A Secondary Xylan-binding Site Enhances the Catalytic Activity of a Single-domain Family 11 Glycoside Hydrolase

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Bacillus circulans xylanase (BcX) is a single-domain family 11 glycoside hydrolase. Using NMR-monitored titrations, we discovered that an inactive variant of this enzyme, E78Q-BcX, bound xylooligosaccharides not only within its pronounced active site (AS) cleft, but also at a distal surface region. Chemical shift perturbation mapping and affinity electrophoresis, combined with mutational studies, identified the xylan-specific secondary binding site (SBS) as a shallow groove lined by Asn, Ser, and Thr residues and with a Trp at one end. The AS and SBS bound short xylooligosaccharides with similar dissociation constants in the millimolar range. However, the on and off-rates to the SBS were at least tenfold faster than those of 
$k_{on}\sim3\times10^{5} M^{-1} s^{-1}$ and $k_{off}\sim1000 s^{-1}$ measured for xylotetraose to the AS of E78Q-BcX. Consistent with their structural differences, this suggests that a conformational change in the enzyme and/or the substrate is required for association to and dissociation from the deep AS, but not the shallow SBS. In contrast to the independent binding of small xylooligosaccharides, high-affinity binding of soluble and insoluble xylan, as well as xylododecaose, occurred cooperatively to the two sites. This was evidenced by an ~100-fold increase in relative $K_{d}$ values for these ligands upon mutation of the SBS. The SBS also enhances the activity of BcX towards soluble and insoluble xylan through a significant reduction in the Michaelis $K_M$ values for these polymeric substrates. This study provides an unexpected example of how a single domain family 11 xylanase overcomes the lack of a carbohydrate-binding module through the use of a secondary binding site to enhance substrate specificity and affinity.

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Introduction

Enzymes involved in the hydrolysis of plant cell-wall cellulose and hemicellulose (xylan) are typically modular, consisting of conserved catalytic domains and carbohydrate-binding modules (CBMs), joined by flexible linker sequences. In general, the constituent domains of these glycoside hydrolyases are structurally independent and exhibit some aspects of their respective functions when separated. Binding modules facilitate catalysis by associating the catalytic domain with its substrates, as well as through possible disruptive effects on the structures of the polysaccharides within these...
systems. Accordingly, CBMs typically increase the activity of cellulases and xylanases towards insoluble or polymeric substrates, but not soluble oligosaccharides. There are also cases, such as the cellulase E4 from *Thermomonospora fusca*, in which the catalytic domain and CBM are effectively fused to one another. This may facilitate processivity by directing a bound cellulose strand into the active site of the enzyme.

While it is very common that glycoside hydrolases have structurally independent CBMs, there are only a few examples of ancillary substrate-binding sites within the catalytic domain itself. In α-amylases, such sites have been proposed to increase the affinity of the enzyme towards granular starch, leading to a dramatic increase in net affinity, and hence a significant improvement of catalysis through a reduction of the $K_M$ values for these substrates. This provides an unexpected mechanism for the enhanced activity of a single-domain glycoside hydrolase towards its cell-wall substrates.

**Results**

**BcX binds xyloooligosaccharides at two independent sites**

As part of an ongoing thermodynamic and kinetic investigation of xyloooligosaccharide binding to BcX, we used $^1$H-$^15$N heteronuclear single quantum correlation (HSQC) spectroscopy to monitor the titration of the inactive, nucleophile-lacking E78Q-

![Figure 1. A portion of the superimposed 600 MHz $^1$H-$^15$N HSQC spectra of $^{15}$N-E78Q-BcX (initially 0.69 mM) titrated with X4 at 25 °C (blue peaks changing to yellow with X4 concentrations of 0, 1.1, 3.0, 7.4, and 21.6 mM). Main-chain amides from the SBS (bold labels) show monophasic titrations in the intermediate-fast exchange regime (progressive chemical shift changes without significant line broadening). Due to a larger $\Delta\omega_0$, some exchange broadening is observed for the side-chain amides of the SBS, such as N54 (Supplementary Data Figure S1). Amides from the AS (italics, underlined) show monophasic titrations in the fast exchange limit (progressive chemical shift changes without significant line broadening). This prompts the question of how these glycoside hydrolases recognize and efficiently catalyze the hydrolysis of hemicellulose, particularly when they lack a CBM, as is the case for the majority of family 11 xylanases.

To address this question, we have characterized the binding and hydrolysis of xyloooligosaccharides and polymeric xylans by BcX. Through NMR-monitored titration studies of a BcX mutant that was inactivated by replacing the catalytic nucleophile Glu78 with glutamine, we discovered an ancillary or secondary binding site (SBS) for these substrates lying across the “knuckles” of the hand-like protein. The active site (AS) and SBS of BcX bind xyloooligosaccharides independently with dissociation constants ($K_M$) in the millimolar range, and the SBS does not contribute to hydrolysis of these small substrates. In contrast, soluble and insoluble polymeric xylans are bound cooperatively at both sites, leading to a dramatic increase in net affinity, and hence a significant improvement of catalysis through a reduction of the $K_M$ values for these substrates. This provides an unexpected mechanism for the enhanced activity of a single-domain glycoside hydrolase towards its cell-wall substrates.

†http://www.cazy.org/fam/acc_GH.html
BcX variant with xylotetraose (X4). $^1$H-$^{15}$N chemical shifts are highly sensitive to even subtle structural changes accompanying ligand binding, and thus provide a powerful route for studying protein–ligand interactions. Unexpectedly, residues showing significant main-chain amide, side-chain amide, and/or indole chemical shift perturbations ($\Delta \delta$) upon X4 binding were located both within the AS of the enzyme, as well as on a surface region distal to the AS (Figures 1 and 2; Supplementary Data Figure S1). Furthermore, whereas the NMR signals from AS residues exhibited substantial exchange broadening at partially saturating X4 concentrations (i.e. $k_{ex} \sim \Delta \omega$), the backbone amides of surface residues generally exhibited fast exchange behavior over the course of the titration (i.e. $k_{ex} N \Delta \omega$, yielding progressive chemical shift changes without significant line broadening).35 The observation of such distinct line shape changes, combined with structural considerations (Figure 3), suggested strongly that the chemical shift perturbations of amides in this distal region were due to X4 binding independently at a secondary binding site (SBS), rather than arising indirectly through conformational changes induced by binding to the AS.

To confirm the existence of the SBS, we used the covalent inhibitor 2',4'-dinitrophenyl 2-deoxy-2-fluoro-$\beta$-xylobioside (DNP2FX2) to block the AS of WT-BcX by forming a long-lived glycosyl-enzyme intermediate, 2-deoxy-2-fluoro-$\beta$-xylobiosyl-BcX (2FX2-BcX). Previous NMR spectroscopic and X-ray crystallographic analyses of wild-type (WT) and 2FX2-BcX demonstrated that only active site residues are perturbed structurally, dynamically, and spectroscopically due to the covalent attachment of this inhibitor to Glu78.22,36 Upon titration of 2FX2-BcX with X4, residues within the SBS exhibited similar chemical shift perturbations in the fast exchange limit as seen with the E78Q-BcX variant, while the blocked AS residues remained completely unaffected (Figure 2). To perform the complementary experiment, we disrupted the putative SBS by substituting alanine for three residues, N141, N181, and T183, which showed large main chain $^1$H-$^{15}$N and side-chain $^1$H-$^{15}$N chemical shift perturbations in the presence of X4 (Supplementary Data Figure S1). The polar side-chains of these residues are on the surface of BcX and, in principle, capable of forming hydrogen bonds to a xylooligosaccharide. Titration of the inactive version of this mutant, E78Q-AAA-BcX, with X4 produced chemical shift perturbations, along with line broadening, exclusively for AS residues (Figure 2). Thus, E78Q-AAA-BcX is no longer capable of binding X4 outside of its AS. Furthermore, as summarized in Figure 2, the chemical shift perturbations of the SBS residues and the AS residues in E78Q-BcX are essentially the same as seen for the corresponding residues in 2FX2-BcX and E78Q-AAA-BcX, respectively. Together, these results prove that BcX binds X4 at a previously unrecognized SBS, which is structurally independent of its AS.

