Influence of the Aglycone Region of the Substrate Binding Cleft of *Pseudomonas* Xylanase 10A on Catalysis

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Received November 27, 2000; Revised Manuscript Received March 7, 2001

ABSTRACT: *Pseudomonas cellulosa* xylanase 10A (Pc Xyn10A) contains an extended substrate binding cleft comprising three glycone (−1 to −3) and four aglycone (+1 to +4) subsites and, typical of retaining glycoside hydrolases, exhibits transglycosylation activity at elevated substrate concentrations. In a previous study [Charnock, S. J., et al. (1997) *J. Biol. Chem. 272*, 2942−2951], it was demonstrated that the −2 subsite mutations E43A and N44A caused a 100-fold reduction in activity against xylooligosaccharides, but did not influence xylanase activity. This led to the proposal that the low activity of these mutants against xylooligosaccharides was due to nonproductive complex formation between these small substrates and the extended aglycone region of the active site. To test this hypothesis, key residues at the +2 (Asn182), +3 (Tyr255), and +4 (Tyr220) subsites were substituted for alanine, and the activity of the mutants against polysaccharides and oligosaccharides was evaluated. All the aglycone mutants exhibited greatly reduced or no transglycosylating activity, and the triple mutants, E43A/Y220A/Y255A and E43A/N182A/Y255A, had activity against xylotriose similar to that of E43A. The aglycone mutations caused an increase in both k_{cat} and K_m against xylan, with N182A/Y220A/Y255A and N182A/Y255A exhibiting 25- and 15-fold higher k_{cat} values, respectively, than wild-type Pc Xyn10A. These data indicate that Glu43 plays a role in binding xylooligosaccharides, but not xylan, suggesting that the mechanisms by which Pc Xyn10A binds polysaccharides and oligosaccharides are distinct. The increased k_{cat} of the mutants against xylan indicates that the aglycone region of wild-type Pc Xyn10A restricts the rate of catalysis by limiting diffusion of the cleaved substrate, generated at the completion of the k_2 step, out of the active site.

Xylanases are endo-acting enzymes that cleave β-1,4-xylose polymers. The two major classes of xylanases, which belong to glycoside hydrolase families 10 and 11, cleave glycosidic bonds with net retention of anomeric configuration (*1*). Two potential catalytic outcomes of retaining glycoside hydrolases are depicted schematically in Figure 1. In the first catalytic step, the glycosidic bond is cleaved via protonation of the intersidic oxygen by the acid−base catalyst, and attack at C1 by the catalytic nucleophile (k_2). The glycosyl−enzyme covalent intermediate is then attacked at C1 either by an activated water molecule leading to hydrolysis (k_3) or by another substrate molecule, deprotonated at its nonreducing end (C4-OH), in which case a new glycosidic bond is formed via a transglycosylation reaction [Figure 2 (*2*)].

The crystal structures of several family 10 xylanases have been determined (*3*−*9*). They have a common $(\alpha/\beta)_8$ barrel fold with the catalytic acid and/or base and nucleophile residues located at the end of β-strands 4 and 7, respectively. Details of the interactions between the xylanases and xylose units at the −1 and −2 subsites were provided by crystal structures of two of the enzymes covalently attached to the mechanistic inhibitor 2′-fluoro-2′-deoxy-β-xylobioside (*8*, *9*).

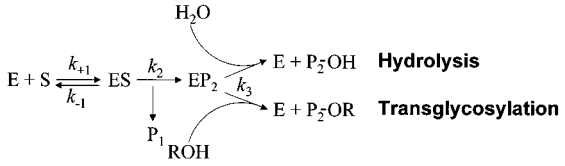

FIGURE 1: Kinetics of a typical retaining glycoside hydrolase. Once the enzyme (E) has bound to its target substrate (S), glycosidic bond cleavage occurs (k_2), during which the glycone sugar becomes covalently attached to the enzyme to form the glycosyl−enzyme intermediate (EP_2) with concomitant release of the aglycone saccharide (P_1). The glycosyl enzyme intermediate is then cleaved (k_3) either by an activated water molecule, resulting in hydrolysis, or by an activated sugar (ROH), leading to transglycosylation.

The active sites of family 10 xylanases comprise an open cleft which can randomly bind xylan polymers, which explains their endo mode of action. The cleft contains several subsites, each one capable of binding a xylose moiety. *Pseudomonas cellulosa* xylanase 10A (Pc Xyn10A)[1] is a typical example of a family 10 xylanase that contains seven xylose binding subsites, four aglycone of the site of bond cleavage (*10*). The −2 subsite exhibits the highest affinity for substrate monomer units, a feature common to the majority of glycoside hydrolases (*10*, *11*). This phenomenon

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[1] Abbreviations: X1, xylose; X2, xylobiose; X3, xylotriose; X4, xylotetraose; X5, xylopentaose; X6, xylohexaose; Pc Xyn10A, xylanase 10A from *P. cellulosa*; *xyn10A*, gene encoding xylanase 10A.

