Identification of two acidic residues involved in the catalysis of xylanase A from *Streptomyces lividans*

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On the basis of similarities between known xylanase sequences of the F family, three invariant acidic residues of xylanase A from *Streptomyces lividans* were investigated. Site-directed-mutagenesis experiments were carried out in *Escherichia coli* after engineering the xylanase A gene to allow its expression. Replacement of Glu-128 or Glu-236 by their isosteric form (Gln) completely abolished enzyme activity with xylan and *p*-nitrophenyl β-D-cellobioside, indicating that the two substrates are hydrolysed at the same site. These two amino acids probably represent the catalytic residues. Immunological studies, which showed that the two mutants retained the same epitopes, indicate that the lack of activity is the result of the mutation rather than misfolding of the protein. Mutation D124E did not affect the kinetic parameters with xylan as substrate, but D124N reduced the K_m 16-fold and the $V_{max.}$ 14-fold when compared with the wild-type enzyme. The mutations had a more pronounced effect with *p*-nitrophenyl β-D-cellobioside as the substrate. Mutation D124E increased the K_m and decreased the $V_{max.}$ 5-fold each, while D124N reduced the K_m 4.5-fold and the $V_{max.}$ 75-fold. The mutations had no effect on the cleavage mode of xylopentaose.

INTRODUCTION

Xylan is the major hemicellulosic component of graminaceous plants, as well as of hardwoods. Xylanases (1,4-β-D-xylan xylanohydrolases; EC 3.2.1.8) hydrolyse xylan to short xylo-oligosaccharides. By comparing all known xylanase sequences by hydrophobic cluster analysis, xylanases have been classified into two families of glycanases (F and G) (Morosoli et al., 1990; Shareck et al., 1991).

Xylanase A from *Streptomyces lividans*, along with the other high-molecular-mass/acidic xylanases, belong to the F family. The G family encompasses the low-molecular-mass/basic xylanases, most of which have been isolated from *Bacillus* species (Wong et al., 1988). Xylanases are potentially related to lysozyme and cellulases which also hydrolyse β-1,4 linkages (Matthews et al., 1981; Baird et al., 1990). The catalytic mechanism of lysozyme is well documented, suggesting for xylanases a model involving two acidic residues (Matthews et al., 1981). Resolution of the crystalline structure of the xylanase of *Bacillus pumilus* IPO implicated two glutamic acid residues in the active site, and this was confirmed by site-directed mutagenesis (Katsube et al., 1989). However, these data only relate to the structure of the xylanases belonging to the G family. Despite the lack of structural information for the F-family xylanases, sequence identities among these xylanases have proved to be useful for targeting presumptive important catalytic residues. Comparisons of known xylanase amino acid sequences indicate the presence of 26 highly conserved amino acid residues distributed along five specific regions (Morosoli et al., 1990; Shareck et al., 1991). Thus we have modified selected residues by site-directed mutagenesis in order to identify the catalytic residues. The presence of a catalytic triad involving three acidic residues has previously been observed in some amylases, glucoamylases and one cyclomaltodextrinase (Sierks et al., 1990; Takase, 1993; Podkovyrov et al., 1993). In the present paper, we report that Glu-128 and Glu-236 are essential residues in the active site of xylanase A from *S. lividans* and that the carboxy group of Asp-124 also contributes to the catalytic mechanism.

EXPERIMENTAL

Materials

Escherichia coli CJ236: *dut-1, ung-1, thi-1, rel A-1*; pCJ105 (Cm^r) and MC1061: *hsdR2 hsdM*$^+$ *hsdS*$^+$ *araD139* $\Delta(ara\text{-}leu)_{7697}$ $\Delta(lac)_{x74}$ *galE15 galK16 rpsL* (Str^r) *mcrA mrcB1*, used in site-directed mutagenesis experiments, were purchased from Bio-Rad Laboratories, Life Science Group, Melville, NY, U.S.A. Phagemid pTZ19U was also from Bio-Rad.

