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Characterization of active-site aromatic residues in xylanase A from *Streptomyces lividans*

Martin Roberge[1], François Shareck, Rolf Morosoli, Dieter Kluepfel and Claude Dupont[2]

Centre de Microbiologie et Biotechnologie, INRS–Institut Armand-Frappier, Laval, Quebec, H7N 4Z3, Canada

[1]Present address: Protein Engineering Department, Genentech Inc., South San Francisco, CA 94080, USA

[2]To whom correspondence should be addressed.
E-mail: claude_dupont@iaf.uquebec.ca

The role of four aromatic residues (W85, Y172, W266 and W274) in the structure–function relationship in xylanase A from *Streptomyces lividans* (XlnA) was investigated by site-directed mutagenesis where each residue was subjected to three substitutions (W85A/H/F; W266A/H/F; W274A/H/F and Y172A/F/S). These four amino acids are highly conserved among family 10 xylanases and structural data have implicated them in substrate binding at the active site. Far-UV circular dichroism spectroscopy was used to show that the overall structure of XlnA was not affected by any of these mutations. High-performance liquid chromatographic analysis of the hydrolysis products of birchwood xylan and xylopentaose showed that mutation of these aromatic residues did not alter the enzyme's mode of action. As expected, though, it did reduce the affinity of XlnA for birchwood xylan. A comparison of the kinetic parameters of different mutants at the same position demonstrated the importance of the aromatic nature of W85, Y172 and W274 in substrate binding. Replacement of these residues by a phenylalanine resulted in mutant proteins with a K_M closer to that of the wild-type protein in comparison with the other mutations analyzed. The kinetic analysis of the mutant proteins at position W266 indicated that this amino acid is important for both substrate binding and efficient catalysis by XlnA. These studies also demonstrated the crucial role of these active site aromatic residues for the thermal stability of XlnA.

Keywords: family 10 hydrolase/glycosyl hydrolase/site-directed mutagenesis/structure–function/xylanase

Introduction

Xylanases (EC 3.2.1.8) catalyze the hydrolysis of xylan, a complex heteropolymer found in plant cell walls. On the basis of sequence homology of their catalytic domains, xylanases have been classified into families 10 and 11 of the 66 families of glycosyl hydrolases (Henrissat and Bairoch, 1998). Xylanase A from *Streptomyces lividans* (XlnA) is a 47 kDa secreted protein composed of two domains: a 33 kDa catalytic domain responsible for the xylanolytic activity and a 14 kDa xylan-binding domain involved in the specific binding to the insoluble substrate (Dupont *et al.*, 1998). The catalytic domain of XlnA belongs to family 10 of glycosyl hydrolases (Shareck *et al.*, 1991) and folds into an $(\alpha/\beta)_8$ motif (Derewenda *et al.*, 1994). The catalytic domain of XlnA, along with members of 11 other different families of glycosyl hydrolases, is also classified into clan GH-A on the basis of structural and mechanistic information (Henrissat and Bairoch, 1998). Xylanases hydrolyze β-1,4-glycosidic bonds with net retention of the configuration of the anomeric carbon via a double displacement mechanism (Sinnott, 1990). The side chains of two glutamic acids are involved, one acting as a nucleophile and the other as an acid–base catalyst. These two amino acids were identified in XlnA by site-directed mutagenesis (Moreau *et al.*, 1994) and confirmed with the structure of the catalytic domain (Derewenda *et al.*, 1994).

Available structural information on family 10 xylanases has allowed the identification of the residues present in the active site along with their possible involvement in the catalytic function of this type of protein. For example, substrate binding in the active site of family 10 xylanases has been investigated with the use of co-crystal structures. In one of these studies, the structure of XynA from *Pseudomonas fluorescens* subsp. *cellulosa* has been obtained in the presence of xylopentaose (Harris *et al.*, 1994). This permitted the identification of the residues forming six substrate-binding subsites (–2 to +4; Davies, *et al.* 1997) of this enzyme. In another study, White *et al.* (1996) obtained the crystal structure of Cex from *Cellulomonas fimi* covalently complexed with a 2-fluoro-cellobioside, an analogue of the catalytic intermediate in the double displacement mechanism. They identified many interactions which could be of crucial importance in the binding and stabilization of the catalytic intermediate in the active site of family 10 xylanases. All these structural data show the implication of different aromatic residues in the binding of the substrate and/or the catalytic intermediate. The aromatic amino acids would allow specific and stable binding of the carbohydrate moieties by partial stacking of the sugar ring (Quiocho, 1986) and/or by hydrogen bonding to hydroxyl groups of the pyranose ring.

The role of aromatic residues in the function of xylanases has been studied through chemical modification. For example, the inactivation of a family 11 xylanase from *Streptomyces* T_7 with *N*-bromosuccinimide suggested the importance of a tryptophan side chain in the active site of the enzyme (Keskar *et al.*, 1989). Moreover, for family 11 xylanase XynA from *Schizophilum commune*, chemical modification of tyrosyl side chains by tetranitromethane suggested that Y97, a conserved residue in this family of enzyme, plays an essential role in substrate binding (Bray and Clarke, 1995).

