Acta Crystallographica Section D
Biological Crystallography
ISSN 0907-4449

Oligosaccharide binding to family 11 xylanases: both covalent intermediate and mutant product complexes display $^{2,5}B$ conformations at the active centre

Elisabetta Sabini,[a]† Keith S. Wilson,[a] Steffen Danielsen,[b] Martin Schülein[b] and Gideon J. Davies[a]*

[a]Department of Chemistry, Structural Biology Laboratory, University of York, Heslington, York YO10 5DD, England, and [b]Novozymes A/S, Smoermosevej 25, DK 2880 Bagsvaerd, Denmark

† Current address: The Scripps Research Institute, MB-31, 10550 North Torrey Pines Road, La Jolla, California 92037, USA.

Correspondence e-mail: davies@ysbl.york.ac.uk

Received 4 April 2001
Accepted 28 June 2001

PDB References: E94A mutant of Xyn11 with xylotriose, 1h4h; 2-fluoro-2-deoxy-xylkobiosyl–enzyme intermediate, 1h4g.

The glycoside hydrolase sequence-based classification reveals two families of enzymes which hydrolyse the β-1,4-linked backbone of xylan, xylanases, termed families GH-10 and GH-11. Family GH-11 xylanases are intriguing in that catalysis is performed *via* a covalent intermediate adopting an unusual $^{2,5}B$ (boat) conformation, a conformation which also fulfils the stereochemical constraints of the oxocarbenium ion-like transition state. Here, the 1.9 Å structure of a nucleophile, E94A, mutant of the Xyn11 from *Bacillus agaradhaerens* in complex with xylotriose is presented. Intriguingly, this complex also adopts the $^{2,5}B$ conformation in the −1 subsite, with the vacant space provided by the Glu→Ala mutation allowing the sugar to adopt the α-configuration at C1. The structure of the covalent 2-deoxy-2-fluoroxylobiosyl-enzyme intermediate has been extended to atomic (1.1 Å) resolution.

1. Introduction

Xylanases hydrolyse the β-1,4-linked D-xylose backbone of xylan, a major component of the plant cell wall. They fall into two families in the glycoside hydrolase sequence-based classification, termed families GH-10 and GH-11 (see http://afmb.cnrs-mrs.fr/~pedro/CAZY/db.html; Coutinho & Henrissat, 1999). Three-dimensional structures of members of both families have now been solved. The two families display different three-dimensional structures. Family GH-10 enzymes are $(\beta/\alpha)_8$ barrels with the two essential catalytic residues displayed on strands β-4 and β-7 (see, for example, White *et al.*, 1994; Ducros *et al.*, 2000), whereas the family GH-11 enzymes are β-jelly-roll enzymes in which the long substrate-binding groove is formed by the concave face of one of the β-sheets (see, for example, Campbell *et al.*, 1993; Sabini *et al.*, 1999).

Both family GH-10 and GH-11 enzymes perform catalysis with net retention of the anomeric configuration. Such a mechanism involves the formation and subsequent breakdown of a covalent glycosyl-enzyme intermediate *via* oxocarbenium ion-like transition states (Koshland, 1953; reviewed in Davies, Sinnott *et al.*, 1997). Family GH-11 enzymes are of particular interest as the covalent glycosyl-enzyme intermediate adopts a $^{2,5}B$ (boat) conformation, in contrast to the $^4C_1$ (chair) conformation observed for other β-glycosidases. Whilst the secondary isotope effects measured on some systems led to the proposal of such a conformation for the transition state (Hosie & Sinnott, 1985), it was the structure determinations of the *B. circulans* xylanase 2-deoxy-2-fluoroxylobiosyl-enzyme intermediate (Sidhu *et al.*, 1999) and a similar *B. agaradhaerens* Xyn11 complex (Sabini *et al.*, 1999) that first revealed a $^{2,5}B$ conformation for a covalent intermediate species. Here, we present the structure of the $^{2,5}B$ intermediate at atomic resolution, together with the structure of the nucleophile E94A mutation in complex with xylotriose, which also displays this unusual conformation, the vacant space provided by the Glu→Ala mutation allowing the sugar to adopt the α-configuration at C1.