Construction of the pIAF217 vector

The xylanase A gene from *S. lividans* 1326, located on a 3.2 kb *Sph*I fragment (Mondou et al., 1986), was inserted into the *Sph*I site of the phagemid pTZ19U to generate pAM19.1. However, the xylanase A gene was poorly expressed in *E. coli*. In order to improve its expression, a 900 bp deletion of the upstream region of the structural gene as well as the introduction, by site-directed mutagenesis, of a good translation-initiation site for *E. coli* (underlined below) were performed. This was carried out with a 51-mer oligo-nucleotide 5′-GGCGT-AGGAGCCCATAATTTTTTCCTCCAAGAA-TTCGCA-TGCAAGCTTTCC-3′ which contains 21 nucleotides from the cloning cartridge of pTZ19U and 15 nucleotides downstream from the ATG start codon of the xylanase A gene. This construction (pIAF217) allowed expression of the xylanase A gene from the *lacZ* promoter (Figure 1). Strain CJ236 harbouring plasmid pIAF217 was used to prepare uracil-containing single-stranded DNA for mutagenesis experiments.

Abbreviations used: i.u., international unit; *p*-NPC, *p*-nitrophenyl β-D-cellobioside; Xln A, wild-type xylanase A; Xln D124E, mutation in Xln A, Asp-124 replaced by Glu; Xln D124N, mutation in Xln A, Asp-124 replaced by Asn.

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Site-directed mutagenesis

Mutagenesis was carried out by the method of Kunkel (1985) using a commercial kit (Bio-Rad). Oligonucleotides for mutagenesis were synthesized on a Gene Assembler (Pharmacia). The oligonucleotide primers used to mutate codons Asp-124, Glu-128 and Glu-236 were the following: D124E 5′-GTTCACGACCTCCCACTGGAC-3′, D124N 5′-GTTCACGACGTTCCACTGGAC-3′, E128Q 5′-GGCGAAGGCCTGGTTCACGAC-3′ and E236Q 5′-GATGTCCAGCTGGGTGATGGC-3′ (underlining indicates the substituted nucleotide). Plasmid DNA was isolated from presumptive mutants (Birnboim and Doly, 1979) and hybridized with the ^{32}P-labelled mutagenic primers in 6 × SSC (1 × SSC is 0.15 M NaCl, 0.015 M sodium citrate) at 42 °C, overnight. Then the dot-blots were washed twice in 3 M tetramethylammonium chloride, at 61 °C for 20 min (Wood et al., 1985). This base-composition-independent method permits the detection of single-base alterations generated by *in vitro* mutagenesis. Subsequently, clones were sequenced using the dideoxy-chain-termination method with [[^{35}S]thio]dATP (Sanger et al., 1977).

Screening of mutant transformants and enzyme assays

Mutant transformants were screened on Luria broth plates containing ampicillin (100 μg/ml) and 0.15% RBB-xylan [oat spelt xylan (Sigma Chemical Co., St. Louis, MO, U.S.A.) covalently linked to Remazol Brilliant Blue (Aldrich Chemical Co., Milwaukee, WI, U.S.A.)]. Xylanase activity was detected by the appearance of a clearing zone around the colony. *E. coli* mutant transformants were grown overnight in 25 ml of 2 × TY (16 g of tryptone, 10 g of yeast extract and 5 g of NaCl per litre) containing 0.5% xylose and ampicillin (100 μg/ml) at 37 °C with shaking. Xylanase activity was measured in supernatants and in periplasmic cell extracts by the dinitrosalicylic acid method and with the RBB-xylan plate assay (Biely et al., 1985; Kluepfel, 1988). One international unit (i.u.) of enzyme activity is defined as the amount of enzyme required to liberate 1 μmol of reducing sugars (expressed as xylose) in 1 min.