10.1021/bi002704r CCC: $20.00
Published on Web 05/30/2001

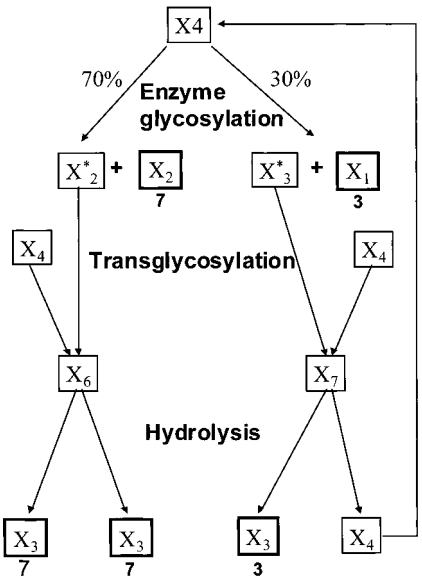

FIGURE 2: Products of xylotetraose transglycosylation by Pc Xyn10A. The predicted products generated by transglycosylation reactions mediated by Pc Xyn10A using xylotetraose (X4) as the substrate. The sugars surrounded by bold boxes are the end products of the transglycosylation reaction, and the numbers below these boxes indicate the proportions of these sugars at the completion of the reaction. Sugars with asterisks form covalent glycosyl−enzyme intermediates with Pc Xyn10A.

is consistent with the requirement for substrate distortion at the −1 subsite.

Site-directed mutagenesis studies of Pc Xyn10A (*10*, *12*), in conjunction with the crystal structure of the enzyme complexed with xylopentaose (*5*), showed that Tyr220, Tyr255, Asn182, Phe181, and Glu43 played key roles in the interaction of subsites +4, +3, +2, +1, and −2, respectively, with xylose moieties, while Asn44 bound sugars at both the −3 and −2 subsites. Biochemical analyses of E43A and N44A showed that disruption of the −2 subsite caused a much larger decrease in the activity of Pc Xyn10A against xylooligosaccharides than against xylan. It was suggested that this phenomenon was the result of the oligosaccharides forming dead-end complexes with distal subsites of the enzyme (*12*). In contrast, if the highly polymeric substrate, xylan, bound to the distal subsites of the xylanase, it was likely that the −1 and −2 subsites would also be occupied by this substrate, and thus, nonproductive complexes were less likely to exist (*12*).

To investigate the hypothesis that nonproductive complexes between xylooligosaccharides and the distal regions of the substrate binding cleft of Pc Xyn10 are responsible for the biochemical properties of E43A, mutations were introduced into the aglycone region of Pc Xyn10A, and the influence of these modifications on catalysis was assessed. The data indicate that Pc Xyn10A binds xylooligosaccharides and xylan by distinct mechanisms and that the rate of diffusion of the cleaved substrate, at the completion of the k_2 step, limits the rate of catalysis.

MATERIALS AND METHODS

Bacterial Strains, Culture Conditions, and Plasmids. The *Escherichia coli* strain used to express Pc Xyn10A and its derivatives was JM83 (*13*). Recombinant *E. coli* strains were grown in Luria broth supplemented with 100 μg/mL ampicillin, at 37 °C with shaking (200 rpm). The plasmid pRS16 comprises the region of *xyn10A* encoding the catalytic domain of Pc Xyn10A, cloned into *Sma*I- and *Eco*RI-restricted pUC19 (*14*). This places truncated *xyn10A* in-frame with the translational start codon of *lacZ*′ present in pUC19 (*14*).

Generation of Pc Xyn10A Mutants. Derivatives of Pc Xyn10A were generated as follows. E43A, N182A, Y255A, and Y220A were produced previously (*10*, *12*). To generate the other derivatives of the xylanase, appropriate mutations were introduced into *xyn10A* using the Transformer mutagenesis kit supplied by Clontech Laboratories Inc. The mutagenic primers (antisense strand of *xynA*) and template DNA that were employed were as follows: Y220A/Y255A, 5′-GGCGATGGACGGA**GC**GTCGTTCATGAC-3′ using pSA1 (encodes Y255A) as the template DNA; N182A/Y255A, 5′-ATTTTCTTCCGTG**GC**AAATCGTTGTAC-3′ using pSA1 as the template DNA; E43A/N182A/Y255A, 5′-CTTCATAATATTT**G**CGGCAGTGATCTG-3′ using pSA2 (encodes N182A/Y255A) as the template DNA; N182A/Y220A/Y255A, 5′-GGCGATGGACGGA**GC**GTCGTTCATGAC-3′ using pSA2 as the template; and E43A/Y220A/Y255A, 5′-CTTCATAATATTT**G**CGGCAGTGATCTG-3′ using pSA3 (encodes Y220A/Y255A) as the template. The nucleotides in bold are the mutations incorporated into *xyn10A*.