2. Methods

2.1. Protein production

Restriction endonucleases and T4 DNA ligase were from New England Biolabs Inc., MA, USA. Pwo polymerase was from Boehringer Mannheim. The Xyn11 variant E94A was produced using the megapriming method (Sarkar & Sommer, 1990) using the following primers: first-round PCR (megaprimer production), 5′-GGCTTTACGCCCGATTG-CTG and 5′-ATCGACAATATAATA**AGC**-GACAAGAGGGTCAAC (mutation in bold). Second-round PCR megaprimer and 5′-CTGAACCGAAAGAGATAAGGATT PCR was performed using Pwo polymerase and pPL2630, a *B. subtilis* plasmid carrying the wild-type Xyn11, as template. The amplified second-round PCR product containing the mutated Xyn11 gene (760 base pairs) was restricted with *Pst*I and *Bst*EII, purified and ligated into *Pst*I and *Bst*EII restricted pPL2630. Competent *B. subtilis* SHA273 was transformed with the ligated DNA and spread

on LB plates containing kanamycin (10 mg ml^{-1}). Plasmid DNA from transformants was isolated and sequenced to verify that the mutation had been incorporated. Production of Xyn11–E94A was performed by fermenting the recombinant strain for 5 d at 303 K, 250 rev min^{-1} in 1 l PS-1 media. Protein was purified essentially as described previously (Sabini *et al.*, 1999).

2.2. Crystallization

Crystals of wild-type Xyn11 were grown using the hanging-drop vapour-phase diffusion method. Initial drops consisted of 2 µl protein solution (10 mg ml^{-1} in 100 m*M* sodium acetate buffer at pH 6.0) and 1.0 µl reservoir solution (100 m*M* MES buffer, 30% ammonium sulfate pH 6.5 and 100 m*M* NaCl). For the E94A mutant the reservoir solution contained 0.8 *M* $K_2HPO_4.3H_2O$/NaH_2PO_4, 10%(*v*/*v*) MPD and 100 m*M* MES buffer pH 6.5, whilst the drops contained 1 µl of protein solution (27 mg ml^{-1} in H_2O) together with 1 µl of this reservoir solution. Mutant crystals appeared after five months at 289 K.

2.3. Complex formation

Crystals of wild-type Xyn11 were soaked in a reservoir solution consisting of mother liquor with the addition of powdered 2′,4′-dinitrophenyl 2-deoxy-2-fluoro-β-D-xylobioside (2FDNP-X2) overnight as described previously (Sabini *et al.*, 1999). Crystals of the E94A mutant were likewise soaked in a reservoir-solution based mother liquor with the addition of xylotetraose to a final concentration of 10 m*M*. MPD to a concentration of 30%(*v*/*v*) in this mother liquor was included as the cryoprotectant for subsequent data collection.

2.4. Data collection

Data for the Xyl11 complex with 2FDNP-X2 were collected at the EMBL Hamburg outstation on beamline BW7B using a MAR345 image-plate detector to a final resolution of 1.1 Å. Data for the E94A–xylotetraose complex were collected at the European Synchrotron Radiation Facility on beamline ID14-4 using an ADSC Quantum-4 CCD detector to a final resolution of 1.9 Å. All data were processed and reduced with the *HKL* suite of programs (Otwinowski & Minor, 1997). All further computation used the *CCP*4 suite unless otherwise stated (Collaborative Computational Project, Number 4, 1994).

2.5. Structure solution and refinement

The native *B. agaradhaerens* Xyn11 structure was used as the starting model. The same cross-validation subset of reflections for the intermediate resolution refinements were maintained and extended to the full resolution limit of the data. Monitoring of the cross-validation reflections suggested that inclusion of riding H atoms and anisotropic modelling (Murshudov *et al.*, 1999) of the thermal displacement parameters was appropriate. Refinement was performed using *REFMAC* (Murshudov *et al.*, 1997). Data for the intermediate extend to a nominal resolution of 1.1 Å resolution. Although the R_{merge} between 1.3 and 1.1 Å was high, the low overall free *R* value (0.18) and small difference between R_{cryst} and R_{free} (0.158 *versus* 0.180 overall; 0.24 *versus* 0.26 in outer resolution shell) suggested that these data were nevertheless being correctly weighted by the maximum likelihood.