Proteins and Western-blot analysis

The protein content of enzyme preparations was determined using Bradford's (1976) method with a commercial kit (Bio-Rad). Proteins from the periplasmic space of *E. coli* cells were extracted by the chloroform shock method (Ames et al., 1984). Proteins from culture supernatants were precipitated by addition of 0.2 vol. of a solution containing 50% trichloroacetic acid and 0.5% sodium deoxycholate. The mixtures were centrifuged for 10 min at 10000 *g* and the protein pellets were dissolved in Tris/glycine electrophoresis buffer, pH 3.3. Proteins were analysed by SDS/PAGE (Laemmli, 1970) followed by Western blotting (Towbin et al., 1979). Xylanase-related polypeptides were identified with immunopurified anti-xylanase A antibodies coupled to ^{125}I-Protein A (Amersham Canada Ltd., Oakville, Ont., Canada). After exposure, the autoradiograms were scanned by a laser scan XL (LKB-Pharmacia) to determine the amount of xylanase present in each preparation. Calibration standards were made with various concentrations (50–500 ng) of pure xylanase A isolated from *S. lividans* and electrophoresed using the same conditions.

Determination of kinetic parameters

Michaelis–Menten constants were determined on birchwood xylan (Sigma lot 110H0464) and *p*-nitrophenyl β-D-cellobioside (*p*-NPC) (Sigma). The hydrolysis conditions were 20 min at 60 °C in 1 ml of 50 mM citrate buffer, pH 6.0, containing 0.2 i.u. of xylanase and concentrations of xylan varying from 0.5 to 16 mg/ml. Liberation of reducing sugars was measured by the dinitrosalicylic acid method. Hydrolysis of *p*-NPC was performed in 1 ml of 50 mM citrate buffer, pH 6.0, containing 0.05 i.u. of xylanase and various concentrations of *p*-NPC (0.5–32 mM). After a 30 min incubation at 50 °C, 1 ml of 2.0 M Na_2CO_3 was added to stop the reaction. The absorbance at 405 nm was measured. One unit of enzymic activity is defined as the quantity of enzyme required to liberate 1 μmol of *p*-nitrophenol/min at 50 °C.

Mutant enzyme production and purification

Mutant Xln E128Q and Xln E236Q were produced with a cellulase- and xylanase-negative mutant, strain *S. lividans* 10-164, as host. Transformation of *S. lividans* 10-164 was as described by Hopwood et al. (1985). The wild-type xylanase gene was replaced in pIAF18 by its mutated counterparts as follows: the *Not*I–*Eco*RI restriction fragments containing the mutation in pIAF217/E128Q and in pIAF217/E236Q were purified and subcloned into pIAF19.1 deleted from its *Not*I–*Eco*RI wild-type DNA fragment (Mondou et al., 1986). After digestion, the resulting 3.2 kb *Sph*I fragments containing the mutations were cloned into pIAF11 to reconstitute pIAF18/E128Q and pIAF18/E236Q. Purification of the mutant E128Q and E236Q xylanases was performed as described previously for the wild-type xylanase A (Morosoli et al., 1986).

Determination of bond-cleavage frequencies

Bond-cleavage frequencies were determined by the method of Biely et al. (1981). The reaction was carried out at 50 °C in a volume of 10 μl of 50 mM citrate buffer, pH 6.0, containing 0.002 i.u. of xylanase, 0.1 mM xylopentaose (Megazyme Pty. Ltd., North Rocks, N.S.W., Australia) and 2×10^5 c.p.m. of [1-^{3}H]xylopentaose as a tracer (gift from Dr. Biely, Bratislava, Slovakia). At various time intervals, 1.5 μl portions of the mixture were removed, immediately spotted on cellulose thin layers (Merck, Darmstadt, Germany) and chromatographed in ethyl acetate/acetic acid/water (3:2:2, by vol.). The chromatograms were cut up and the radioactivity in each band was counted in a liquid-scintillation counter.