**Structural features of the SBS**

The SBS site was qualitatively identified by mapping the amide and indole chemical shift perturbations due to X4 binding onto the structure of BcX (Figure 3). Residues showing the largest

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**Figure 2.** Main-chain $^1$H$^N$ and $^{15}$N chemical shift perturbations $\Delta \delta$ (equation (1)) of four BcX variants upon titration with xylotetraose (X4) and cellopentaose (G5). E78Q-BcX binds X4 at both the AS and SBS. 2FX2-BcX binds X4 at the SBS only, due to covalent blockage of the AS. E87Q-AAA-BcX binds X4 at the AS only, due to disruption of the SBS by triple mutation. WT-BcX binds G5 extremely weakly at its AS (note the 20-fold expanded scale). The $\Delta \delta$ values are taken from titration points of 0.5 mM protein without and with 7 mM oligosaccharide. The secondary structure elements of BcX, defined by Promotif,70 are identified by arrows ($\beta$-strands, sheet A in black, sheet B in white) and a coil ($\alpha$-helix). The “thumb” loop connects strands 9 and 10.
spectral changes clustered to a region roughly corresponding to the “knuckles” of the hand-like protein. In contrast to the pronounced cleft of the AS, the SBS is a relatively flat region with flanking asparagine (N25, N54, N141, and N181), serine (S2, S27, and S140), and threonine (T3, T143, and T183) residues, along with tryptophan (W185) at one end. Such residues are typically found in carbohydrate-binding sites due to their ability to participate in hydrogen bonding or aromatic stacking interactions, respectively. Unfortunately, because of the relatively weak nature of this interaction, we were unable to use NMR spectroscopy to determine the structure of X4 bound to BcX. Also, binding to this site was not observed in a previous crystallographic study of E172C-BcX soaked with X4, possibly due to ligand hydrolysis by residual xylanase activity or packing constraints. Therefore, the importance of several SBS residues for xylan binding was confirmed by reduced retardation of E78Q-BcX in an affinity electrophoresis assay upon introduction of the separate sets of mutations N54A, N141A, N181A/T183A, W185A, or combinations thereof (data not shown). For subsequent binding and kinetic studies, the triple AAA-BcX mutant was used to completely eliminate any interactions at the SBS.

**Binding affinity of BcX for xylooligosaccharides**

The dissociation constants ($K_d$) of the AS and SBS for X4 were determined by a quantitative analysis of the concentration-dependent chemical shift changes measured from $^1$H-$^{15}$N HSQC-monitored titrations (Table 1 and Figure 4). As summarized in Table 1, binding to either site of E78Q-BcX is relatively weak, with the SBS exhibiting only marginally higher affinity ($K_d = 2.2(±0.1)$ mM) for X4 than the AS ($K_d = 3.4(±0.5)$ mM). Importantly, within error, the same $K_d$ values were measured for the SBS in 2FXB-BcX and the AS in E78Q-AAA-BcX, demonstrating further that the two sites function independently in binding xylooligosaccharides.

To characterize the dimensions of the SBS, and to examine the number of subsites within the AS, we also carried out $^1$H-$^{15}$N HSQC-monitored titrations of E78Q-BcX with xylobiose (X2) and xylohexaose (X6), and of WT-BcX with xylose (X1) and X2. The

![Figure 3](http://pymol.sourceforge.net/)
Table 1. Dissociation constants of BcX variants with xylose-based ligands

<table>
<thead>
<tr>
<th>Site</th>
<th>Ligand</th>
<th>WT-BcX</th>
<th>E78Q-BcX</th>
<th>2FX2-BcX</th>
<th>E78Q-AAA-BcX</th>
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<tbody>
<tr>
<td></td>
<td></td>
<td>SBS</td>
<td>AS</td>
<td>Overall</td>
<td>SBS</td>
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<tr>
<td>$K_d$ (mM)</td>
<td>Xylose</td>
<td>221(±330)</td>
<td>44.4(±4.2)</td>
<td>1510(±340)</td>
<td>17.4(±1.4)</td>
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<tr>
<td></td>
<td>Xylobiose</td>
<td>1420(±670)</td>
<td></td>
<td>2.2(±0.1)</td>
<td>3.4(±0.5)</td>
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<tr>
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<td>Xylohexaose</td>
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<td></td>
<td>0.076(±0.008)</td>
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<td>Xyloolectaose</td>
<td></td>
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<tr>
<td></td>
<td>Cellopentaose</td>
<td>300(±200)</td>
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<tr>
<td>$K_{dr}$ (mg/ml)</td>
<td>Soluble xylan</td>
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<td></td>
<td>0.60(±0.04)</td>
<td>0.08(±0.02)</td>
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<tr>
<td></td>
<td>Insoluble xylan</td>
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<td></td>
<td>0.030(±0.001)</td>
<td>0.10(±0.002)</td>
</tr>
<tr>
<td></td>
<td>Avicel</td>
<td></td>
<td></td>
<td>0.100(±0.002)</td>
<td>13.2(±0.2)</td>
</tr>
</tbody>
</table>

Dissociation constants were determined by NMR-monitored titration (xylo- and cello-oligosaccharides, and fractionated soluble birchwood xylan DP~180) or by a sedimentation assay (insoluble birchwood xylan and avicel) in sodium phosphate buffer (pH 6.0 at 25 °C), or as indicated. The site-specific dissociation constants for the AS or the SBS, or overall dissociation constants to the protein.

* NMR spectra were recorded at 500 MHz.

** NMR spectra were recorded at 600 MHz.

See Discussion.

latter are not cleavable by BcX. As summarized in Table 1, the SBS did not show any measurable affinity for X1, bound X2 very weakly, and bound X6 with only twofold higher affinity ($K_d=1.0(±0.1)$ mM) than X4. This indicates that the primary binding determinants of the SBS span three or four xylose units. The slightly tighter binding of X6 may result from additional weak protein–carbohydrate interactions or simply a statistical increase in the effective concentration of protein-binding sites on the longer xylooligosaccharide (i.e. X6 contains three xylotetraose segments). In contrast, similar patterns of AS chemical shift perturbations were observed upon titrations with X1, X2, X4, and X6, indicating specific binding of all of these xylooligosaccharides to the active site of BcX (Supplementary Data Figure S2). Although X1 binds WT-BcX very poorly ($K_d=2200(±330)$ mM), X2 binds E78Q-BcX with an affinity ($K_d=17.4(±1.4)$ mM), comparable to that of X4, especially when considering the higher effective concentration of protein-binding sites within the longer oligosaccharide. Surprisingly, the fit $K_d$ of the AS for X6 (4.7(±0.8) mM) appeared to increase marginally relative to X4. However, deviations from the fast exchange limit reduced the accuracy and precision of the measured $K_d$ values for both xylooligosaccharides. Since exchange broadening was most pronounced for X6 binding to the AS, it may actually bind slightly tighter than the other xylooligosaccharides tested. Also somewhat unexpectedly, the $K_d$ value of X2 with E78Q-BcX was about threefold lower than with the WT enzyme, demonstrating that substitution of the nucleophile E78 with a neutral glutamine increases the affinity of the AS for xylooligosaccharides.

