to PNPC but also an associated reduction in \( K_m \) as the glycosyl-enzyme intermediate accumulates. It is interesting to note that the complete removal of the acid-base residue in mutant E127G causes a further 10-fold reduction in catalytic activity and switches the rate-limiting step in enzymatic action to the deglycosylation step for both PNPC and 2,4DNP. This suggests that Xyla can cleave substrates with moderate or good leaving groups in the absence of protonation, but the enzyme cannot elicit deglycosylation without the capacity to abstract protons from water. Thus, by inference, the N126A mutation does not completely abolish the capability of Glu-127 to accept or donate protons, as glycosylation is still the rate-limiting step in PNPC hydrolysis.

An insight into the role of Asn-126 in the function of Glu-127 can be obtained from the study of White et al. (30), who suggested that this residue forms a hydrogen bond with C-2-OH of the proximal glucose moiety of 2-deoxy-2-fluorocellobiose located at the B site. It is possible that this interaction is important in positioning the glycosidic oxygen in close proximity to Glu-127. The role of Asn-126 in the deglycosylation of 2,4DNPc is not so readily apparent. However, Ducros et al. (31) have suggested that Asn-126 is connected to Glu-127 via Gln-203, and it is possible that disruption of the charge transfer by the N126A mutation could influence the protonation state of Glu-127. It should also be noted that the N126A mutation causes a 2-nm increase in the fluorescence spectrum of Xyla, suggesting an increase in the exposure of certain aromatic residues to a hydrophilic environment, and this subtle modification to the active site could alter the capacity of Glu-127 to accept or donate protons, making the enzyme against oligosaccharides larger than xylotriose and shifted the site of xylotetraose cleavage from the middle glycosidic bond to the terminal linkage. This is in contrast to Moreau et al. (26) who showed that a N173D (Asn-173 is equivalent to Xylasl to Asn-182 in Xyla) mutation of Xylasl increased the rate of xylotriose and xylotetraose release from xylan, altered the transglycosylation products from, and the site of cleavage of, xylotriose, but had no effect on the cleavage of xylotetraose. These differences between the Pseudomonas and Streptomyces xylanases suggest that, in Xyla, Xyla-182 is also on the surface of the active site and is positioned the appropriate distance away from the two key catalytic residues, Glu-127 and Glu-246, to occupy xylene binding site C of Xyla.

**Fig. 6. View of subsites C, D, E, and F of Xyla.** The residues that are the focus of this paper are represented in stick and stick configurations and are appropriately labeled. Glu-43, Asn-44, and Lys-47 are adjacent to each other on the surface of the active site cleft and clearly have the potential to form a xylene binding site. Met-46 is located close to these residues but is not on the surface of the active site. Asn-126 is in close proximity to the acid-base catalyst Glu-127 and thus could play an important role in the function of the catalytic carboxylic acid residue. Trp-83 is positioned between Lys-47 and Asn-126, and thus these two residues could influence the fluorescence of the aromatic amino acid. Asn-182 is also on the surface of the active site and is positioned the appropriate distance away from the two key catalytic residues, Glu-127 and Glu-246, to occupy xylene binding site C of Xyla.

**Importance of Asn-182**—Data presented in this study clearly show that Xyla is an endo-acting xylanase that contains a sixth xylene binding site, designated site F, and exhibits considerably lower affinity for cellulosic substrates than Cex. Site-directed mutagenesis studies provided insights into the roles of several residues located in the F, E, and C sites of the xylanase. The data showed that highly conserved residues do not always play an equivalent role in different xylanases.

**Conclusions**—Data presented in this study clearly show that Xyla is an endo-acting xylanase that contains a sixth xylene binding site, designated site F, and exhibits considerably lower affinity for cellulosic substrates than Cex. Site-directed mutagenesis studies provided insights into the roles of several residues located in the F, E, and C sites of the xylanase. The data showed that highly conserved residues do not necessarily play equivalent roles in different xylanases from the same family and that disruption of ligand binding at subsite F compromised the enzyme's capacity to hydrolyze xylooligosaccharides but not highly polymeric substrates such as xylan.

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