Immunological methods

Studies on antigenic epitope conservation in the negative mutants were performed by e.l.i.s.a. For titration experiments, plates were coated overnight with 250 ng/well wild-type xylanase A (Xln A) and mutant enzymes. Dilutions of rabbit anti-xylanase serum were added and the plates were incubated for 2 h at 37 °C. The reaction was developed using biotinylated goat anti-rabbit antibodies, streptavidin–horseradish peroxidase complex and tetramethyl benzidine by standard procedures. The absorbance at 450 nm was read. End-point value was established at twice the background level. Inhibition studies were also performed by e.l.i.s.a. using a predetermined anti-xylanase serum dilution and increasing concentrations of Xln A and corresponding mutants.

RESULTS AND DISCUSSION

There is no method available of performing site-directed mutagenesis experiments directly in streptomycetes. For this reason the experiments were carried out in *E. coli*. As streptomycetes

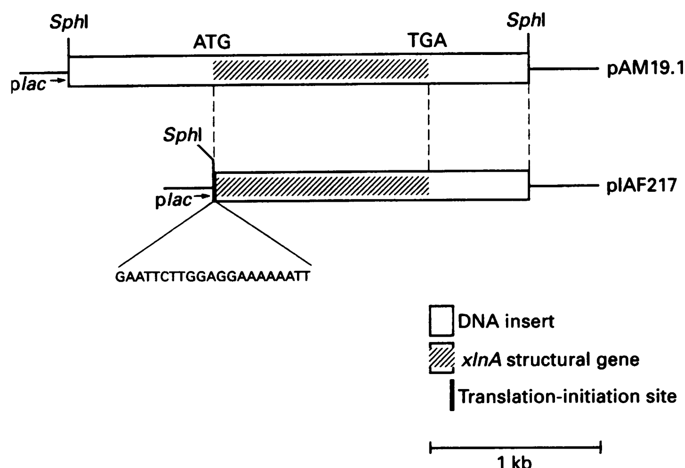

Figure 1 Construction of pIAF217

The 900 bp non-coding region upstream of the *xlnA* gene was removed from pAM19.1, and a specific consensus translation-initiation site for *E. coli* was added in front of the ATG codon in order to put the *xlnA* gene under control of the *lacZ* promoter.

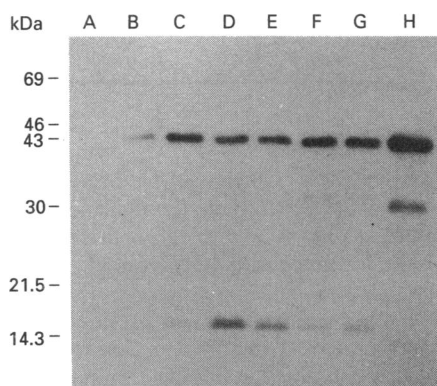

Figure 2 Protein analysis of culture supernatants of *E. coli* MC1061 transformants

Proteins were separated by SDS/PAGE followed by Western transfer. Immunodetection was carried out with specific anti-xylanase A antibodies coupled to ^{125}I-labelled Protein A (80 μg of protein was loaded into each well). Lane A, clone with pTZ19U, as control; lane B, clone with pAM19.1; lane C, clone IAF217; lane D, mutant D124E; lane E, mutant D124N; lane F, mutant E128Q; lane G, mutant E236Q; lane H, native xylanase produced by *S. lividans*.