Cooperative binding of xyloolectaose

The AS and SBS are located on opposite sides of BcX, and thus it is not unexpected that each site acts independently in binding short xylooligosaccharides. This prompted the critical question of whether longer xylooligosaccharides can interact with both sites simultaneously, leading to enhanced binding avidity. To answer this question, we carried out a $^1$H,$^15$N HSQC-monitored titration of E78Q-BcX with glycosynthase-prepared 4'-nitrophenyl xyloolectaose (PNP-X12). Inspection of the structure of BcX (Figure 3) suggested that X12 is long enough to span both binding sites simultaneously. For SBS residues, patterns of $\Delta \delta$ values were observed similar to those seen with shorter xylooligosaccharides, confirming that PNP-X12 binds at this site (Supplementary Data Figure S2). However, fitting these chemical shift changes to a simple dissociation equilibrium indicated a substantial $\sim$30-fold increase in binding affinity ($K_d=0.076(±0.004)$ mM) relative to X4 (Table 1). Note that the SBS amides now exhibited signal broadening, as well as chemical shift perturbations, indicative of PNP-X12 binding in the fast–intermediate exchange regime (not shown); thus, the actual increase in affinity may be greater than this estimated value due to biasing of chemical shifts from a population-weighted average. Also, due to this tighter binding, amides within the AS now exhibited a progressive loss of signal intensity, without substantial chemical perturbations, upon titration with PNP-X12 (i.e. slow exchange). Since only small quantities PNP-X12 were available, we did not reach saturation and could not reliably fit the intensity changes to obtain a second measure of $K_d$ for this ligand. Nevertheless, these data demonstrate that the longer xylooligosaccharide binds cooperatively to both the AS and SBS.

BcX binds xylan with high affinity due to AS and SBS cooperativity

To test the relevance of AS and SBS cooperativity in binding natural substrates, we used a simple sedimentation assay to measure the association of inactive BcX with insoluble birchwood xylan (Supplementary Data Figure S3). The relative $K_d$ values...
of E78Q-BcX and E78Q-AAA-BcX for this xylan at 4 °C were 0.030(±0.001) mg/ml and 3.2(±0.1) mg/ml, respectively (Table 1). Both values increased by three- to fourfold at 37 °C, suggestive of enthalpically driven binding ($\Delta H$. Since the number of accessible binding sites on the insoluble material is unknown, we cannot compare these $K_{dr}$ values directly with the $K_d$ values measured for the xylooligosaccharides. Nevertheless, disruption of the SBS by the triple alanine mutation led to a dramatic ∼100-fold decrease in the relative binding affinity of inactive BcX for insoluble xylan, clearly demonstrating cooperativity of this ancillary site with the AS.

In parallel, we used $^1$H-$^{15}$N HSQC spectroscopy to monitor the binding of BcX to soluble birchwood xylan, fractionated to a chain with a degree of polymerization (DP) of ∼180. The titration spectra of E78Q-BcX occurred in a fast-to-intermediate exchange regime, as evidenced by Asp119 showing chemical shift changes, as well as exchange broadening, leading to decreased signal intensity that was most pronounced near the midpoint of the titration. E78Q-AAA-BcX showed a similar behavior with X4 and soluble xylan as E78Q-BcX did with X4 at the AS, and no binding to the mutated SBS. In contrast, E78Q-BcX bound soluble xylan tightly in the slow exchange regime, leading to decreased signal intensities for all amides, regardless of location, due to formation of a high molecular mass complex (i.e. only signals from the free protein were observed). Superimposed upon this intensity change, W185 (but not D119 or Y105) exhibited significant chemical shift changes, indicative of a faster exchange process at the SBS (see Discussion). Intensities were normalized to the initial intensity of the protein without added ligand, with correction for dilution effects and number of transients collected per spectrum. The lines show fits to equation (2) for X4 and equation (4) for soluble xylan.

![Figure 4](image_url)

**Figure 4.** Summary of the $^1$H-$^{15}$N HSQC monitored titrations of E78Q-BcX (top; containing both the SBS and AS) and E78Q-AAA-BcX (bottom; containing only the AS). Shown are chemical shift ($\Delta \delta$, equation (1)) and relative peak intensity ($I_i/I_0$) changes for representative amides in the AS (D119 ○), the SBS (W185 ●), and a non-binding site (Y105 ▽) upon titration with X4 (left) and fractionated soluble xylan with DP∼180 (right). Titrations were carried out in sodium phosphate buffer (pH 6.0) at 25 °C and 500 MHz (E78Q-AAA-BcX) or 600 MHz (E78Q-BcX).
In striking contrast to E78Q-AAA-BcX, which has only a functional AS, E78Q-BcX, with both binding sites intact, exhibited dramatically different $^1$H-$^{15}$N HSQC spectra when titrated with soluble xylan. That is, the peak intensities of all amides, regardless of their structural location in the protein, decreased upon addition of xylan (Figure 4). The AS and non-binding site amides showed no significant chemical shift change. This is diagnostic of tight binding in the slow exchange regime ($k_{ex} < \Delta \omega$), leading to the formation of a high molecular mass complex with signals broadened beyond detection due to slow molecular tumbling. The high molecular mass may have resulted from several BcX molecules binding to one xylan chain or one BcX molecule binding to two chains, compounded by the tendency of long xylan polymers to self-associate. Fitting the peak intensities of amides in the unbound protein yielded a relative $K_d$ of 0.08(±0.02) mg/ml for the overall binding of E78Q-BcX to soluble xylan. Thus, similar to insoluble xylan, inactive BcX with both the AS and SBS intact binds soluble xylan ∼90-fold better than with only the AS. That is, the two sites are functionally cooperative in binding polymeric substrates. Interestingly, amides within the SBS also showed chemical shift perturbations, as well as decreased intensities, over the course of the titration of E78Q-BcX with soluble xylan (Figure 4). Fitting the shift changes yielded a $K_{dr}$ value of 0.60(±0.04) mg/ml for the SBS, or about eightfold higher than that measured for all (even the SBS) residues by intensity changes (Table 1). The significance of this observation is addressed in Discussion.