The active site of XlnA contains many aromatic residues, four of which are exposed to solvent and could be involved in substrate binding (W85, Y172, W266 and W274). These residues are highly conserved in family 10 glycosyl hydrolases, suggesting their importance in the function of this type of enzyme (Figure 1). Moreover, the equivalents of these amino acids were shown to be part of the substrate-binding subsites surrounding the cleavage site that contain the two catalytic residues, –1 and +1 (Harris *et al.*, 1994). Site-directed muta-

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                W85              Y172               W266    W274
                 ↓                 ↓                  ↓       ↓
A    QVRGHTLAWHS  …  AKLCYNDYNVENW  …  GITVWGVRDSDSWRS-
B    LIRGHTLVWHS  …  AKLYINDYNLDSA  …  GITVWGVADPDSWRA-
C    LIRGHTLVWHS  …  AKLYINDYNLDSA  …  GITVWGVADPDSWRS-
D    LIRGHTLLWHS  …  AKLYINDYNLDKS  …  GITVWGVSDKDSWRP-
E    QVRGHTLAWHS  …  AKLCYNDYNIENW  …  GITVWGVRDTDSWRS-
F    EVRGHTLAWHS  …  AKLCYNDYNIENW  …  GITVWGVRDTDSWRS-
G    TVHGHALVWHS  …  VILYYNDYNIE-Q  …  GISVWGTTDANTWLDG
H    NVHGHALVWHS  …  AILYYNDYNID-Q  …  GISVWGTTDANTWLT-
I    GVRGHTLVWHN  …  ALLFYNDYNECFP  …  NVTFWGIADDHTWLDH
J    KLRGHTFVWHN  …  AKLFYNDYNNEMP  …  SVTLWGISDRHTWKDN
K    AIRGHNLL-HQ  …  AQLFYNDYSTENP  …  SVSFWGLKDDYSWLQ-
L    SLRGHTLVWHQ  …  AKLFYNDYSTEDP  …  SVTFWGLKDDYSWLR-
M    GIRGHTLVWHN  …  AKLFYNDYNTEIS  …  SVTFWGLKDDYSWLR-
N    KVRGHTLVWHN  …  AKLFYNDYNLEDP  …  GVTFWGVADDYTWLYF
O    VIRGHTLVWHN  …  AKLFYNDYNTFEP  …  NVTFWGLKDDYSWRAT
P    VVRGHTLVWHN  …  AKLFYNDYNTFEP  …  NVTFWGLKDDYSWRAT
Q    DIRFHTLVWHS  …  IKLYMNDYNTEVE  …  NVTFWGIADNHTWLDS
R    ELRFHTLVWHS  …  AKLYINDYNTEVP  …  SVTFWGIADNHTWLDG
S    ELRFHTLVWHN  …  IKLYINDYNTDDP  …  AVVFWGISDKYSWLNG
T    KMRGHTLLWHN  …  MKLFINDYNIENN  …  AVVFWGVSDDVTWLS-
U    KMRGHTLLWHN  …  MKLFINDYNIENN  …  AVVFWGVSDDVTWLS-
V    AIIGHCLIWHS  …  AELYYNDYSMAQP  …  RVTLWGVADQNSWRND
W    TVHGHCLVWHS  …  AELYYNDYSMSIP  …  RVTVWGVDDGSSWLNG
X    CFNFHVLLWGA  …  TRLMINDYGILSS  …  ----------------
Y    CFNFHVLLWGA  …  TRLMINDYGILSS  …  PAMVWLREFMEAYRES
Z    ELYGHTLVWHS  …  AKLCINDYNVEGI  …  GVTVWGITDKYSWVP-
AA   IVHGHTLVWHN  …  AILIYNDYNIEEI  …  AIQFWGFTDKYSWVPG
AB   QMRGHTLIWHN  …  ALLFYNDYNIEDL  …  -FVMWGFTDKYTWIPG
AC   TVHGHALVWHP  …  AELYYNDFNTEEN  …  GITVWGIADPDSWLYT
AD   LLRGHNICWDS  …  MKLCINDYNIETV  …  GVSIWQFADPTSWIPG
AE   KMRGHVLVWHN  …  VALFYNDYNVFLP  …  CVTVFGICDDYPLYKN
AF   AMRGHTLVWHN  …  VSLFYNDYETAQP  …  SVTFWNLLDENSWLSG
AG   AVRGHTLVWHS  …  CKLYYNDYNEYWD  …  AVCVWGPNDANTWLGS
AH   KMRGHTLVWHS  …  VKLFYNDYNTY--  …  CITWWGPSDAETWIRN
AI   RARGHCVFWST  …  AALFVNDYNVECG  …  -IVFWGIMQGKMWRKD
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Fig. 1. Amino acid sequence alignment of family 10 xylanases. Only the corresponding regions of the equivalents of W85, Y172, W266 and W274 of *S.lividans* XlnA are shown. The four highly conserved aromatic residues are highlighted in bold. Sequences are identified as follows with the NCBI accession numbers in brackets: A, *Streptomyces lividans* XlnA [322292]; B, *Penicillum chrysogenum* XylP [731178]; C, *Aspergillus niger* XynA [83659]; D, *Fusarium oxysporum* GunF [1170139]; E, *Actinomadura* sp.FC7 Xyl1 [478982]; F, *Streptomyces thermoviolaceus* Xyn1 [2055278]; G, *Pseudomonas fluorescens cellulosa* XynF [2120656]; H, *Cellvibrio mixtus* XynB [2144184]; I, *Bacillus stearothermophilus* XynA [2126856]; J, *Caldocellum saccharolyticum* XynA [279947]; K, *Anaerocellum thermophilum* Xyn [1208895]; L, *Caldicellulosiruptor* XynA [311189]; M, *Caldocellum saccharolyticum* CelB[121812]; N, *Dictyoglomus thermophilum* XynA [2494332]; O, *Thermotoga maritima* XynA [2120364]; P, *Thermotoga neapolitana* XynA [603892]; Q, *Bacillus stearothermophilus* Xyn [2126857]; R, *Bacillus* sp. C125 XynA [80173]; S, *Clostridium stercorarium* XynB [729101]; T, *Thermoanaerobacterium saccharolyticum* XynA [538957]; U, *Clostridium thermocellum* XynX [586269]; V, *Bacteroides ovatus* XynA [1364168]; W, *Prevotella ruminicola* XynA [806576]; X, *Rhodothermus marinus* Xyn2 [1332581]; Y, *Rhodothermus marinus* Xyn1 [1877399]; Z, *Cellulomonas fimi* Cex [121856]; AA, *Thermotoga neapolitana* XynB [2494335]; AB, *Clostridium thermocellum* XynZ [80546]; AC, *Pseudomonas fluorescens cellulosa* XynA [77715]; AD, *Filobasidium floriforme* Xyn [83855]; AE, *Caldocellum saccharolyticum* ORF4 [139864]; AF, *Butyrivibrio fibrisolvens* XynB [97313]; AG, *Clostridium thermocellum* XynY [1084235]; AH, *Butyrivibrio fibrisolvens* XynA [97316]; AI, *Horde vulgare* XynII [1718238].