The complete sequences of the *xyn10A* mutants were determined using an ABI 377 DNA sequenator and the M13 forward and reverse primers, to confirm that only the desired mutations had been introduced.

Protein Purification. Pc Xyn10A and its derivatives were purified essentially as described previously (*14*). Appropriate recombinant strains of *E. coli* JM83 were grown to stationary phase, and the periplasm was prepared by the osmotic shock method (*14*). Pc Xyn10A was prepared from the periplasm by ion exchange chromatography using a DEAE-TrisAcryl Plus M column (Sigma Chemical Co.; 300 mm × 25 mm) and a 400 mL linear 0 to 500 mM NaCl gradient in 10 mM Tris/HCl buffer (pH 8.0) to elute the enzyme. The purity of Pc Xyn10A was evaluated by SDS−PAGE (*15*).

Assays. Xylanase activity was determined using soluble oat spelt xylan (Sigma Chemical Co.) as the substrate. Enzyme was incubated with substrate in 50 mM sodium phosphate/12 mM citrate (PC) buffer (pH 6.5) containing 1 mg/mL BSA, at 37 °C for 10 and 20 min, and the amount of reducing sugar that was released was determined as described by Miller (*16*). To measure the rate of xylotriose (X3) hydrolysis, the substrate at 50 μM was incubated with 50−500 nM enzyme for up to 3 h in PC buffer containing 1 mg/mL BSA at 37 °C. At regular time intervals, aliquots were removed, boiled for 20 min to inactivate the enzyme, and then subjected to HPLC analysis as described previously (*12*). Progress curves of oligosaccharide cleavage were used to determine the k_{cat}/K_m values of the reaction using eq 1 as described by Matsui et al. (*17*)

$$kt = \ln\frac{[S_0]}{[S_t]} \quad (1)$$

where $k = (k_{cat}/K_m)$[enzyme], t represents time, and $[S_0]$ and $[S_t]$ represent the substrate concentrations at time 0 and t, respectively.

To determine the extent to which transglycosylation was occurring, xylotetraose (X4) was incubated with native and mutant forms of Pc Xyn10A, until approximately 80% of the substrate had been hydrolyzed. The products that were generated were xylose (X1), xylobiose (X2), and xylotriose (X3). The ratio of mono- to trisaccharide gave an indication of the extent to which transglycosylation had occurred. When no transglycosylation had occurred, the ratio of X1 to X3 was 1:1. If X1 and X3 were generated, at least in part, through the hydrolysis of transglycosylation products, then more X3 would be produced than X1, as illustrated in Figure 2. The amount of enzyme that was employed ensured that insignificant hydrolysis of the X3 product occurred during the time course of the reaction. Pc Xyn10A is approximately 100 times more active against X4 than X3, and thus, if 20% of the X4 substrate remained at the end of the reaction, no significant hydrolysis of the X3 product would have occurred.

Protein concentrations were determined by measuring the A_{280} according to the method of Stoscheck (*18*). The molar extinction coefficient of wild-type Pc Xyn10A and single and double tyrosine mutants were 54 000, 52 700, and 51 400 M^{-1} cm^{-1}, respectively.

RESULTS AND DISCUSSION

Transglycosylation by Pc Xyn10A. Pc Xyn10A, typical of retaining glycoside hydrolases, catalyzes transglycosylation as well as hydrolytic reactions (*10*). To characterize the transglycosylation activity of Pc Xyn10A, the enzyme was incubated with increasing concentrations of X4 and the products that were generated were analyzed by HPLC. The data showed that at low concentrations of the tetrasaccharide (50 μM) the X1:X2:X3 molar ratio, X1−X3 being the end products of hydrolysis, was 3:14:3 (Table 1). When higher substrate concentrations were used, the X3:X1 ratio progressively increased to a value of 9:2 at 500 μM X4. As illustrated in Figure 2, when X4 acts as the nucleophile in the deglycosylation reaction (cleaves the glycosyl−enzyme covalent intermediate), the products that are generated are ultimately hydrolyzed to X3:X1 molar ratios of >1. These results indicate, albeit indirectly, that Pc Xyn10A catalyzes significant transglycosylation reactions, in addition to hydrolysis, at X4 concentrations of >100 μM.

To obtain direct evidence that Pc Xyn10A catalyzes transglycosylation reactions, the enzyme was incubated with a high concentration (16 mM) of xylohexaose (X6), and the initial products of the reaction were analyzed by HPLC. The rationale for using X6 was that it is hydrolyzed at a rate similar (approximately 3 times more slowly) to that of polymers and greater than or equal to that of xyloheptaose (*12*), and thus, the initial transglycosylation products are sufficiently stable to be detected. The data (Figure 4) revealed that significant amounts of oligosaccharides larger than X6 were synthesized during the first 30 min of the reaction. In addition, there was an accumulation of significant amounts of xylopentaose (X5) and X4, which were not associated with the production of X1 or X2, as would be expected if X6 was exclusively hydrolyzed. It is apparent, therefore, that under these conditions unmodified Pc Xyn10A performs extensive transglycosylation reactions.