**Binding kinetics**

Amides of the AS and SBS in E78Q-BCX exhibited distinctly different spectroscopic behavior during NMR titrations (Figures 1 and 4). With short xylo-oligosaccharides such as X4, backbone amide signals of the SBS displayed changes only in chemical shift, but no line broadening; this is typical of weak binding with $K_d$ in the millimolar range. In contrast, residues in the AS displayed pronounced line broadening upon addition of X4. Since the $K_d$ value for X4 to the AS is also in the millimolar range, this indicates that the rates of association and dissociation to this site must both be significantly lower than to the SBS. To quantify these rates, $^{15}$N relaxation dispersion spectroscopy was applied to E78Q-BcX without ligand and at partial (6%) saturation with X4 (Figure 5). This NMR technique measures the millisecond to microsecond timescale exchange contributions, $R_{ex}$ to transverse $^{15}$N relaxation. In the absence of X4, 11 AS residues with $R_{ex} > 3$ Hz were identified in E78Q-BcX. Global fitting of the relaxation dispersion profiles for these residues to a two-site exchange process yielded $k_{ex} = 830(±70)$ s$^{-1}$ between major and minor (0.8(±0.04) %) conformational states. We are currently investigating the hypothesis that this exchange reflects motions of the AS thumb region. More importantly, as exemplified by S117 in Figure 5, dramatically increased $R_{ex}$ terms were detected for 16 AS residues in E78Q-BcX upon addition of X4. A global fit of the dispersion profiles for these 16 residues, with the population of the minor bound state fixed at 6% yielded a common $k_{ex}$ of 1070(±40) s$^{-1}$. A more detailed analysis of the relaxation dispersion data is significantly complicated by the fact that an exchange process is already present in the free protein. Nevertheless, the relaxation dispersion of AS residues in the presence of X4 is clearly dominated by conformational exchange between the free and bound states of E78Q-BcX, and values of $k_{on} \sim 3 \times 10^7$ M$^{-1}$ s$^{-1}$ and $k_{off} \sim 1000$ s$^{-1}$ can be estimated from the fit $k_{ex}$ term. In contrast to the AS, no significant (>3 Hz) $R_{ex}$ term was measured for residues in the SBS of E78Q-BcX in the absence or in the presence of X4. This is exemplified in Figure 5 by the flat relaxation dispersion of G56 (left) and S117 (right) in 0.7 mM E78Q-BcX recorded $B_0 = 14.1$ T without (triangles, broken lines) and with 0.33 mM X4 (squares, continuous lines). The lines represent the best per-residue fits of the data, and error bars (1–2%) are smaller than the symbols. G56 is located in the SBS and shows no $R_{ex}$ contributions due to X4 binding in the fast exchange limit. In contrast, S117 in the AS shows a small $R_{ex}$ of 1.9 Hz in the free E78Q-BcX, which increases significantly to 20.5 Hz in the presence of X4. On the basis of a $K_d$ of 3.4 mM, 6% of the E78Q-BcX AS is in the bound state under these conditions.

![Figure 5](image-url)
persion profile of G56, which is an SBS residue that displays a large amide $^{15}$N chemical shift change upon X4 binding (Figure 1). However, this change occurs in the fast exchange limit and thus is not detectable by relaxation dispersion measurements (i.e. $k_{ex}$$>10^4$ s$^{-1}$). With this limit in mind, the association and dissociation rate constants for X4 to the SBS must both be more than tenfold greater than to the AS, given the similar $K_d = k_{off}/k_{on}$ values for the two binding sites.

**Binding specificities of the SBS and AS**

Thus far, we have demonstrated that the SBS and AS bind small xylooligosaccharides independently, but bind longer xylan polymers cooperatively. To examine the specificity of the SBS and AS for other possible hemicellulosic species, we utilized affinity electrophoresis as a qualitative binding assay (Figure 6). In gels containing xylans from three plant sources, E78Q-BcX (AS and SBS) showed the largest retardation, followed by E78Q-AAA-BcX (AS only) and 2FX2-BcX (SBS only) with similar intermediate levels of retardation, and AAA-2FX2-BcX (no binding site) with an unaffected electrophoretic mobility relative to an external reference. This provides a clear visual confirmation of the presence of two binding sites within BcX. Upon closer inspection, small changes in the mobility patterns between E78Q-AAA-BcX and 2FX2-BcX were seen with xylans from birchwood, larchwood and, most pronounced, oat spelts. This likely reflects variations in the binding specificities of the AS and SBS for the three differentially substituted xylans. In contrast, the four proteins were not retarded by xyloglucan or $\beta$-D-glucan, and those with an intact SBS showed very slight retardation by hydroxyethyl cellulose.

The binding of inactive BcX to cellulosic substrates was also tested. As summarized in Table 1, E78Q-BcX and E78Q-AAA-BcX bound avicel (a mixture of crystalline and amorphous cellulose) very weakly in sedimentation assays. In contrast to the cooperativity seen with xylan, mutation of the SBS increased the overall $K_d$ value of BcX for Avicel by only about twofold. Also, extremely weak binding of the AS of WT-BcX to cellopentaose (G5; $K_d$$\sim0.3$ M) was observed in NMR-monitored titrations. Although small, measurable chemical shift perturbations were observed for AS residues, whereas SBS residues remained essentially unchanged (Figure 2).

**SBS enhances catalysis by reducing the $K_m$ value of BcX towards xylan**

To determine the role of the SBS in catalysis, we undertook comparative kinetic studies of WT-BcX and AAA-BcX, measuring $K_m$ and $k_{cat}$ against a xylobioside derivative 2'-nitrophenyl $\beta$-xylobioside (ONPX2), soluble xylan, and insoluble xylan (Figures 7 and 8; Table 2). In all cases, linear Lineweaver–Burk plots were obtained, indicating a lack of detectable transglycosylation activity at elevated substrate concentrations. Similar $K_m$ values within error (6.3(±0.8) mM, 7.5(±0.5) mM) and only slightly different $k_{cat}$ values ($2.6(±0.1)$ s$^{-1}$, 3.1(±0.1) s$^{-1}$) for hydrolysis of ONPX2 by WT-BcX and AAA-BcX, respectively, revealed that the three point
mutations within the SBS do not significantly influence the catalytic properties of the AS towards small substrates. This is consistent with the observed independent binding of xylooligosaccharides at each site. Note also that glycosylation is the rate-limiting step of the double displacement mechanism utilized by BcX,19 and thus $K_M$ values are expected to be greater than or equal to the equilibrium dissociation constants for the equivalent substrate. Since the $K_M$ value of WT-BcX for ONPX2 is about sevenfold less than the $K_d$ of the AS of WT-BcX for X2 binding (44.4 mM), this may reflect additional interactions with the o-nitrophenol leaving group of the synthetic substrate in the AS +1 subsite.

The presence of SBS had marked effects on the hydrolysis of xylan substrates. Using the soluble fraction of birchwood xylan without any further treatment (DP∼90), $K_M$ values of 0.14±0.02 mg/ml and 0.53±0.02 mg/ml were measured for WT-BcX and AAA-BcX. After enrichment for longer polymers by ultrafiltration (DP∼180), the $K_M$ values for WT-BcX decreased significantly to 0.06±0.01 mg/ml, while those for AAA-BcX did not change within error. A similar, approximately tenfold lower $K_M$ value was measured for WT-BcX (0.35±0.05 mg/ml) as compared to AAA-BcX (3.9±0.4 mg/ml) towards insoluble xylan. As with ONPX2, AAA-BcX consistently exhibited ~1.3-fold higher $k_{cat}$ values towards these xylans than WT-BcX. There is no obvious explanation for this result, and thus the mutations must subtly perturb the structure, dynamics, or electrostatic

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Figure 7. The SBS enhances catalysis by reducing the $K_M$ values for xylan hydrolysis. Michaelis–Menten plots for WT-BcX (●) and AAA-BcX (○). Initial rates ($v_0$) are plotted against the initial concentrations of ONPX2, fractionated soluble birchwood xylan (DP∼180), and insoluble birchwood xylan. $k_{cat}$ and $K_M$ values (Table 2) were extracted by non-linear least-squares fitting to the Michaelis–Menten equation. Lineweaver–Burk plots are shown as insets.

(a) Generated reducing sugar ends are plotted as a function of reaction time (initial points expanded in the inset). Data were fit to a continuous line, from which the instantaneous rates, $v_t$, were calculated by differentiation.

(b) The rates, $v_t$, are then plotted versus the calculated average xylan chain length (thick solid (WT-BcX) or thick dotted lines (AAA-BcX); top x-axis) and the extent of reaction (thin continuous or dotted lines; lower x-axis). The initial concentrations of xylan and enzyme were 0.05 mg/ml and 0.005 μM, respectively.
properties of the distant AS. Nevertheless, these measurements demonstrate clearly that cooperative xylan binding by the SBS and AS leads to enhanced catalysis through a significant reduction in $K_M$ values for substrate hydrolysis by BcX.