genesis was used to replace these aromatic residues in XlnA and the purified mutant proteins were characterized.

Materials and methods

Site-directed mutagenesis

Site-directed mutagenesis of *xln*A was performed according to the method of Kunkel (1985) on phagemid pIAF217 or pAM19.1 (Moreau *et al.*, 1994). The following oligonucleotides were used for mutagenesis: W85A, 5′-CTG GGA GTG GGC GGC CAG GGT-3′; W85F, 5′-CTG GGA GTG GAA GGC CAG GGT-3′; W85H, 5′-CTG CTG GGA GTG GTG GGC CAG GGT GTG-3′; Y172A, 5′-CTC GAC GTT GGC GTC GTT GTA-3′; Y172F, 5′-CTC GAC GTT GAA GTC GTT GTA-3′; Y172S, 5′-CTC GAC GTT GGA GTC GTT GTA-3′; W266A, 5′-GCG CAC ACC GGC GAC GGT GAT-3′; W266F, 5′-GCG CAC ACC GAA GAC GGT GAT-3′; W266H, 5′-GCG CAC ACC GTG GAC GGT GAT-3′; W274A, 5′-CTC CGA CCG GGC GGA GTC GCT-3′; W274F, 5′-CTC CGA CCG GAA GGA GTC GCT-3′; W274H 5′-CTC CGA CCG GTG GGA GTC GCT-3′ [underlining indicates the substituted nucleotide(s)]. Screening and subcloning of the mutated genes from pIAF217 or pAM19.1 into plasmids pIAF18 and pIAF18.1, respectively, were done as described previously (Moreau *et al.*, 1994; Roberge *et al.*, 1997a).

Enzyme production and purification

XlnA was obtained as described previously (Bertrand *et al.*, 1989). Proteins in the supernatant of *S.lividans* culture were first concentrated by ultrafiltration with a 3 kDa cut-off membrane (Omega). The concentrated proteins were then precipitated with ammonium sulfate at 65% saturation and, after centrifugation, the precipitate was dissolved in 50 mM sodium citrate buffer, pH 6.0. Samples of 100 mg of protein were loaded on a Phenyl-Sepharose column (Pharmacia) in 50 mM sodium citrate buffer, pH 5.6, containing 1 M ammonium sulfate. Proteins were eluted with a decreasing linear gradient to 0 M ammonium sulfate, followed by an increasing linear gradient to 50% ethylene glycol. Protein concentration was measured by UV absorption at 280 nm. The fractions containing XlnA were pooled, dialysed against Milli-Q water and freeze-dried. Further purification to apparent homogeneity as indicated by SDS–PAGE analysis (>95% by Coomassie Brilliant Blue staining), was achieved by separation on a Superdex HR75 beaded column (3×60 cm) (Pharmacia) with 100 mM sodium citrate, pH 6.0, as the eluent. The purified XlnA-containing fractions were pooled, dialyzed and freeze-dried.

Enzymatic activity determinations

The specific activity was determined by incubating the enzymes with 4.5 mg/ml birchwood xylan (Sigma) in 50 mM sodium citrate buffer, pH 6.0, at 60°C for 10 min. The released reducing sugars were determined by the *p*-hydroxybenzoic acid hydrazide method (Lever *et al.*, 1984) adapted for microtiter plates. For the determination of Michaelis–Menten constants, the initial velocities of the enzymes were measured at 60°C in 50 mM sodium citrate buffer, pH 6.0, with birchwood xylan concentrations increasing from 0.045 to 4.5 mg/ml. The kinetic parameters were calculated with GraFit software version 3.09b. All enzymatic activities were expressed in international units (IU), where 1 IU represents the amount of enzyme releasing 1 μmol/min of reducing sugars using xylose as standard.