promoters are generally not functional in *E. coli* (Bibb and Cohen, 1982), the xylanase gene promoter was deleted by looping-out in order to transcribe the structural *xlnA* gene from the *lacZ* promoter (Figure 1). Expression of the xylanase gene by *E. coli* transformants was readily observed on RBB-xylan plates. Enzyme activity was detected in the periplasmic space (4 i.u./mg of protein) and in the culture medium (1.5 i.u./ml) representing respectively 24 % and 76 % of the total activity expressed by the transformant. The periplasmic and supernatant enzymes produced by *E. coli* had a molecular mass of 43 kDa, which corresponds to that of the mature xylanase secreted by *S. lividans* (Figure 2). This suggested that the signal peptide of 41 amino acid residues had been cleaved off. The enzymes also showed the same properties as that produced by *S. lividans*, such as pH optimum, thermal stability and xylan-hydrolysis pattern (results not shown). These results allowed us to analyse the mutated xylanase gene product with reasonable confidence. One aspartic and two glutamic residues, which are invariant in the F family, were mutated. The following mutations were performed by site-directed mutagenesis: D124E, D124N, E128Q and E236Q. All mutants synthesized a 42 kDa protein which reacted in immunoblots with specific antibodies (Figure 2). In addition, some minor proteins of low molecular mass, detected in Western blots, represented proteolytic degradation of the recombinant xylanase. In order to confirm the mutations, the four mutated genes were completely sequenced and compared with the original sequence. No differences were found except those introduced by the substitutions (results not shown).

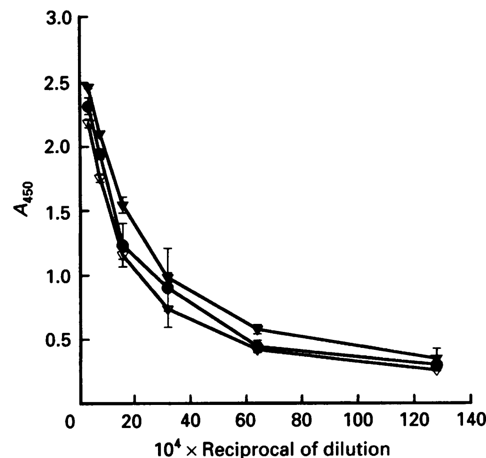

Figure 3 Representative titration of anti-xylanase serum against equally coated (250 ng) Xln A (●), Xln E128Q (▽) and Xln E236Q (▼)

Mutants E128Q and E236Q showed no xylanolytic activity, as revealed by the absence of clearing zones on RBB-xylan plates. No activity was found in either the periplasmic space or the culture supernatant when either xylan or *p*-NPC was used as substrate. The two glutamates in these positions probably represent the catalytic residues of Xln A, which would be similar to that demonstrated, but at a different location, for the G-family xylanases (Katsube et al., 1989).

The lack of activity in Xln E128Q and Xln E236Q could have been the result of incorrect folding of the mutant protein as well as the replacement of residues crucial for the catalytic mechanism. This question was addressed by immunological studies. In order to compare their immunological properties with those of the native enzyme, the E128Q and E236Q xylanases were purified. To improve enzyme production, the mutated genes were inserted into plasmid pIAF11 (Mondou et al., 1986) by replacement of the wild-type structural gene, and the new plasmids were used to transform *S. lividans* 10-164 (a xylanase- and cellulase-negative mutant). After transformation, the presence of the mutation on the plasmid was confirmed by hybridization with the labelled oligonucleotide primer used to generate the mutation. *S. lividans* IAF/E128Q and IAF/E236Q secreted about 1 mg/ml mutated inactive xylanase, as much as IAF 18 secreted of the wild-type enzyme (Bertrand et al., 1989). This yield was at least 100-fold higher than the production by the *E. coli* transformants. Polyclonal anti-xylanase antibodies were used to titrate the mutant enzymes, and there was no difference between the titration curves for Xln A, Xln E128Q and Xln E236Q (Figure 3). Moreover, in

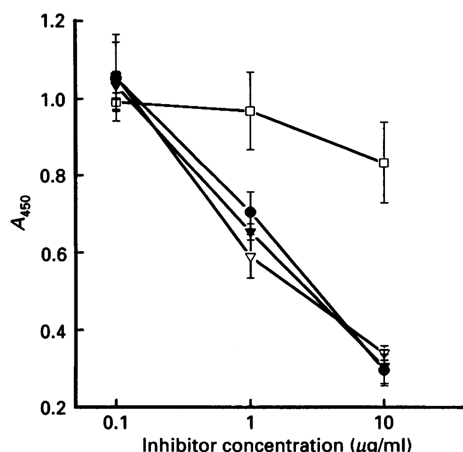

Figure 4 Inhibition of anti-xylanase antibody binding to Xln A in e.l.i.s.a.