**Discussion**

**Xylooligosaccharide binding in the active site of BcX**

The structural bases for specific xylan binding and hydrolysis by BcX and related family 11 xylanases have been characterized in detail by many kinetic and X-ray crystallographic studies of the WT and mutant forms of these enzymes.\(^{18,22,23,26,30-33}\) In particular, the roles of active site residues in hydrogen bonding, aromatic stacking, and van der Waals interactions with substrates bound non-covalently and covalently in the $-2$ and $-1$ subsites are well established. Using NMR spectroscopy to monitor the interactions of xylooligosaccharides with catalytically inactive E78Q-BcX, we have furthered this structural understanding from both thermodynamic and kinetic perspectives.

The site-specific $K_d$ values for xylooligosaccharides binding the AS of inactive E78Q-BcX follow the order $X1 \gg X2 \gg X4 \sim X6$ (Table 1). Consistent with the reported kinetic $K_M$ values for synthetic xylobiose-based substrates with glycosylation as the rate-limiting step, the equilibrium dissociation constants for species longer than xylose are in the low millimolar range. More importantly, X4 and X6 bind with only about fivefold higher affinity than X2, demonstrating that two xylose units provide the majority of the interactions with the AS of E78Q-BcX. These interactions likely occur in the $-2$ and $-1$ subsites, as indicated by several lines of evidence. Family 11 xylanases do not hydrolyze X2, and generally cleave X3 and X4 slowly;\(^{27}\) thus, a disaccharide cannot bind productively across the $-1/+1$ subsites. Rather, AS residues, excluding W9, Y69, Y80, R112, and Y166, showed significant amide and/or indole $\text{H}^{15}$N HSQC chemical shift changes upon titration with the xylooligosaccharides (Figures 2 and 3; Supplementary Data Figure S2). These form the $-2$ and $-1$ subsites of BcX, and are observed by X-ray crystallography to interact extensively with bound substrates.\(^{22,23}\) Furthermore, crystallographic studies of BcX covalently bound to a xylotriose derivative revealed only water-mediated hydrogen bonds between the most distal xylose moiety and any potential $-3$ subsite residues (I. D’Angelo, personal communication). A similar situation occurs with the related *Bacillus agaradhaerens* Xyn11,\(^{33}\) and *Chaelomium thermophilum* Xyn11A.\(^{26}\)

The catalytic activity of family 11 xylanases obviously requires substrate binding to (+) subsites, yet to date, no ligand within this AS region or across the $-1/+1$ subsites has been observed by X-ray crystallography. For example, although soaked with X4, only two xylose units were observable in the $-2$ and $-1$ subsites of E172C-BcX.\(^{23}\) The absence of any detectable electron density for the remaining two units may have resulted from their disorder or from hydrolysis by residual enzyme activity. Similarly ordered binding was observed only within the $-3$, $-2$, and $-1$ subsites for X4 in complex with the nucleophile deletion variant E94A of *B. agaradhaerens* Xyn11,\(^{33}\) and for a thioxylopentaose inhibitor bound to *C. thermophilum* Xyn11A.\(^{36}\) These observations imply that binding in the (+) subsites is energetically unfavorable, at least for short xylooligosaccharides in the crystalline state. Thus, it was somewhat surprising that residues forming the aglycone binding subsites, including Y88, T91, T110, W129, and Y174, exhibited significant NMR spectral perturbations upon titration of E78Q-BcX with xylooligosaccharides (Figure 2). It is possible that these spectral perturbations arose indirectly through subtle, propagated structural changes from binding only in the (-) subsites. However, given that xylan must lie across the (-) and (+) subsites in order to be cleaved by BcX, it seems plausible that we are also detecting xylooligosaccharide binding to both regions of the AS. In support of an indirect perturbation model, fitting the initial portion of the NMR-derived X2 binding isotherm for (+) subsite residues yielded very similar $K_d$ values as fit for the (-) subsite residues, suggestive that each report a common binding event. However, in support of a direct binding model, the (+), but not (-), subsite residues also showed deviations from a simple isotherm at higher concentrations of X2. This is suggestive of weaker binding of X2 to (+) subsites after saturation.

---

**Table 2.** Steady-state kinetic parameters for the hydrolysis of ONPX2 and various birchwood xylan preparations by WT-BcX and AAA-BcX

<table>
<thead>
<tr>
<th></th>
<th>ONPX2</th>
<th>Soluble xylan (DP~90)</th>
<th>Soluble xylan (DP~180)</th>
<th>Insoluble xylan</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$K_M$ (mM)</td>
<td>$k_{cat}$ (s$^{-1}$)</td>
<td>$K_M$ (mg/ml)</td>
<td>$k_{cat}$ (s$^{-1}$)</td>
</tr>
<tr>
<td>WT-BcX</td>
<td>6.3(±0.8)$^a$</td>
<td>2.6(±0.1)$^b$</td>
<td>0.14(±0.02)$^b$</td>
<td>16.7(±0.5)$^b$</td>
</tr>
<tr>
<td>AAA-BcX</td>
<td>7.5(±0.5)</td>
<td>3.1(±0.1)</td>
<td>0.53(±0.02)</td>
<td>22.8(±0.4)</td>
</tr>
</tbody>
</table>

Assays were carried out in sodium phosphate buffer (pH 6.0). The $k_{cat}$ and $K_M$ values were determined by a non-linear, least-squares fit to the standard Michaelis–Menten equation. For xylan substrates, apparent $k_{cat}$ and $K_M$ values are denoted by r for relative.

- $^a$ At 25 °C.
- $^b$ At 37 °C.
- $^c$ Results differ from those published previously due to differences in experimental conditions and protocols used to measure only the initial rates of xylan hydrolysis before significant changes in the DP.\(^{18,23}\)
of the (−) subsites. In either case, since X1, X2, X4, and X6 all caused similar spectral perturbations with E78Q-BcX, binding likely occurs with intramolecular equilibration between multiple positions along the AS. Further studies, including stoichiometry measurements or saturation transfer-type NMR experiment, with BcX variants having subsites blocked by mutation or chemical modification may help resolve the energetics of these complex binding events.

Surprisingly, despite relatively weak binding, amides within the AS of E78Q-BcX exhibited conformational exchange broadening at partial saturation of X6, X4, X2, and even X1. This behavior is indicative of relatively slow on and off rates for complex formation. Using 15N relaxation dispersion measurements, values of $k_{\text{off}}$ ∼ $3 \times 10^7$ M$^{-1}$ s$^{-1}$ and $k_{\text{on}}$ ∼ $100$ s$^{-1}$ for the interaction of X4 with the AS of E78Q-BcX were measured. This $k_{\text{off}}$ rate constant is ∼$10^3$ fold slower than that of simple diffusion-limited bimolecular association (without consideration of orientational or structural constraints).

Co-operative xylan binding by the AS and SBS of BcX

Unexpectedly, NMR-monitored titration studies also revealed that BcX contains an ancillary binding site specific for xylooligosaccharides. This SBS is a shallow groove lying along the knuckles of the BcX hand, and thus distal to the pronounced AS cleft (Figure 3). On the basis of a comparison of chemical shift perturbations, relaxation dispersion profiles, and fit $K_d$ values measured for E78Q-BcX, E78A-AAA-BcX, and 2FX2-BcX, binding of X4 and X6 to the SBS and AS occurs independently at the structural, kinetic, and thermodynamic levels. However, while exhibiting similar $K_d$ values for these xylooligosaccharides, on and off rate constants for X4 with the SBS must both be more than tenfold greater than to the AS, as evidenced by binding in the fast versus intermediate exchange limits, respectively. This is consistent with the structural differences between these two binding sites.