Enzyme stability

For the determination of the stability, solutions of 500 μg/ml of enzyme were incubated at 60°C. Samples were taken at regular intervals and kept on ice for 30 min prior to determination of residual activity. The half-life was obtained by plotting the natural logarithm of the residual activity as a function of incubation time. Stability was also determined in the presence of substrate by incubating the enzyme with 7.2 mg/ml birchwood xylan in 50 mM sodium citrate buffer, pH 6.0. At regular intervals, aliquots were withdrawn and analyzed for their reducing sugar contents. The XlnA half-lives were determined by non-linear analysis of the released reducing sugars versus time plots.

Circular dichroism (CD)

Solutions of 500 μg/ml xylanase in 10 mM sodium phosphate buffer, pH 6.0, were analyzed, at room temperature, using a

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0.05 cm jacketed cell on a Jasco J-710 spectropolarimeter interfaced with an IBM computer. Data were averaged from 10 acquisitions between 250 and 190 nm at a scan rate of 100 nm/min. Thermal denaturation of XlnA was done by heating 500 μg/ml samples in a 0.05 cm jacketed cell at a rate of 0.5°C/min from 50 to 85°C using a Neslab 110 water-bath. Changes in XlnA structure due to unfolding were followed at 210 nm at every 0.2°C. The values were then transformed into fraction of unfolded protein according to the following equation, assuming a two-state unfolding mechanism:

$$f_D = (X - X_n)/(X_d - X_n)$$

where X_n is the value for folded protein, X_d the value for unfolded protein and X the value observed (Pace *et al.*, 1989).

HPLC analysis of hydrolysis products

Wild-type XlnA (14.8 nM) or the mutant proteins (equivalent amount of enzymatic units) were incubated with birchwood xylan (4.5 mg/ml) or xylopentaose (4.5 mM) in 50 mM sodium citrate buffer, pH 6.0, at 60 or 45°C, respectively. Because of their low thermal stability, the hydrolysis of xylan by mutants W266A and W266F was performed at 45°C along with the wild-type enzyme for comparison purposes. At time intervals, aliquots were withdrawn, boiled for 5 min and diluted 5- or 41-fold in water for xylan or xylopentaose hydrolysis, respectively, prior to HPLC analysis. Samples of 100 μl were injected on to a Dionex CarboPac PA1 (4×250 mm) anion-exchange column installed on a Dionex DX-500 HPLC system equipped with a pulsed-electrochemical detector interfaced to an IBM computer and a Thermo Separation Products AS3500 autosampler with 150 mM NaOH (1 ml/min) as the eluent. The oligosaccharides were separated by applying a linear gradient to 500 mM sodium acetate in 150 mM NaOH. The identification and concentration determination of the oligoxylosides produced were achieved by comparison with different concentrations of purified standards (X_1 to X_9) analyzed under the same conditions.

Results

To study the role of W85, Y172, W266 and W274 in XlnA, three mutant proteins were generated at each position: W85A/F/H, Y172A/F/S, W266A/F/H and W274A/F/H. Far-UV CD was first used to determine whether any of these mutations would affect the structure of the enzyme. None of the mutations significantly modified the CD spectrum of the enzyme, indicating that the secondary structure content remained the same after mutation (data not shown). Therefore, loss of function and differences with the wild-type protein characteristics described thereafter may only be due to minor local structural changes undetectable by far-UV CD.

To evaluate the consequences of the mutations on the enzyme, the specific activity against xylan was measured. Only mutation Y172F did not alter the activity and all the others decreased it by more than 74% (Table I). Particularly, the three mutations at position W266 (W266A/F/H) dramatically reduced the specific activity by 99.7, 85.7 and 97.5%, respectively, suggesting that this residue is important for the catalytic properties of the protein (Table I). Most of these mutant proteins retained enough activity for accurate evaluation of their kinetic parameters. The catalytic constant (k_{cat}) of the enzyme was reduced by values similar to the specific activity (Table I). Under the conditions used, the specific activity is a measure of the apparent k_{cat} since a saturating concentration of substrate was used. However, the k_{cat} value provides a more accurate measure of the catalytic efficiency of XlnA, because enzyme stability cannot influence this parameter. As expected, the binding affinity of XlnA for xylan was reduced by most of the mutations, confirming that these residues play a role in substrate binding. Only mutation W85F did not alter this parameter. Interestingly, replacement of the three tryptophan and the tyrosine residues by a phenylalanine is less detrimental to the affinity of XlnA for xylan (lower K_M values) than other mutations (Table I), suggesting that the aromatic nature of these residues is important. The results show that mutation of these aromatic residues caused a decrease of 61.0–99.3% in the specificity of XlnA for xylan, which is reflected by losses in binding energy [$\Delta(\Delta G)$] (Wilkinson *et al.*, 1983) ranging from 2.61 to 13.8 kJ/mol (Table I).