●, Xln A; □, heat-inactivated Xln A; ▽, Xln E128Q; ▼, Xln E236Q.

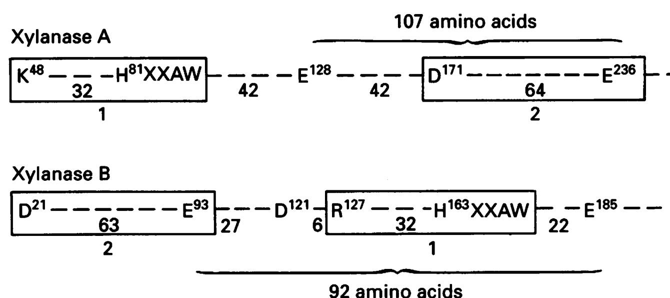

Figure 5 Structural organization of the catalytic domain of the A and B xylanases of *S. lividans* belonging respectively to the F and G families

Table 1 Kinetic constants of Xln A, Xln D124E and Xln D124N with xylan and *p*-NPC as substrates

	Xln D124E	Xln D124N	Xln A
Xylan			
K_m (mg/ml)	0.9	0.06	1.0
$V_{max.}$ (i.u./mg)	556	44	616
p-NPC			
K_m (mM)	39.4	1.7	7.6
$V_{max.}$ (mM/mg)	0.0015	0.0001	0.0075

e.l.i.s.a., inhibition of anti-xylanase antibody binding to Xln A, by Xln A, Xln E128Q or Xln E236Q was similar, whereas a reduced immunological reaction was observed when heat-inactivated Xln A was used (Figure 4). These immunological studies demonstrated preservation of the structural integrity of the antigenic epitopes of the mutants. Therefore the lack of xylanase activity in the two mutant enzymes is unlikely to be caused by misfolding of the protein but rather is the result of the mutations themselves. However, the e.l.i.s.a. method might not be sensitive enough to detect minor structural changes inside the protein, which might affect the catalytic activity.

Studies of hydrolysis of β-1,4-glycosidic bonds have been greatly influenced by the results obtained with hen egg white lysozyme. Site-directed-mutagenesis experiments have attributed to Glu-35 and Asp-52 the role of catalytic residues acting in concert to hydrolyse the substrate (Malcolm et al., 1989). In two endo-β-1,4-glucanases from *Bacillus*, changing one glutamic acid residue to glutamine, its isosteric form, resulted in loss of activity (Baird et al., 1990). Recently, crystallographic and site-directed-mutagenesis data for a xylanase from *B. pumilus* IPO, belonging to the G family, demonstrated the importance of two glutamic residues, Glu-93 and Glu-182 (Katsube et al., 1989). Our mutagenesis results show that two glutamic acid residues, Glu-128 and Glu-236, are essential for the enzymic activity of xylanase A from *S. lividans*. The complete loss of activity towards xylan and *p*-NPC confirms that these two substrates are hydrolysed at the same site. This is in agreement with previous results obtained with a xylanase from *Cellovibrio gilvus*, where competition experiments carried out between xylan and *p*-NPC indicated that the two substrates are hydrolysed at the same catalytic site (Haga et al., 1991). In the exoglucanase from *Cellulomonas fimi*, which also exhibits xylanase activity and belongs to the F family of glycanases, Glu-274 was shown to be the nucleophile (Tull et al, 1991). Accordingly, Glu-236 of Xln A probably plays this role.