Although we were unable to determine by NMR methods the structure of a xylooligosaccharide bound to the SBS, chemical shift perturbation mapping defines this site as a shallow groove flanked by asparagine, serine, and threonine residues, and capped at one end by a tryptophan ring (Figure 3(b)). The importance of several of these residues (N54, N141, N181, T183, and W185) towards X4 binding was confirmed by site-directed mutagenesis. Such amino acids, typically found in CBMs specific for small sugars and single-chain glycans, facilitate carbohydrate binding through hydrogen bonding and aromatic stacking interactions. In contrast, CBMs specific for the surfaces of insoluble polysaccharides generally bind through hydrophobic interactions mediated by exposed aromatic side-chains. Interestingly, simple modeling suggested that a single xylose should be able to stack on W185, while also forming hydrogen bonds to N54, N141, and T183. However, X1 did not bind to the SBS with any measurable affinity, whereas the $K_d$ values for xylooligosaccharides followed the trend X2 ≫ X4 ~ X6. Therefore, the SBS appears to span three or four xylose units, and is longer than might be suggested by perturbation mapping of only the residues showing the largest chemical shift changes during titration experiments (Figure 3(a)). Indeed, as seen in Figure 3(c), the SBS and AS are aligned with one another and connect via a small shallow groove, which may facilitate co-operative binding of longer xylan polymers (Figure 3(c)). Residues 23 ~ 26 along this groove exhibited moderate chemical shift perturbations, especially in titrations with PNP-X12 and soluble xylan (Supplementary Data Figure S2). In this respect, the SBS might be viewed as an extension of the AS around the BcX hand. Furthermore, the observation that PNP-X12 binds cooperatively to BcX suggests that xylooligosaccharides in the SBS and AS have the same orientation with respect to this extended groove (i.e. based on distance considerations, X12 likely binds in a straight rather than hairpin fashion). Regardless, the two sites are clearly independent for binding of small xylooligosaccharides.
of the SBS (Table 1). Due to limited quantities of DNP2FX2 and the possibility of reactivation of the trapped glycosyl-enzyme intermediate (t_{1/2}~20 h), we did not carry out parallel studies with 2FX2-BcX to quantitatively measure binding to only the SBS in the context of a covalently blocked AS. Nevertheless, using affinity electrophoresis as a qualitative indicator of binding, BcX variants with only an SBS or an AS showed similar levels of retardation in the presence of birchwood and larchwood xylans (Figure 6). Thus, combined with their similar K_{d} values for short xylooligosaccharides, it is reasonable to assume that the AS and SBS also exhibit comparable site-specific affinities for xylan polymers.

A simple model for the multivalent interaction of xylan substrates with BcX is presented in Figure 9. By virtue of their small sizes, xylooligosaccharides can bind independently only to the AS and SBS. On the other hand, longer polymeric substrates can bind to either or both sites. Given the heterogeneous nature of naturally occurring xylans, it is likely that mixed modes of binding occur, i.e. binding of one polymer chain to both sites or separate chains to each site. However, once a xylan polymer is bound at one site via an intermolecular association event, binding of the same chain at the other site is favored due to its increased local concentration and a reduced entropic penalty for intramolecular association. Following the approach taken by Linder et al., we used the equilibrium association constants of X4 and X6 binding to calculate the free binding energy of the individual sites (ΔG_{AS}, ΔG_{SBS}) and of PNP-X12 binding to the combined sites (ΔG_{AS,SBS}). The estimated coupling or “synergy” free energy ΔG^{\text{c}} = ΔG_{AS,SBS} - (ΔG_{AS} + ΔG_{SBS}) for the latter process is approximately +6 kJ/mol, indicating that binding to both sites occurs with a less favorable net free energy change than expected from the sum of the free energy changes for binding to the individual sites. Thus, binding is co-operative, but ΔG_{AS,SBS} appear to be partially offset by unfavorable energetic terms, such as possible conformational changes in either PNP-X12 or BcX required for simultaneous association of the xylooligosaccharide at both the AS and SBS.

Due to the different NMR exchange behaviors, and hence kinetics, of substrate association/dissociation to the AS and SBS residues, interpreting the titration curves of E78Q-BcX with PNP-X12 and soluble xylan is very difficult. In particular, fitting the intensity decreases for all residues in E78Q-BcX to the SBS of BcX. As discussed in the text, binding can occur to either of the sites individually, or to both sites simultaneously in a cooperative manner.

SBS enhances xylan hydrolysis by BcX

The SBS significantly enhances the catalytic activity of BcX towards polymeric xylan through improved substrate binding. This is evident from an approximately tenfold increase in the K_{d} values for soluble and insoluble xylan, but not a xylobiose derivative, upon disruption of the SBS. Notably, however, this effect is smaller than the ~100-fold increase in K_{d} values for these xylans due to the same mutations. This difference may arise because the equilibrium dissociation constant involves all modes of BcX binding to xylan (i.e. via either or both the AS and SBS). In contrast, the kinetic Michaelis constant reflects only binding at the AS, either independently or cooperatively with the SBS. Furthermore, birchwood xylan is heterogeneous, and thus some binding modes may not be catalytically productive. Also, K_{d} measurements were carried out with inactive E78Q-BcX, which binds X2 with about threefold higher affinity than the WT enzyme (Table 1).

The cooperative effects of SBS binding primarily facilitate hydrolysis of longer xylans by BcX. For example, fractionating soluble xylan to increase the average DP from ~90 to ~180 led to an approximately twofold decrease in the apparent K_{M} value for BcX, but not AAA-BcX, towards this substrate. This conclusion is supported further by comparing
the instantaneous rates of xylan cleavage by BcX and AAA-BcX over the full timecourse of digestion. As shown in Figure 8, hydrolysis by AAA-BcX was initially slower than by WT-BcX, but remained relatively insensitive to polymer length. In stark contrast, after only ~10% of the hydrolyzable bonds in soluble xylan (initial DP~180) were cleaved, the rate of catalysis by the WT enzyme dropped dramatically to a plateau level comparable to that of AAA-BcX. The simplest interpretation of these results is that longer polymers are bound preferentially by both the SBS and AS, reducing the $K_M$ value for hydrolysis. However, this is somewhat surprising, given that PNP-X12 binds the two sites in E78Q-BcX cooperatively. Thus, shorter polymers may not associate in a catalytically productive mode, perhaps due to structural constraints for binding to both sites. Alternatively, this effect may result from the extensive modifications present in naturally occurring xylans. The sharp dependence of $K_M$ on polymer length also implies that the effects of the SBS may not be observable experimentally unless great care is taken to study only the initial stages of xylan hydrolysis, and that the tenfold decrease in $K_M$ value cited above may be an underestimation as shorter polymers were likely present in the soluble xylan fractionated to a DP~180. Finally, the results illustrated by Figure 8 confirm that xylan hydrolysis by BcX is endolytic but non-processive.

Concluding remarks

We have identified and characterized a non-catalytic xylan-binding site on the surface of BcX. The SBS functions cooperativity with the AS, leading to higher-affinity substrate binding and enhanced catalysis. This demonstrates an effective strategy by which a single-domain xylanase can compensate for the lack of a CBM. It remains to be established how common such a strategy is among xylanases, or glycoside hydrolases in general. The specific SBS residues in BcX, defined by mutational studies and chemical shift perturbation mapping, are only moderately conserved among family 11 xylanases, and the equivalent of tryptophan at position 185 occurs infrequently. However, as a rather nondescript shallow groove with exposed polar side-chains, we would not have identified the SBS by simply inspecting the crystallographic structure of this enzyme. Since this site is located at the edge of a well-known Ser/Thr-rich surface present in most family 11 members, it is very possible that other regions of this surface in related enzymes also function as ancillary binding sites. Moreover, the observation of xylooligosaccharides bound to non-active site surface regions of family 8 and 10 xylanases suggest that the SBS is not a unique feature for BcX. Although such secondary sites may bind small oligosaccharides weakly, in synergy with active site residues they can significantly enhance the specificity and affinity of a glycoside hydrolase for its natural cell-wall substrates.