Aglycone Mutants of Pc Xyn10A Inhibit Transglycosylation Reactions. Previous studies on Pc Xyn10A have shown that

Table 1: Ratio of X3 and X1 Released from Xylotetraose by Wild-Type and Mutant Forms of Pc Xyn10A[a]

Pc Xyn10A	[X4] (μM)	X3:X1 molar ratio
wild-type	50	0.9
wild-type	100	2.1
wild-type	375	3.1
wild-type	500	4.5
E43A	50	1.4
E43A	100	3.4
E43A	500	4.9
Y220A	100	1.0
Y220A	375	1.2
Y220A	500	2.1
Y220A	1000	3.8
Y220A	5000	4.9
Y255A	100	0.9
Y255A	375	1.1
Y255A	500	1.8
Y255A	1000	3.2
Y255A	5000	4.8
N182A	2000	1.0
N182A	5000	1.8
Y220A/Y255A	500	1.0
Y220A/Y255A	1000	1.1
Y220A/Y255A	5000	2.1
N182A/Y255A	5000	1.0
E43A/Y220A/Y255A	500	1.2
E43A/Y220A/Y255A	1000	1.6
E43A/Y220A/Y255A	2000	3.1
E43A/N182A/Y255A	5000	1.0
N182A/Y255A/Y220A	5000	1.0

[a] Wild-type and mutant forms of Pc Xyn10A (20 nM) were incubated with X4; aliquots were removed at regular time intervals and analyzed by Dionex HPLC as described in Materials and Methods, and the amount of X3 and X1 produced was quantified. The X3:X1 ratio was evaluated when approximately 80% of the substrate had been hydrolyzed, at which point no significant degradation of X3 would have occurred.

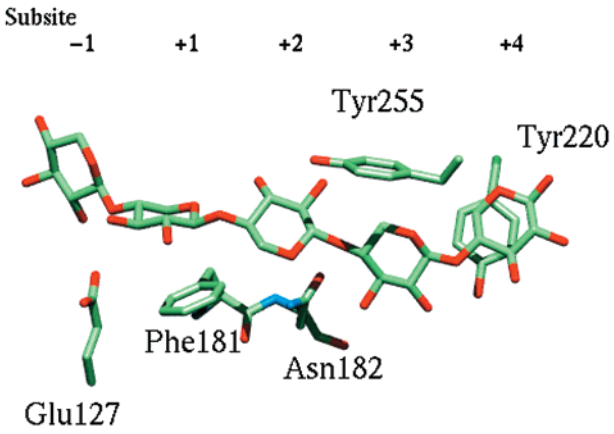

FIGURE 3: Topology of the aglycone region of the substrate binding cleft of Pc Xyn10A. Region of the Pc Xyn10A substrate binding cleft containing xylopentaose (shown in green) bound from subsite −1 to +4. The residues that play a critical role in ligand binding at the different subsites are shown. Tyr220 and Tyr255 form important hydrophobic interactions with xylopyranose moieties at the +4 and +3 subsites, respectively, while Asn182 interacts with a saccharide unit at the +2 subsite.

Asn182, Tyr255, and Tyr220 play a critcal role in sugar binding at the +2, +3, and +4 aglycone subsites, respectively [Figure 3 (*10*)]. To investigate the importance of the aglycone region of the active site in the transglycosylation activity of the enzyme, appropriate mutants of the xylanase were generated and their capacity to catalyze synthetic reactions was evaluated. Data displayed in Figure 4 show that when the triple mutant N182A/Y255A/Y220A was