Table I. Specific activity and kinetic parameters of the mutant xylanases determined with birchwood xylan as substrate

Enzyme	Specific activity (IU/mg)	k_{cat} (s^{-1})	K_M (mg/ml)	k_{cat}/K_M (s^{-1}.ml/mg)	$\Delta(\Delta G)$[a] (kJ/mol)
Wild-type	174	134	0.10	1327	–
W85A	29.4	32.3	0.16	202	5.2
W85F	43.9	50.1	0.10	511	2.6
W85H	31.1	34.1	0.30	114	6.8
Y172A	45.2	16.7	0.51	41.8	9.6
Y172F	180	124	0.24	517	2.6
Y172S	24.8	20.2	0.80	25.2	11.0
W266A	0.6	nd[b]	nd	nd	nd
W266F	24.9	46.0	2.12	21.7	11.4
W266H	4.4	nd	nd	nd	nd
W274A	10.2	8.3	0.91	9.12	13.8
W274F	21.6	22.4	0.18	124	6.6
W274H	21.2	21.2	0.54	39.3	9.8

[a] $\Delta(\Delta G) = -RT[(k_{cat}/K_M)_{mutant}/(k_{cat}/K_M)_{wild\text{-}type}]$.
[b] nd, parameter not determined.

The role of these aromatic residues for the thermal stability of XlnA was investigated by activity measurements and CD. The transition temperatures (T_m) of wild-type and mutant XlnA were determined from the thermal unfolding curves obtained from CD measurements at 210 nm, the wavelength where the difference in ellipticity between the native and unfolded states of XlnA is maximal. Under these conditions, this experiment provides a way to quantify the effects of the point mutations on the stability of the secondary structures present in XlnA. The three mutations introduced at each position have different effects on the thermal stability of XlnA. Mutations W85H, Y172F and W274F/H did not significantly modify the T_m of XlnA, while mutations W85A, Y172S and W274A increased it by 2.0, 1.6 and 4.1°C, respectively. All the other mutations decreased the T_m by values ranging from 3.3 to 7.3°C (Table II). These variations in T_m translate into differences in free energy of unfolding ranging from a stabilization of 9.5 kJ/mol to a destabilization of 16.7 kJ/mol. However, if one generalizes these results, mutations at positions W85, Y172 and particularly W266 tend to decrease the thermal stability of XlnA, whereas mutations at position W274 have no effect or slightly increase the T_m of the protein (Table II).

The thermal stability of mutated proteins was also evaluated by measuring the half-life of the xylanolytic activity at 60°C in absence or in presence of xylan. Since the activity of the enzyme is measured, these assays provide a way to evaluate the effect of the mutation on the thermal stability of the active site loops of XlnA. Therefore, these results yield additional

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Table II. Stability parameters of the mutant xylanases

Enzyme	T_m (°C)	$\Delta(\Delta G)^a$ (kJ/mol)	Half-life at 60°C (min)	
			Without substrate	With substrate
Wild-type	68.0	–	131	318
W85A	70.0	4.5	39	278
W85F	64.0	–9.2	4	229
W85H	67.0	–2.2	22	216
Y172A	63.0	–11.6	4	5
Y172F	67.1	–2.1	21	238
Y172S	69.6	3.6	5	42
W266A	63.3	–10.8	12	166
W266F	64.7	–7.6	2	2
W266H	60.7	–16.7	5	122
W274A	72.1	9.5	57	159
W274F	68.8	1.8	27	357
W274H	68.2	0.6	27	236

$^a\Delta(\Delta G) = \Delta H_{Tm\ wt}(T_{m\ wt} - T_{m\ mut})$ (Pace *et al.*, 1989).

information on the role of these amino acids located in the active site of XlnA. In absence of substrate, the half-life of XlnA at 60°C was decreased by all the mutations. Replacement of W266 had the worst effect, reducing the half-life by 91–98.5%, whereas mutations at position W274 had a less pronounced effect than the others decreasing the half-life of the enzyme by 56–79% (Table II). In presence of xylan, no protection against thermal inactivation of XlnA was observed for mutations Y172A and W266F, while the other enzymes were protected to levels ranging from 13 to 112% of the wild-type value (Table II). The degree of protection against thermal inactivation provided by the presence of xylan, in comparison with the wild-type protein, is dependent on the enzyme affinity for xylan, i.e. the half-life in the presence of substrate is proportional to the affinity for xylan, as suggested previously (Roberge *et al.*, 1998). This, however, cannot explain the subtle variations observed for some mutations such as Y172A. In that particular case, one would expect Y172A to be more stable than Y172S since its K_M is lower, but this is not the case (Table II). This deviation from the general rule could be explained by the important decrease in stability of the structure of that mutant as measured by CD, probably caused by local structural perturbations (Table II). Nevertheless, these and previous results (Roberge *et al.*, 1998) support the fact that stabilization provided by the presence of xylan is dependent on the affinity of the enzyme for its substrate.

In order to verify whether the mode of action of XlnA was affected by any of the mutations studied, the hydrolysis products of birchwood xylan and X_5 were analyzed by HPLC. Figure 2 shows the results obtained with wild-type XlnA. In our HPLC analysis, oligoxylosides with a degree of polymerization <10 were well resolved and linearly produced throughout the experiment. The major oligoxylosides produced in the hydrolysis of birchwood xylan by XlnA are X_3 and X_4 (Figure 2A). No xylose is seen after 30 min of hydrolysis, while XlnA also produced many other oligoxylosides such as X_2, X_5–X_8 and other oligoxylosides with degrees of polymerization up to 20 (Figure 2A). From the HPLC analysis at different intervals of incubation, a time course of oligoxylosides production was constructed and the results for wild-type XlnA are shown in Figure 2C. Oligoxylosides with a degree of polymerization <9 were analyzed and their rates of production decreased in the order $X_3 > X_4 > X_5 > X_2 = X_6 > X_7 > X_8$. Using the same method, hydrolysis of xylan by the different mutant proteins was also analyzed. No major differences from the wild-type profile were observed (data not shown). However, X_2 was produced by all the mutant proteins at a rate 20–60% slower than that of the wild-type XlnA.