For the E94A–oligosaccharide complex, the structure was first solved by molecular replacement using *AMoRe* (Navaza & Saludijan, 1997). A four-molecule solution was found, 5% of the reflections were set aside for cross-validation analysis and the structure was refined with *REFMAC*, initially including tight non-crystallographic symmetry restraints. The 'unbiased' electron-density map revealed density for a xylotriose moiety in the −3 to −1 subsites (nomenclature described by Davies, Wilson *et al.*, 1997). In order to avoid bias in the −1 subsite, xylobiose was modelled in the −3 and −2 subsites only and the refinement was continued. Only when the electron density was unambiguous at the −1 subsite was the xylose ring modelled.

3. Results and discussion

3.1. Covalent 2-fluoro-2-deoxyxylobiosyl-enzyme intermediate

The structure of the covalent intermediate for family 11 xylanases has previously been reported for both the *B. circulans* enzyme at 1.8 Å (Sidhu *et al.*, 1999) and the *B. agaradhaerens* Xyn11 at 2.0 Å resolution (Sabini *et al.*, 1999). Crystals of the *B. agaradhaerens* enzyme belong to space group $P2_12_12_1$, with unit-cell parameters a = 72.1, b = 75.1, c = 78.3 Å and two molecules in the asymmetric unit. Data were collected to 1.1 Å (outer resolution shell, 1.12–1.10 Å; statistics for this shell are given in parentheses); they are 99.9% (99.0%) complete with an R_{merge} of 0.055 (0.71), a mean multiplicity of observation of 4.5 (3.8) and a mean $I/\sigma(I)$ of 18 (2.0). Refinement using data to the nominal resolution of 1.1 Å generated a model with a final R_{cryst} and R_{free} of 0.158 and 0.180, respectively. The model consisted of 1647 protein atoms and 18 ligand atoms per chain and 607 water molecules. It displays deviations from stereochemical target values of 0.016 Å for 1–2 bonds and 0.039 Å for the 1–3 angle distance. The 2-fluoro-2-deoxy-xylobiosyl-enzyme intermediate, at 1.1 Å, provides the structure of a covalent glycosyl-enzyme intermediate with high precision (Fig. 1*a*). In particular, the location of a potentially hydrolytic water molecule 3.4 Å from the anomeric C1 atom and 2.74 Å from the catalytic acid/base Glu184 is seen in the perfect position for 'in-line' nucleophilic attack with an $OW_{wat}-C1_{Xyl}-OE2_{Glu184}$ angle of 168°. This is in contrast to the *B. circulans* trapped intermediate structure (PDB code 1bvv), which has no similarly placed water molecule (Sidhu *et al.*, 1999).

3.2. E94A–xylotetraose complex

Crystals of the E94A mutant in complex with xylotetraose belong to space group $P2_1$, with unit-cell parameters a = 74.3, b = 78.9, c = 76.3 Å, β = 91.9°. Data were collected to 1.9 Å; they are 100.0% complete to 2.2 Å, falling off linearly to 77.7% completeness at 1.9 Å. The R_{merge} is 0.051 (0.157) with a mean multiplicity of observation of 4.6 (1.7) and a mean $I/\sigma(I)$ of 22 (5.0). Refinement of the four-molecule molecular-replacement solution generated a model with R_{cryst} and R_{free} of 0.18 (0.22) and 0.24 (0.32), respectively, which consisted of 6539 protein atoms, 84 ligand atoms and 492 water molecules. Deviations from stereochemical target values are 0.018 Å for 1–2 bonds and 0.043 Å for the 1–3 angle distance.