Materials and Methods

Cloning, mutagenesis and protein purification

The synthetic gene encoding WT-BcX was cloned into the pCW plasmid system under the control of an inducible lac promoter. The gene encoding E78Q-BcX was generated by site-directed mutagenesis using the Kunkel method. To create the plasmids expressing N54A-BcX, N141A-BcX, N181A/T183A-BcX, W185A-BcX, N54A/N181A/T183A-BcX, N54A/W185A-BcX, N141A/N181A/T183A-BcX (AAA-BcX), N141A/N181A/W185A-BcX, and E78Q/N141A/N181A/T183A-BcX (E78Q-AAA-BcX), the WT-BcX gene was sub-cloned from pCW into the NdeI/HindIII sites of pET22b (Novagen, EMD Biosciences) followed by QuikChange site-directed mutagenesis (Stratagene Cloning System, La Jolla, CA). The resulting plasmids were verified by DNA sequencing.

Protein expression and purification was done as described. The WT-BcX and E78Q-BcX were expressed from the pCW plasmid in Escherichia coli strain BL21 (DE3). Other BcX variants were expressed from the pET22b plasmid in E. coli Tuner (DE3) pLysS (Novagen, EMD Biosciences). Cells were grown at 30 °C in LB medium or in M9 medium with 1 g/l of 15NH4Cl and/or 3 g/l of 13C6-glucose (Spectra Stable Isotope Inc.), induced with IPTG (1 mM for pCW plasmids and 0.1 mM for pET22b plasmids) at $A_{600}$~0.6, and harvested after 20 h. Proteins were purified using SP-Sepharose ion-exchange chromatography (10 mM sodium phosphate buffer (pH 6.0), elution with a 0–1 M NaCl gradient) followed by Sephacryl S-100 size-exclusion chromatography (10 mM sodium phosphate buffer (pH 6.0), 150 mM NaCl) (GE Healthcare). Covalently inhibited 2FX2-BcX and 2FX2-AAA-BcX were prepared by incubation with a twofold molar excess of DNP2FX2. The purity and integrity of the protein preparations were confirmed by SDS-PAGE and mass spectrometry. Protein concentrations were determined by absorbance spectroscopy with a Biochrom Ultraspec 3000 spectrophotometer using a predicted ε280 value of 81,790 M$^{-1}$ cm$^{-1}$ for WT-BcX.

Substrates for binding and enzyme kinetic studies

ONPX2 and DNP2FX2 were synthesized according to published procedures. PNP-X12 was prepared using a glycosynthase engineered from a Cellulomonas fini retaining xylanase and purified by HPLC. X2, X4, X6, and xyloglucan (amylloid, from tamarind seed) were purchased from Seikagaku Co. (Tokyo, Japan). D-xylene, hydroxyethyl cellulose, birchwood xylan, larchwood xylan, oat spelts xylan, barley β-D-glucan, and avicel were obtained from Sigma-Aldrich (Oakville, Ontario).

To produce soluble and insoluble fractions of birchwood xylan, 5 mg of xylan powder was suspended in 50 ml of water and shaken for 5 h at room temperature. After centrifugation the pellet was washed several times with 100 mM sodium phosphate buffer (pH 6.0) and then with water until the supernatant was xylan-free, as measured by a reducing sugar assay (see below). The water was removed by lyophilization and the resulting insoluble xylan used for sedimentation binding and enzyme kinetic assays. The soluble xylan in the initial 

‡ http://ca.expasy.org/tools/protpar-ref.html
survivant, with an average DP of ~90, was either
collected by lyophilization and used for affinity electrophoresis and enzyme kinetics assays, or fractionated further by ultrafiltration through a 10 kDa cutoff membrane ( Pall Co., Ann Arbor, USA). The ultrafiltration concentrate, with a DP of ~180 and a brownish color due to heterogeneous modifications or other impurities, was washed several times with water until the flow-through was xylan-free, lyophilized, and used for kinetic assays. The initial xylan-containing flow-through from the 10 kDa cutoff membrane was subsequently concentrated with a 1 kDa cutoff membrane and further fractionated by Sephadex G-25 size-exclusion chromatography (GE Healthcare) in water at 50 °C to remove shorter xylo-oligosaccharides. The collected, colorless fractions were lyophilized and those with a DP ~180 were used for NMR binding assays. The DP values of the soluble xylan fractions were estimated by measuring reducing sugars before and after total hydrolysis by Bx, with xylotriose assumed to be the main product. Xylan and xylooligosaccharide concentrations were based on dry weight.

**NMR spectroscopy**

NMR spectra were recorded using Varian 500 Unity and 600 Inova spectrometers, processed with NMRPipe, and analyzed with Sparky software. The spectral assignments of WT-Bx and 2FX2-Bcx have been reported.

Along with consideration of these data, the main-chain 1H-15N signals of 0.8 mM 13C/15N-labeled E78Q-Bcx, in 10 mM sodium phosphate (pH 6.0) and 10% 2H2O were assigned from sensitivity-enhanced 1H-15N HSQC spectroscopy. Weighted chemical shift perturbation values (Δδi) were calculated according to equation (1) and (2) to obtain a site-specific dissociation constant (Ki) for formation of a 1:1 protein-ligand complex:

\[
\Delta \delta_i = \sqrt{(5\Delta \delta_{115i})^2 + (\Delta \delta_{215i})^2}
\]

\[
\Delta \delta_i = \frac{[P]_i + [L]_i + K_d - \sqrt{[P]_i + [L]_i + K_d - 4[P]_i[L]_i}}{2[P]_i}
\]

where \(\Delta \delta_i\) is the fit total chemical shift change upon saturation, and \([P]_i\) and \([L]_i\) are the total protein and ligand concentrations, corrected for dilution, at each point i. Since this equation is valid in the fast exchange limit with population-weighted chemical shifts, the \(K_d\) values for the AX were determined using titration data recorded at 500 MHz to minimize deviations from this limit due to conformational exchange broadening (with the exception of PNP-X12, which was titrated at 600 MHz due to sensitivity issues). Furthermore, to analyze the titration data of E78Q-Bcx, which binds X4 and X6 independently at two sites, amides with chemical shifts dependent upon binding at only the AS or only the SBS (as identified from parallel studies of E78Q-AAA-Bcx and 2FX2-Bcx, respectively) were treated as two separate groups. The \(\Delta \delta_i\) data from each group were reiteratively fit to equation (2), with \([L]_i\) corrected for the amount of ligand bound to the other site, using the averaged \(K_d\) value of that site, until the \(K_d\) values of both groups converged to their respective final values. To analyze the G5 titration curves, \(\Delta \delta_i\) was assumed to be the same as for X4. Tabulated \(K_d\) values are the averages and standard deviations of the fit values for at least seven residues in either the AS and SBS.