It is interesting to point out that about 100 residues separate the two catalytic glutamic acids in the F and G families (Figure 5). The F family also shares two motifs, illustrated by boxes 1 and 2, with the G family, but here they are located in the reverse order. However, there is no similarity between the boxes, except the distribution of some highly conserved residues at similar locations in each box. Nevertheless, the conserved glutamic acid residue in box 2 always represents the nucleophile (Katsube et al., 1989; Tull et al., 1991). This suggests that the two families, classified as retaining glycanases (Gebler et al., 1992), may have some structural similarities despite the lack of sequence identity.

The presence of a conserved aspartate residue (Asp-124) close to the putative catalytic Glu-128 is a characteristic of the F family, as no such organization has been found in the G family. This conserved acidic residue should play some role in catalysis and was thus analysed by site-directed mutagenesis. The specific activity, as determined on birchwood xylan for recombinant Xln A, Xln D124E and Xln D124N, was respectively 377 i.u., 331 i.u. and 26 i.u. per mg of enzyme, calculated from the amount of enzyme detected on the immunoblots. The kinetic parameters of Xln D124E and Xln D124N were compared with those of Xln A using xylan and *p*-NPC (Table 1). With xylan, Xln D124E had almost the same K_m and $V_{max.}$ as Xln A. However, with *p*-NPC, the K_m value was 5-fold higher and the $V_{max.}$ 5-fold lower than those of Xln A. The D124N mutation had a more pronounced effect on the catalytic properties of the enzyme. With xylan, the K_m and $V_{max.}$ values were 16-fold and 14-fold respectively lower, and with *p*-NPC as substrate, the K_m and $V_{max.}$ were respectively 4.5-fold and 75-fold lower than the Xln A values. However, the values for Xln D124E were extrapolated for *p*-NPC as substrate, because this compound is soluble in 70% dimethylformamide and a concentration above 20% inhibits enzyme activity. Therefore the *p*-NPC concentration could not exceed 32 mM in the test.

Replacement of Asp-124 by Glu corresponds to a conservative change in terms of function, but an additional carbon to the amino acid side chain could create a steric hindrance for the substrate in the active-site pocket. The K_m was increased and $V_{max.}$ was reduced for *p*-NPC but no change was observed for xylan. This could be due to the large size of the xylan molecule

which, by binding to several subsites on the enzyme, may neutralize the effect of the mutation. On the other hand, mutation D124N represents a change of function, but it is conservative in terms of size. There is not much difference in K_m, but there is a considerable decrease in $V_{max.}$, which could possibly reflect a modification in the pK_a of the active cleft or a modification of the hydrogen-binding network affecting the local structure.

The overall decrease in activity observed in the two mutant xylanases could be due to perturbation of the cleavage mode of the β-1,4 linkages in the substrate. For this reason the bond-cleavage frequencies of xylopentaose were determined as initial-product-degradation ratios of [1-^{3}H]xylopentaose by Xln A, Xln D124E and Xln D124N. The three enzymes exhibited the same cleavage mode, liberating 54% labelled xylobiose and 46% labelled xylotriose, indicating that the second and third β-1,4 linkages from the reducing end of xylopentaose were cleaved (results not shown). Therefore neither mutation seems to favour the specific formation of a productive enzyme–substrate complex (Biely et al., 1993). As shown for the D124N xylanase, the $V_{max.}$ and K_m were reduced for *p*-NPC. From biochemical data, it was suggested that the substrate-binding site of the *Cryptococcus albidus* xylanase, which is comparable with xylanase A of *S. lividans*, is composed of four subsites, and only two of them, the outer subsites +II and −II, show a strong affinity for binding xylosyl residues (Biely et al., 1981). It was proposed that subsite −II could also accommodate glycosyl residues, and this could explain why xylanase hydrolyses *p*-NPC. One would then be tempted to localize the −II subsite in the vicinity of Asp-124, as its replacement by Glu increases the K_m for the substrate, probably by causing steric hindrance.

In conclusion, site-directed mutagenesis of cloned xylanases has provided highly incisive probes into the roles of individual residues in enzyme mechanisms, and crystallographic data on the F-family xylanases are now needed to validate these results.

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