The density map reveals three β-1,4-linked xylosyl units in the −3 to −1 subsites. The crystals were soaked in xylotetraose and the lack of density for the fourth sugar moiety presumably results from disorder; there is weak density beyond the O-4 atom of the −3 subsite sugar which eventually peters out, but this could not appropriately be modelled as a fourth sugar unit (Fig. 1*b*). The xylose units in the −2 and −3 subsites adopt an undistorted $^4C_1$ chair conformation. The *B* values for all sugar rings are in the range observed for the surrounding protein atoms (around 20–28 Å^2). In the crucial −1 subsite the pyranoside rings display *B* values around 20 Å^2 in all four molecules. The −1 sugar adopts the $^{2,5}B$ (boat) conformation previously observed for the covalent intermediate (Fig. 1*b*). The vacant space available from the Glu→Ala mutation allows the sugar to bind as its

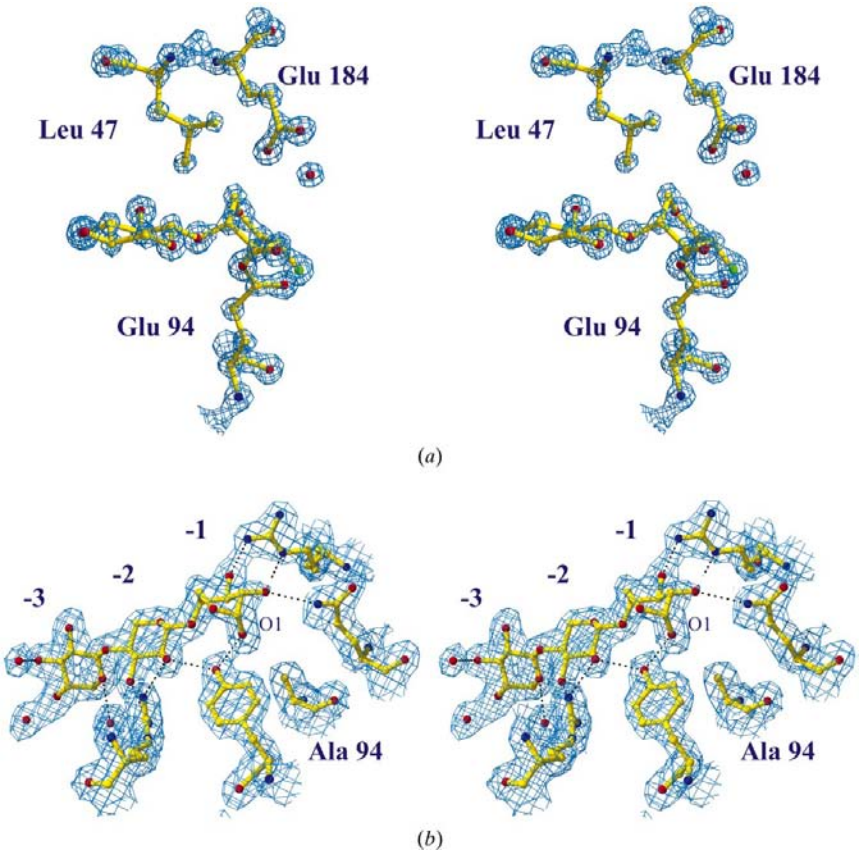

Figure 1
(*a*) Electron density for the covalent 2-fluoro-2-deoxyxylobiosyl-enzyme intermediate at 1.1 Å resolution in divergent (wall-eyed) stereo. The density map is a maximum-likelihood-weighted $2mF_{obs} - F_{calc}$ synthesis contoured at 1.6 e Å^{-3} (approximately 4σ). (*b*) Observed electron density for the E94A–xylotriose complex. The map shown is a maximum-likelihood weighted $2mF_{obs} - F_{calc}$ synthesis contoured at 0.3 e Å^{-3} (approximately 1σ). This figure was drawn with *BOBSCRIPT* (Esnouf, 1997). Atoms are colour coded (yellow, carbon; red, oxygen; blue, nitrogen; green, fluorine).