Hydrolysis of X_5 mainly produces X_3 but also significant amounts of X_2 and X_4 and only very little X_1 is observed after 30 min of hydrolysis (Figure 2B). Incubation of XlnA with a saturating amount of X_5 also gives rise to transglycosylation products with degrees of polymerization up to 9 (Figure 2D, inset). In the hydrolysis of X_5, the increase in the concentrations of released oligoxylosides was not linear over time during the 30 min period used in our experiment (Figure 2D) and it was impossible to calculate the rate of production for each oligoxyloside as in the case with the hydrolysis of xylan. The amount of the transglycosylation products X_7–X_9 increased in the first 5 min and was stable for the remainder of the 30 min period, while the concentration of X_6 increased with time during the whole period (Figure 2D). Since X_1 is not readily produced from the hydrolysis of X_5, the transglycosylation product X_6 was not observed at first, but was probably generated by the assembly of two X_3 or one X_2 and one X_4 in the course of the reaction. Neither the hydrolysis nor the transglycosylation products of the reaction with X_5 were significantly altered by any of the mutations studied, indicating that the mode of action of XlnA was not affected (data not shown). However, when X_5 was hydrolyzed with the same quantity of enzymatic units, mutations Y172A, Y172S, W266F W274A and W274H caused a reduction in hydrolysis efficiency by 88, 72, 91, 73 and 51%, respectively (data not shown).

Discussion

S.lividans XlnA is a well characterized member of family 10 glycosyl hydrolases for which the three-dimensional structure of the catalytic domain is known (Derewenda *et al.*, 1994). There are over 30 enzymes in this family, four of which have known three-dimensional structures (Henrissat and Bairoch, 1998). Thus, primary and tertiary structure comparisons are very useful tools in the identification of potentially important residues. These comparisons have shown the presence of conserved aromatic residues in the active site of this family that could be important in substrate binding. Site-directed mutagenesis was used to replace specifically four of these aromatic amino acids shown to be located in subsites –1 and +1 of the active site of family 10 xylanases (Harris *et al.*, 1994). While mutation of either of these four active residues did not alter the hydrolysis pattern of the enzyme with either xylan or xylopentaose as substrates, significant variations in kinetic and stability parameters from the wild-type were observed, allowing a better understanding of the roles of these four aromatic residues in the structure–function relationships of family 10 xylanases.

Role of tryptophan 85

In the structure of XlnA, W85 (Nε1) is hydrogen-bonded to E128 (Oε1), the acid–base catalyst and to N127 (Oδ1) (Figure 3). This pattern was also observed in the structures of other family 10 xylanases, which suggested its importance for catalysis by the enzyme (Dominguez *et al.*, 1995; Harris *et al.*, 1996; White *et al.*, 1996). From the kinetic results, the 70% average loss in activity due to the three mutations tested (Table I) could be attributed to secondary effects involving one or both of these residues, which have been shown to be very

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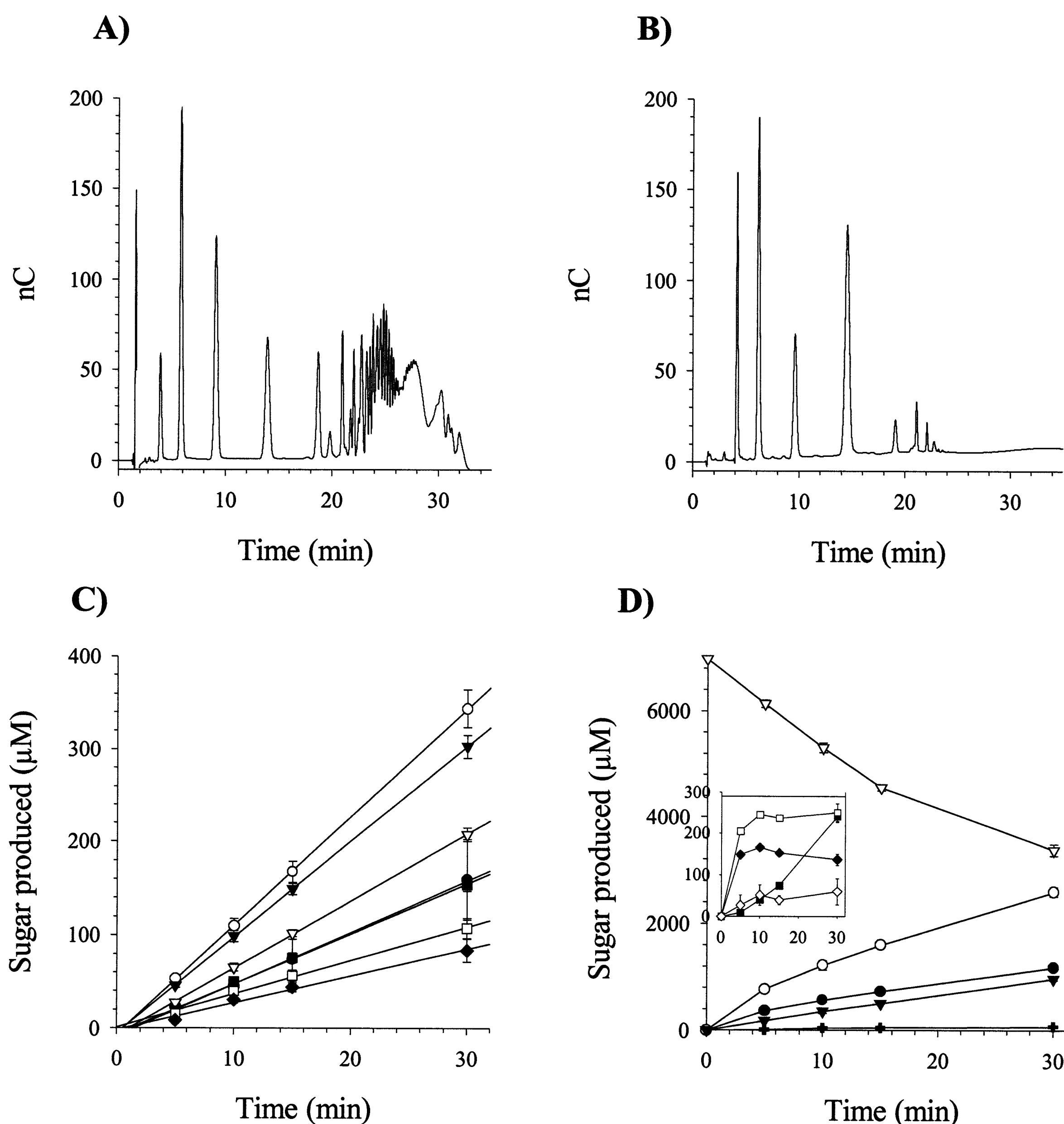