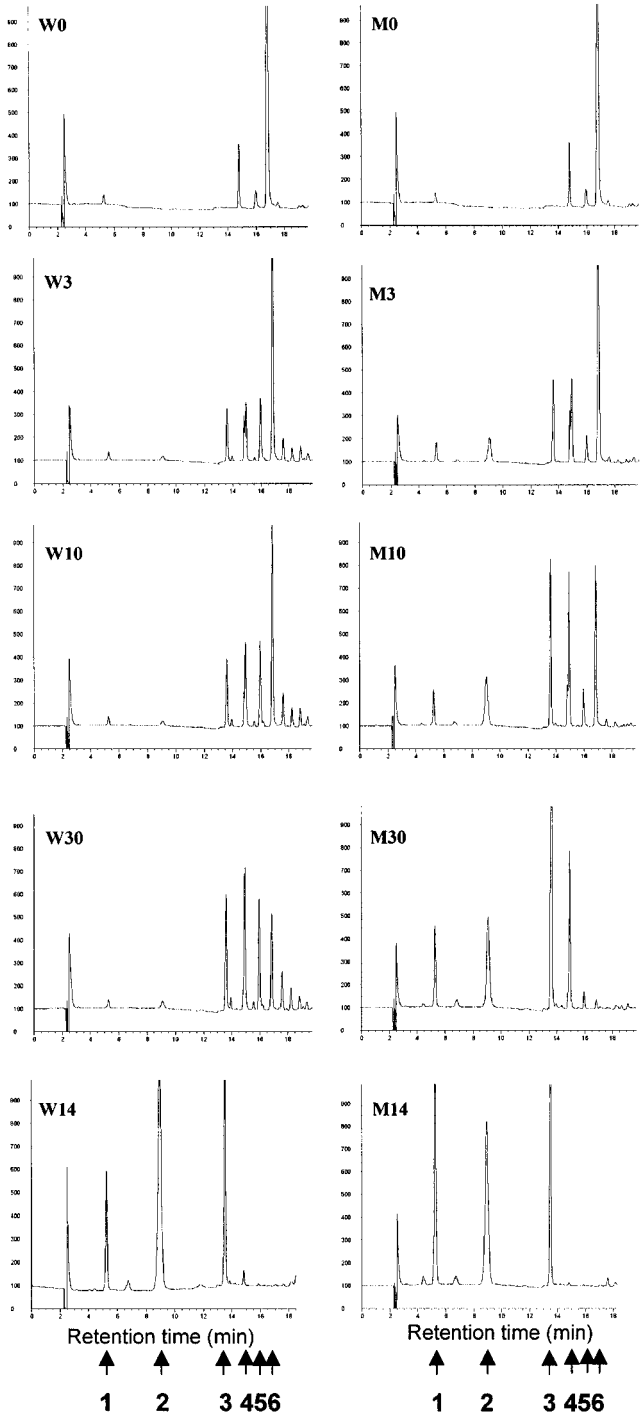

FIGURE 4: HPLC analysis of transglycosylation products generated by wild-type Pc Xyn10A and N182A/Y255A/Y220A. Wild-type Pc Xyn10A (W) and the N182A/Y255A/Y220A mutant (M) were incubated with 16 mM xylohexaose for 0 (0), 3 (3), 10 (10), and 30 min (30) and 14 h (14), and the sugars that were generated were analyzed by Dionex HPLC as described in Materials and Methods. The retention times of the standards are denoted with arrows: xylose (1), xylobiose (2), xylotriose (3), xylotetraose, (4), xylopentaose (5), and xylohexaose (6).

incubated with 16 mM X6 only trace amounts of oligosaccharides with a dp of >6 were detected; the primary products were X3 and equimolar amounts of X4 and X2 (Figure 4). These results indicate that the triple mutant displays very little transglycosylation activity under conditions in which the wild-type enzyme catalyzes significant glycosidic bond formation.

Table 2: Relative Activity of Wild-Type and Mutant Forms of Pc Xyn10A against Xylotriose[a]

Pc Xyn10A	relative activity against X3
wild-type	1.0[b]
Y220A/Y255A	1.1
N182A/Y255A	1.3
E43A	0.01
E43A/N182A/Y255A	0.03
E43A/Y220A/Y255A	0.02

[a] The different forms of Pc Xyn10A were incubated with 50 μM X3; the rate of hydrolysis of the trisaccharide was evaluated by removing aliquots at regular intervals, and the amount of substrate remaining was determined by HPLC. [b] The activity of wild-type Pc Xyn10A against X3 was defined as 1.0.

To compare the transglycosylating capacity of the wild type and the aglycone mutants of Pc Xyn10A, these enzymes were incubated with different concentrations of X4, and the ratio of reaction products X3 and X1 was determined by HPLC. The data, displayed in Table 1, show that reducing the level of ligand binding in the aglycone region of the active site greatly inhibited transglycosylation reactions, as evidenced by X3:X1 ratios significantly reduced compared to that of the wild-type enzyme (transglycosylation reactions generate X3:X1 molar ratios of >1). Of the single mutations, N182A had the biggest affect on transglycosylation, with synthetic reactions occurring only at a concentration of X4 50-fold higher than for the native enzyme. This result indicates that the +2 subsite plays a central role in the transglycosylation process. However, the observation that both Y220A and Y255A required 10-fold higher levels of X4 than native Pc Xyn10A, before transglycosylation occurred, supports the view that subsites +2, +3, and +4 all contribute to the transglycosylating activity of the enzyme. With the triple mutant N182A/Y220A/Y255A, and the double mutant N182A/Y255A, no transglycosylation was evident, even at substrate concentrations as high as 5 mM.