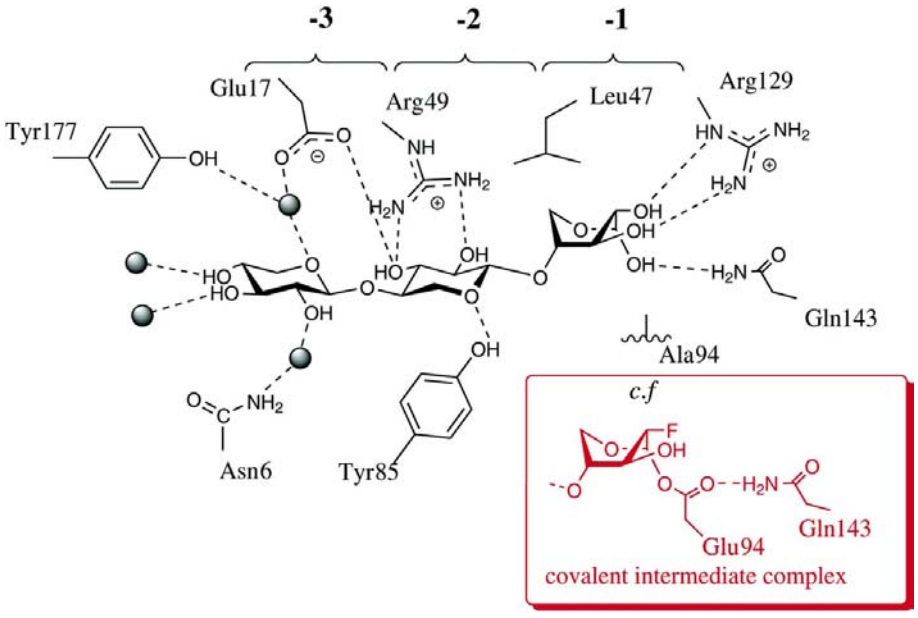

Figure 2
Interactions of the Xyn11 E94A mutant with xylotriose. The −1 subsite of the covalent 2-fluoro-2-deoxyxylobiosyl-enzyme intermediate (Sabini *et al.*, 1999) is included for reference.

α-anomer. Nucleophile mutant product complexes of β-glycoside hydrolases have previously been observed to bind the α-anomer should enough space be available (see, for example, Hadfield *et al.*, 1994; both the E197A and E197S mutants of the *Humicola insolens* Cel7B also bind the α-anomer preferentially, unpublished observations); indeed, such binding has been harnessed for synthesis using activated α-F-glycosides using the 'glycosynthase' technology (Fort *et al.*, 2000; Mackenzie *et al.*, 1998). The interactions of the E94A mutant complex are extremely similar to that described for the covalent intermediate complex, with the addition that the interactions in the −3 subsite of the enzyme can now be assigned (Fig. 2). Indeed, the −3 subsite sugar makes no direct links to the protein and is associated only by virtue of three solvent-mediated hydrogen bonds with Asn6, Glu17 and Tyr177.

3.3. Discussion

Previous medium-resolution analyses of covalent intermediate structures for family 11 enzymes had revealed an unusual $^{2,5}B$ conformation for the glycosyl-enzyme intermediate (Sabini *et al.*, 1999; Sidhu *et al.*, 1999), a conformation not observed in the equivalent trapped covalent-enzyme intermediate structures from other glycoside hydrolase families. This conformation is important because it obeys the stereochemical constraints of the oxocarbenium ion-like transition-states (C-5, O-1, C-1 and C-2 coplanar), suggesting that the transition state may also display such a conformation. Catalysis would then presumably proceed along a $^{4}C_1 \rightarrow {}^{2}H_3 \rightarrow {}^{2}S_0 \rightarrow {}^{2,5}B$ itinerary. This $^{2,5}B$ conformation also discriminates against binding of a glucopyranoside owing to 'bowsprit' interactions between the C-5 hydroxymethyl substituent and H-2 and may therefore be partially responsible for the specificity of family GH-11 xylanases for xylosyl units, in contrast to the more promiscuous behaviour of family 10 xylanases, which utilize a $^{4}C_1$ chair conformation for the intermediate (Andrews *et al.*, 2000; Ducros *et al.*, 2000). Furthermore, the cellulases from family GH-12, which are structurally closely related to the family GH-11 xylanases described here, utilize the $^{4}C_1$ chair intermediate which presumably reflects the greater energetic penalties for conformational change in gluco- as opposed to xylo-pyranosides (Sulzenbacher *et al.*, 1999). The observation that xylotriose in complex with a nucleophile mutant of Xyn11 also adopts the $^{2,5}B$ conformation, without the constraint of a covalent link to the

protein, gives further credence to arguments that this conformation is favoured by the enzyme and thus may be adopted at the oxocarbenium ion-like transition state.