Fig. 2. HPLC analysis and time course of the products obtained from the hydrolysis of, respectively, birchwood xylan (A and C) and xylopentaose (B and D) by wild-type XlnA. The oligoxylosides are identified as follows with their retention times in parentheses: X_1, + (2.9 min); X_2, ○ (4.2 min); X_3, ● (6.0 min); X_4, ▼ (9.3 min); X_5, ▽ (14.2 min); X_6, ■ (18.9 min); X_7, □ (21.1 min); X_8, ◆ (22.2 min); X_9, ◇ (22.9 min).

important in the catalytic efficiency of XlnA (Moreau *et al.*, 1994; Roberge *et al.*, 1997b). Moreover, the aromatic nature of W85 appears to play a role in substrate binding, as expected. Increases in K_M were obtained by replacing W85 by non-aromatic residues (A and H), while the mutant enzyme W85F maintained its affinity for xylan (Table I). This suggests that W85 is involved in stacking interactions with the substrate. Furthermore, this residue is important for the thermal stability of the protein, as shown by the decrease in mutant enzyme half-lives at 60°C in the absence of substrate (Table II). In that case, the aromatic nature of W85 is probably not the only factor involved since the phenylalanine substitution seems to have the worst effect on that parameter. These results suggest that hydrogen bonding involving W85 is important for the stability of the protein.

Role of tyrosine 172

The hydroxyl group of Y172 is involved in hydrogen-bonding interactions with D132 (Oδ1), R139 (Nη1) and an active-site water molecule (Figure 3). However, these interactions do not seem to be important for enzyme activity since Y172F mutant protein, without hydrogen-bonding possibility, is as active as the wild-type protein (Table I). The aromatic nature of this residue is important in substrate binding, since its replacement by non-aromatic residues decreases the affinity for xylan (Table II) and the apparent rate of hydrolysis of xylopentaose, while the Y172F mutation behaves similarly to the wild-type. Our results also show that Y172 is important for enzyme stability, particularly in the absence of substrate at 60°C (Table II). Again, the Y172F replacement is less detrimental to enzyme stability in the presence of substrate, strengthening the hypo-

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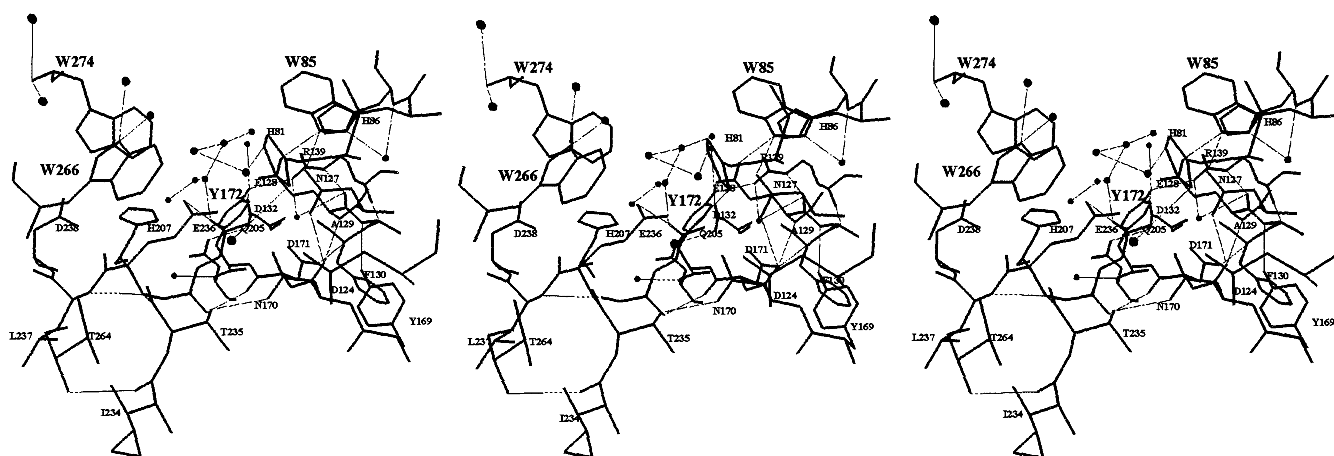