Analyzing Dead-End Complex Formation with E43A. Charnock et al. (*10*) showed that reduction in the level of substrate binding at the −2 subsite of Pc Xyn10A, through the introduction of the E43A or N44A mutation, resulted in a substantial decrease in activity against oligosaccharides but not against xylan. It was proposed that the −2 subsite played a key role in catalysis of small substrates by ensuring they formed productive complexes with the xylanase through occupation of the −1 and +1 subsites. However, disruption of the −2 subsite increased the frequency with which these oligosaccharides bound to distal subsites, forming nonproductive complexes with the enzyme. Enzyme-bound xylan, because of its size, was unlikely to form a nonproductive complex, as the polysaccharide would invariably occupy the −1 and +1 subsites, even if the initial interaction with the xylanase is with the distal region of the substrate binding cleft. To test this hypothesis, two triple mutants of Pc Xyn10A were generated, E43A/Y220A/Y255A and E43A/N182A/Y255A, that do not bind oligosaccharides efficiently (aglycone) to the site of bond cleavage (Table 1 and Figure 4). Thus, if the nonproductive complex hypothesis is tenable, the triple mutants should be more efficient at hydrolyzing X3 than E43A. The data, presented in Table 2, showed that although E43A/Y220A/Y255A and E43A/N182A/Y255A were more active than E43A against X3, the 2−3-fold increase in activity was not sufficient to explain the 100-

fold reduction in catalytic activity of E43A against xylooligosaccharides, compared to that of native Pc Xyn10A. It would appear, therefore, that the formation of nonproductive complexes of small substrates with the aglycone region of the active site did not explain why mutations to the −2 subsite caused a substantial reduction in the rate of oligosaccharide catalysis, but had little affect on xylan cleavage.

An alternative explanation for the substrate specificity of E43A is that xylan and xylooligosaccharides make different interactions with the −2 subsite of Pc Xyn10A. It is possible that Glu43 forms a hydrogen bond with the C2-OH group of xylose moieties from xylooligosaccharides situated in the −2 subsite, but does not make the equivalent interaction with xylan. For this hypothesis to be tenable, the secondary structure of xylans and xylooligosaccharides would have to be different. Intrapolymer interactions occur via hydrogen bonds between the C3-OH group of one xylose residue and the ring oxygen of the next monomer unit, imparting a left-handed 3-fold helical structure on xylan (*19*). It is possible that because of the distortion of xylose at the −1 subsite into a semichair or boat configuration during catalysis, the C3-OH−ring oxygen interaction may be lost, and thus, the orientation of xylose in the −2 subsite would change. In contrast, the highly polymeric nature of xylan may increase the stability of its helical structure, and thus, the sugar at the −2 subsite of this molecule could interact differently with the enzyme than the equivalent xylose unit in xylooligosaccharides, and thus, Glu43 may only interact with xylose when present in "flexible" xylooligosaccharides.

Recent studies, together with this paper, demonstrate differences in the capacity of active site mutants of glycoside hydrolases to cleave polysaccharides and oligosaccharides. Mutations of the *P. cellulosa* mannanase Man26A, which reduce the level of substrate binding at either the −2 or +1 subsite, cause a substantial reduction in activity against mannooligosaccharides but not against mannan (*20*). Similarly, mutations to the −6 subsite of amylase AMY1 from *Saccharomyces cerevisiae* reduced the activity against maltooligosaccharides but not against insoluble starch (*21*). It would appear, therefore, that there are differences in the binding mode of oligosaccharides and polysaccharides in the active site of the mannanase and amylase, and these differences could be invoked to explain the catalytic properties of all three glycoside hydrolases.

Catalytic Activity of Aglycone Mutants of Pc Xyn10A against Xylan. The data presented in Table 1 show that the rate of transglycosylation is substantially reduced in the aglycone mutants of Pc Xyn10A, indicating that substrate binding is greatly diminished in the +2, +3, and +4 subsites of the xylanase. To investigate how this decrease in substrate affinity influences the activity of the enzyme against highly polymeric substrates, the kinetics of xylan cleavage by native and mutant forms of Pc Xyn10A were determined. The data, presented in Table 3**,** show that the K_m for xylan hydrolysis was significantly increased by all the mutations, and this was particularly apparent in Pc Xyn10A derivatives containing N182A. These results are entirely consistent with the view that these mutations substantially reduced the affinity of the enzyme for substrate in the aglycone region of the active site.