This work was supported by the BBSRC, the European Union, Novozymes A/S and the University of York. GJD is a Royal Society University Research Fellow. Both the EMBL and ESRF provided support for data collection.

References

Andrews, S. R., Charnock, S. J., Lakey, J. H., Davies, G. J., Claeyssens, M., Nerinckx, W., Underwood, M., Sinnott, M. L., Warren, R. A. J. & Gilbert, H. J. (2000). *J. Biol. Chem.* **275**, 23027–23033.

Campbell, R. L., Rose, D. R., Wakarchuk, W. W., To, R., Sung, W. & Yaguchi, M. (1993). *Proceedings of the second Tricel symposium on Trichoderma reesei cellulases and other hydrolases*, edited by P. Suominen & T. Reinikainen, Vol. 8, pp. 63–72. Espoo: Foundation for Biotechnical and Industrial Fermentation Research.

Collaborative Computational Project, Number 4 (1994). *Acta Cryst.* D**50**, 760–763.

Coutinho, P. M. & Henrissat, B. (1999). *Recent Advances in Carbohydrate Engineering*, edited by H. J. Gilbert, G. J. Davies, B. Svensson & B. Henrissat, pp. 3–12. Cambridge: Royal Society of Chemistry.

Davies, G., Sinnott, M. L. & Withers, S. G. (1997). *Comprehensive Biological Catalysis*, edited by M. L. Sinnott, Vol. 1, pp. 119–209. London: Academic Press.

Davies, G. J., Wilson, K. S. & Henrissat, B. (1997). *Biochem. J.* **321**, 557–559.

Ducros, V., Charnock, S. J., Derewenda, U., Derewenda, Z., Dauter, Z., Dupont, C., Shareck, F., Morosoli, R., Kluepfel, D. & Davies, G. J. (2000). *J. Biol. Chem.* **275**, 23030–23036.

Esnouf, R. M. (1997). *J. Mol. Graph.* **15**, 133–138.

Fort, S., Boyer, V., Greffe, L., Cottaz, S., Moraz, O., Davies, G. J., Christiansen, L., Schülein, M. & Driguez, H. (2000). *J. Am. Chem. Soc.* **122**, 5429–5437.

Hadfield, A. T., Harvey, D. J., Archer, D. B., MacKenzie, D. A., Jeenes, D. J., Radford, S. E., Lowe, G., Dobson, C. M. & Johnson, L. N. (1994). *J. Mol. Biol.* **243**, 856–872.

Hosie, L. & Sinnott, M. L. (1985). *Biochem J.* **226**, 437–446.

Koshland, D. E. (1953). *Biol. Rev.* **28**, 416–436.

Mackenzie, L. F., Wang, Q., Warren, R. A. J. & Withers, S. G. (1998). *J. Am. Chem. Soc.* **120**, 5583–5584.

Murshudov, G. N., Vagin, A. A. & Dodson, E. J. (1997). *Acta Cryst.* D**53**, 240–255.

Murshudov, G. N., Vagin, A. A., Lebedev, A., Wilson, K. S. & Dodson, E. J. (1999). *Acta Cryst.* D**55**, 247–255.

Navaza, J. & Saludijan, P. (1997). *Methods Enzymol.* **276**, 581–594.

Otwinowski, Z. & Minor, W. (1997). *Methods Enzymol.* **276**, 307–326.

Sabini, E., Sulzenbacher, G., Dauter, M., Dauter, Z., Jørgensen, P. L., Schülein, M., Dupont, C., Davies, G. J. & Wilson, K. S. (1999). *Chem. Biol.* **6**, 483–492.

Sarkar, G. & Sommer, S. S. (1990). *Biotechniques*, **8**, 404–407.

Sidhu, G., Withers, S. G., Nguyen, N. T., McIntosh, L. P., Ziser, L. & Brayer, G. D. (1999). *Biochemistry*, **38**, 5346–5354.

Sulzenbacher, G., Mackenzie, L. F., Wilson, K. S., Withers, S. G., Dupont, C. & Davies, G. J. (1999). *Biochemistry*, **38**, 4826–4833.

White, A., Withers, S. G., Gilkes, N. R. & Rose, D. R. (1994). *Biochemistry*, **33**, 12546–12552.