Fig. 3. Environment of the two catalytic residues in *S.lividans* XlnA showing the positions of the aromatic amino acids. Hydrogen bonds are represented by dashed lines and water molecules by filled spheres.

thesis that the protection gained against thermal inactivation is highly dependent on substrate affinity (Roberge *et al.*, 1998). However, results for the T_m of the proteins suggest that hydrogen-bonding interactions are not important for the structural stability of XlnA, since similar T_m values were obtained for both the Y172F mutant and wild-type proteins (Table II). In this case, it is difficult to draw conclusions on the role of the aromatic nature of Y172 without knowing the structure of the mutant protein, since the Y172S mutation did not decrease the T_m of XlnA while significantly reducing the half-lives with and without substrate (Table II).

Role of tryptophan 266

Amino acid W266 is also highly conserved in family 10 xylanases. Its Nε1 atom is hydrogen-bonded to two active-site water molecules. However, W266 is also stacked to E236, the residue acting as a nucleophile in the hydrolysis of substrate in XlnA (Figure 3). This interaction was proposed to be of importance in other families of glycosyl hydrolases using the same mechanism of action (Dominguez *et al.*, 1995). Our results confirm that these structural features are indeed important for the function of this type of enzyme and comparison of the specific activity of the different mutant proteins at this position suggests that the aromatic nature of W266 is involved in the activity of the protein.

The equivalents of residue W266 were shown to be involved in substrate binding in two other family 10 xylanase structures when complexed with substrates (Harris *et al.*, 1996; White *et al.*, 1996). Our kinetic analysis of W266F mutant protein clearly shows that this amino acid is involved in substrate binding and supports the previously published structural analysis. While mutant W266F retains a certain activity level, it is still 66% less active than the wild-type protein (Table I), indicating that W266 probably serves to position the nucleophile in the active site for efficient binding and catalysis. The large variation in affinity for xylan and the important reduction in activity towards xylopentaose exhibited by mutant W266F probably reflect a bad positioning of E236. This would weaken one of the major stabilizing interactions between Oε2 of E236 and OH-2 of the substrate at the transition state (White *et al.*, 1996). Moreover, the modest effect on the k_{cat} of the enzyme caused by the W266F mutation suggests that the hydrogen bond observed between Nε1 of the W266 equivalent and the catalytic intermediate analog in *C.fimi* Cex (White *et al.*, 1996; Notenboom *et al.*, 1998), which is missing in W266F, plays a minor role in the stabilization of the intermediate. Finally, W266 is very important in the thermal stability of XlnA, as shown by the large decreases in half-lives at 60°C and in T_m exhibited by all the mutant proteins studied (Table II). Again this supports the concept that the residues forming the complex hydrogen bonding network in the active site of the enzyme are involved in interactions crucial for the integrity of the structure of family 10 xylanases.

Role of tryptophan 274

In contrast to the three other amino acids studied here, W274 is not involved in hydrogen bonding interactions with other residues (Figure 3). In fact, W274 (N) is only hydrogen bonded to two active-site water molecules. Our kinetic analysis of the three mutant proteins at position W274 showed that this residue is involved in substrate binding (Table I). This supports the observations that the equivalent of W274 in the binding of xylopentaose at subsite –1 in XynA from *P. fluorescens* subsp. *cellulosa* has a high *B* value in the refined structure of this enzyme (Harris *et al.*, 1994). The authors suggested that this residue could be important for the binding of large xylan substrates (Harris *et al.*, 1994, 1996). Our results support this hypothesis, although the significant decrease in activity towards xylopentaose caused by mutations W274A and W274H, but not by W274F, indicates that the aromatic nature of this residue is also important for efficient catalysis of smaller oligoxylosides. Comparing the hydrolysis patterns of both xylan and xylopentaose by the three mutants showed that the aromatic nature of this residue plays a large role in substrate binding, suggesting that stacking interactions between W274 and the substrate are involved. From the decreases in k_{cat} obtained (Table I), W274 also seems to play a role in the stabilization of the catalytic intermediate. This result was expected after analysis of the complexed structures of other family 10 xylanases that suggested W274 to be important for the specificity between glucosyl and xylosyl substrates (White *et al.*, 1996; Notenboom *et al.*, 1998). Finally, our results on the T_m values have shown that W274 is not crucial to the stability of the XlnA structure. Still, replacement of this active site residue reduced the half-life in absence of substrate by up to 79% (Table II), suggesting that the active site environment is affected by these mutations.

In conclusion, this study on four conserved aromatic residues

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in family 10 xylanases using site-directed mutagenesis has demonstrated their role in substrate binding and catalysis by this type of enzyme. As expected for sugar-binding proteins, the aromatic nature of W85, Y172 and W274 is particularly important in substrate binding. W266 is also involved in the catalytic efficiency of the protein and the four residues were shown to be important for the thermal stability of XlnA. This kinetic evidence supports the structural observations that were made on other family 10 xylanases and thus improves our knowledge of the structure–function relationship of this type of protein.

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