The k_{cat} parameters for the aglycone mutants were significantly higher than for wild-type Pc Xyn10A with the two double mutants N182A/Y255A and Y220A/Y255A and the triple mutant N182A/Y230A/Y255A displaying k_{cat} values that were 24-, 17-, and 20-fold higher, respectively, than that of the native xylanase. One could argue that the increase in k_{cat} reflects a reduction in the level of transglycosylation and thus an increase in the rate of reducing sugar production, as hydrolysis of the glycosyl−enzyme covalent intermediate generates a new reducing sugar, while transglycosylation does not. However, even at the maximum substrate concentration used for the wild-type enzyme, the concentration of free xylan C4-OH groups was only 50 μM (assuming an average dp of 500), a concentration at which no transglycosylation occurred when X4 was the substrate (Table 1). The most likely explanation for the increase in k_{cat} is, therefore, that the diffusion of the product of the glycosylation step (P_1; Figure 1) out of the aglycone region of the active site, after initial bond cleavage (k_2), is rate-limiting for overall catalysis. A reduction in substrate affinity in the aglycone region will increase the rate at which cleaved xylan can leave the active site, and thus increase the rate at which water can diffuse into the +1 subsite and complete the hydrolytic reaction. If this hypothesis is correct, then k_2 should be larger than k_3 for xylan hydrolysis. This is consistent with the data presented by Charnock et al. (*10*) that showed k_2 was greater than k_3 for xylooligosaccharides.

Table 3: Catalytic Activity of Wild-Type and Mutant Forms of Pc Xyn10A against Soluble Xylan[a]

Pc Xyn10A	k_{cat} (s^{-1})	K_m (mg/mL)	k_{cat}/K_m (s^{-1} mL mg^{-1})
wild-type	497 ± 21	1.4 ± 0.12	355
N182A	836 ± 65	4.0 ± 0.54	424
Y220A	1465 ± 31	2.7 ± 0.42	542
Y255A	836 ± 85	2.1 ± 0.31	398
Y220A/Y255A	8370 ± 1206	15 ± 1.8	558
N182A/Y255A	12027 ± 1427	20 ± 2.7	601
N182A/Y220A/Y255A	10213 ± 1326	31 ± 5.9	329

[a] Xylanase assays were performed as described in Materials and Methods. The concentrations of substrate that were used were optimized for the different enzymes, in the range of 0.2−5 times K_m wherever possible. Initial rates were determined from linear plots comprising three time points, and the amount of enzyme used ensured <10% of the substrate was cleaved during the reaction.

In a recent study, Schmidt et al. (*22*) used xylooligosaccharides as cryoprotectants during low-temperature X-ray crystallography studies of a family 10 xylanase from *Penicillium simplicissimum*. The authors observed that X5 and X4 were cleaved when soaked into crystals of the enzyme. Released X3 bound tightly to the glycone region of the active site, while the weak electron density for X1 (from X4 cleavage) and X2 (from X5 hydrolysis) suggested that sugars bound weakly to the aglycone region of the substrate binding cleft. The authors concluded that the two catalytic residues of the xylanase divide the binding cleft into a "substrate recognition area" (the glycone binding region) with strong and specific xylan binding and a "product release area" (aglycone binding region) with considerably weaker and less specific binding. This is in contrast to the data presented in this report, which indicate that detachment of the cleaved substrate from the aglycone region of the active site has a rate-limiting effect on overall catalysis. It is possible that the aglycone region of the fungal enzyme contains fewer subsites than Pc Xyn10A, and thus, the cleaved substrate

(generated by the k_2 step) can be more easily released from the active site of this enzyme than Pc Xyn10A. Alternatively, despite xylooligosaccharides interacting relatively weakly with the aglycone region of the active site of the fungal xylanase, the release of the hydrolyzed substrate (after the k_2 step) from the enzyme could still be rate-limiting.

CONCLUSIONS

In this report, we have shown that mutating residues, important in aglycone sugar binding, decreases the propensity of Pc Xyn10A to perform transglycosylation reactions. In addition, aglycone mutants display an increase in both their k_{cat} and K_m values against xylan, although the k_{cat}/K_m ratio is not greatly changed. We propose that the elevated activity of the aglycone mutants, at high substrate concentrations, reflects the increased rate at which the cleaved substrate can diffuse out of the aglycone region of the active site. This hypothesis suggests diffusion of the cleaved substrate out of the aglycone region of the active site, which at the completion of the k_2 step limits the maximum catalytic activity of Pc Xyn10A against xylan. It is unclear, however, whether diffusion of the cleaved product out of the aglycone region limits the activity of other family 10 xylanases, in which the aglycone binding region is smaller than that of Pc Xyn10A (*9*, *10*). Finally, this report shows that the difference in activity of the −2 subsite mutant against xylooligosaccharides and xylan cannot be explained by the formation of dead-end complexes. It is interesting to note that this phenomenon is an emerging trend with mutations in other glycoside hydrolases that have a much greater affect on oligosaccharide hydrolysis than polysaccharide cleavage (*21*, *22*). We propose that the properties of these mutants reflect fundamental differences in the mechanism by which oligosaccharides and polysaccharides bind to the substrate binding cleft of Pc Xyn10A and other hydrolases.

NOTE ADDED IN PROOF

The authors have omitted to include the references of three papers describing the structures of family 10 xylanases. They are now included as refs *23*−*25*.

REFERENCES

1. Davies, G. J., and Henrissat, B. (1995) *Structure 3*, 853−859.
2. Moreau, A., Shareck, F., Kluepfel, D., and Morosoli, R. (1994) *Eur. J. Biochem. 219*, 261−266.
3. White, A., Withers, S. G., Gilkes, N. R., and Rose, D. R. (1994) *Biochemistry 33*, 12546−12552.
4. Derewenda, U., Swenson, L., Green, R., Wei, Y., Morosoli, R., Shareck, F., Kluepfel, D., and Derewenda, Z. (1994) *J. Biol. Chem. 269*, 20811−20814.
5. Harris, E., Jenkins, J. A., Connerton, I., Cummings, N., Lo Leggio, L., Scott, M., Hazlewood, G. P., Laurie, J. I., Gilbert, H. J., and Pickersgill, R. W. (1994) *Structure 2*, 1107−1114.
6. Schmidt, A., Schlacher, A., Steiner, W., Schwab, H., and Kratky, C. (1998) *Protein Sci. 7*, 2081−2088.
7. Dominguez, R., Souchon, H., Spinelli, S., Dauter, Z., Wilson, K. S., Chauvaux, S., Béguin, P., and Alzari, P. M. (1995) *Nat. Struct. Biol. 2*, 569−576.
8. Notenboom, V., Birsan, C., Warren, R. A. J., Withers, S. G., and Rose, D. R. (1998) *Biochemistry 37*, 4751−4758.
9. Ducros, V., Charnock, S. J., Derewenda, U., Derewenda, Z. S., Dauter, Z., Dupont, C., Shareck, F., Morosoli, R., Kluepfel, D., and Davies, G. J. (2000) *J. Biol. Chem. 275*, 23020−23026.
10. Charnock, S. J., Spurway, T. D., Xie, H., Beylot, H.-M., Virden, R., Warren, R. A. J., Hazlewood, G. P., and Gilbert, H. J. (1998) *J. Biol. Chem. 273*, 32187−32199.
11. Biely, P., Kluepfel, D., Morosoli, R., and Shareck, F. (1993) *Biochim. Biophys. Acta 1162*, 246−254.
12. Charnock, S. J., Lakey, H. J., Virden, R., Hughes, N., Sinnott, M. L., Hazlewood, G. P., Pickersgill, R., and Gilbert, H. J. (1997) *J. Biol. Chem. 272*, 2942−2951.
13. Norrander, J., Kempe, T., and Messing, J. (1983) *Gene 26*, 101−106.
14. Hall, J., Hazlewood, G. P., Huskisson, N. S., Durrant, A. J., and Gilbert, H. J. (1989) *Mol. Microbiol. 3*, 1211−1219.
15. Laemmli, U. K. (1970) *Nature 227*, 680−685.
16. Miller, G. L. (1959) *Anal. Chem. 31*, 426−428.
17. Matsui, I., Ishikawa, K., Matsui, E., Miyairi, S., Fukui, S., and Honda, S. (1991) *J. Biochem. 106*, 566−569.
18. Stoscheck, C. M. (1990) *Methods Enzymol. 182*, 50−68.
19. Atkin, E. D. A. (1991) in *Xylan and Xylanases* (Visser, J., Beldman, G., Kusters-van Someren, M. A., and Voragen, A. G. J., Eds.) pp 39−50, Elsevier, Amsterdam.
20. Hogg, D., Woo, E.-J., Bolam, D. N., McKie, V. A., Gilbert, H. J., and Pickersgill, R. W. (2000) *J. Biol. Chem.* (submitted for publication).
21. Svensson, B., Bak-Jensen, K. S., Mori, H., Sauer, J., Jensen, M. T., Kramhoft, B., Gottschalk, T. E., Christensen, T., Sigurskjold, B. W., Aghajari, N., Haser, R., Payre, N., Cottaz, S., and Driguez, H. (1999) in *Recent Advances in Carbohydrate Bioengineering*, pp 272−281, Royal Society of Chemistry, Cambridge, U.K.
22. Schmidt, A., Gubitz, G. M., and Kratky, C. (1999) *Biochemistry 38*, 2403−2412.
23. Natesh, R., Bhanumoorthy, P., Vlthayathil, P. J., Sekar, K., Ramakumar, S., and Viswamitra, M. A. (1999) *J. Mol. Biol. 288*, 999−1012.
24. Fujimoto, Z., Kuno, A., Kaneko, S., Yoshida, S., Kobayashi, H., Kusakabe, I., and Mizuno, H. (2000) *J. Mol. Biol. 14*, 575−585.
25. White, A., Tull, D., Johns, K., Withers, S. G., and Rose, D. R. (1996) *Nat. Struct. Biol. 3*, 149−154.

BI002704R