**NMR-monitored titrations with soluble birchwood xylan**

Titrations were performed under the same conditions as for xylooligosaccharides using a 50 mg/ml solution of birchwood xylan, fractioned by size-exclusion chromatography to a DP of ~180. In the case of E78Q-Bcx with binding in the slow exchange limit, dilution-corrected 1H-15N HSQC peak intensities of amides from the unbound protein at each point i relative to the starting intensity, I_i, were used to determine the concentrations of free (\([P]_i\) = \([P]_{i-1}\) · I_i/1) and bound (\([P]_i\) = \([P]_{i-1}\) · \([P]_i\)) protein, from which relative dissociation constants (\(K_d\)) were derived by non-linear least-squares fitting to equation (3):

\[
\frac{[L]_i}{[P]_i} = \frac{K_d}{[P]_i} + \frac{\alpha}{[N]_i}
\]

Here, \([L]_i\) is the total ligand concentration (g/l) at point i, \([N]_i\) is the concentration of binding sites in the absence of protein (mol/g), and \(\alpha\) is the number of lattice units occupied by a single protein molecule. The final two terms cannot be separated in this analysis. Note that equation (2) expresses the fraction of protein bound in terms of the free oligosaccharide concentration, whereas equation (3) expresses the fraction of xylan bound in terms of the concentration of free protein. This latter treatment is necessary because \([N]_i\) and \(\alpha\) are unknown.

In parallel, since residues from the SSs of E78Q-Bcx also showed progressive chemical shift changes (as well as decreasing intensities), their observed \(\Delta \delta_i\) values were non-linear least-squares fit to equation (4) to obtain \(\Delta \delta_e\) (but not \(K_d\) as the molarity of binding sites on the xylan is unknown). This corresponds to equation (2) without correction of \([L]_i\) for bound ligand.

\[
\Delta \delta_i = \Delta \delta_{e} + \frac{[L]_i}{K_d + [L]_i}
\]

Here, \([SBS]_i\) is the concentration of bound ([SBS]_i = \([P]_i\) · \(\Delta \delta_i/ \Delta \delta_{e}\)) and free (\([SBS]_i = \([P]_i\) − [SBS]_i)) secondary binding sites were calculated and non-linear least-squares fit to equation (3) to obtain an apparent relative \(K_d\) for the SBs. Note that \([P]_i\) was derived from
[P]_i by subtracting the concentration of protein bound in slow exchange, i.e. assuming that fast exchange behavior reflects binding at the SBS by a population of E78Q-BcX without xylan cooperatively bound in slow exchange to its AS and SBS. A similar treatment could not be made for the AS, due to xylan binding in the slow exchange limit. For E78Q-AAA-BcX, which binds soluble xylan only at its AS with approximate fast exchange behavior, Δδ_i values were obtained by fitting Δδ_i to equation (4), and then used to calculate [P]^on_1 and [P]^on_2 from which k_dr values were determined using equation (3).

In all cases, the tabulated k_dr values are the averages and standard deviations of the fit values for at least seven residues in either the AS or SBS.

Relaxation dispersion

Amide 15N relaxation dispersion data were recorded with 0.7 mM E78Q-BcX in the absence and in the presence of 0.33 mM X4 at 25 °C and with B0 fields of 11.75 T (500 MHz) and 14.1 T (600 MHz).64,65 The constant time delay T_CP was set to 40 ms, and spectra were collected in an interleaved fashion with duplicate experiments using the indicated CPMG frequencies (νCPMG in s⁻¹): 50 (×2), 100, 150, 200, 250, 300, 500, 500 (×2), 600, 700, 800, 900, 1000 (×2). The relaxation dispersion data were fit initially on a per residue basis to obtain R_rel values.66,67 Briefly, the relaxation dispersion profiles ([R]_i=1)/([R]_0) versus νCPMG, where I is the signal intensity at a given CPMG frequency and I_0 is the signal intensity of the reference experiment (for both static fields is fit on a per residue basis to a flat line, and the fast exchange equation, and F-test statistics were used to assess whether the use of the fast exchange equation was justified. Subsequently, data for residues passing the fast exchange equation were justified. Subsequently, data for residues passing the F-test were fit using the full Carver–Richards equation valid for all timescales. Again, F-statistics were used to decide whether the use of the more complicated model was justified. Significant R_ex terms (<3 Hz at 14.1 T) were observed only for AS residues (E78Q-BcX: T44, S46, S74, L76, T91, T109, T110, R112, T123, Y128, S162, E78Q-BcX + X4: T44, T71, W73, Y79, T91, T109, R110, S117, D119, T123, T124, T126, W129, S162, Y166, S176). The data for these residues were subsequently fit simultaneously to obtain a global exchange rate constant k_ex from which values of k_on and k_off were derived using equations (5)–(7):

L + P^[on]_1 → L[P]^on

k_d = [L][P]/[PL] = k_off/k_on

k_ex = k_on[L] + k_off = k_on[L]/P[PL]

where [L], [P], and [PL] denote the equilibrium concentrations of X4, E78Q-BcX, and the complex, respectively. Using the K_d data in Table 1 to account for binding to both the AS and SBS, [L] was calculated to be 0.22 mM, and the fraction of the protein with the AS in the bound state, p_{PL}, was calculated to be 0.06.

Affinity electrophoresis

The binding of inactive BcX variants to soluble polysaccharides was evaluated qualitatively by affinity electrophoresis.42 The polysaccharides (0.5%, w/v) were polymerized in native 12.5% (w/v) polyacrylamide gels with 100 mM sodium phosphate buffer (pH 6.0). Samples of protein (5 μL, 15 mg/mL) in 100 mM sodium phosphate buffer (pH 6.0), 5% (v/v) glycerol were run using a Mini PROTEAN 3 system (Bio-Rad, Mississauga, Ontario) at a constant voltage of 140 V with ice-water cooling for 4 h, followed by staining with Coomassie brilliant blue. A reference gel, polymerized without any polysaccharides, was run under the same conditions.

Adsorption isotherm measurements

The affinity of inactive BcX variants for insoluble substrates was measured using a sedimentation assay.42,65 A range of concentrations (0.1–100 mg/mL) of washed birchwood xylan or avicel were incubated with E78Q-BcX or E78Q-AAA-BcX (1–500 μM) in 100 mM sodium phosphate buffer (pH 6.0) at 4 °C or 37 °C for 2–3 h while rotating end-over-end. The samples were centrifuged twice (16,000g) for 10 min at the incubation temperature. The concentration of unbound protein [P]^off, remaining in the supernatant was measured spectrophotometrically. The data were analyzed according to equation (3) to obtain relative K_dr values.

Enzyme kinetics

Michaelis–Menten steady-state parameters with ONPX2 as a substrate were determined in 100 mM sodium phosphate buffer (pH 6.0). 0.1% (w/v) bovine serum albumin at 25 °C as described.18,20 Initial ONPX2 concentrations [S] were varied from 1.0 mM to 2.0 mM. After 15 min of preincubation, 20 μL of enzyme (0.25 μM final concentration, [E]^off) was added to 180 μL of assay solution. The initial rate of enzymatic hydrolysis, v_0, was determined by monitoring 2-nitrophenol release at 400 nm using a Cary 4000 spectrophotometer. The difference in molar absorptivity between the phenol and its xylobioside (Δε) at pH 6.0 is 1.07 mM⁻¹ cm⁻¹. Enzyme concentrations and reaction times were chosen such that less than 10% of the total substrate was hydrolyzed over the course of the experiment. Experimental rates measured at each given substrate concentration were non-linear least-squares fit to the standard Michaelis–Menten equation (8) to obtain the parameters k_cat and K_M:

v_0 = k_cat[E]^off[S] / (K_M + [S])

Michaelis–Menten steady-state parameters for soluble and insoluble xylan substrates, prepared as described above, were determined in 100 mM sodium phosphate (pH 6.0), 0.1% bovine serum albumin at 37 °C. Xylan concentrations were varied from 0.02 mg/mL to 10 mg/mL. After 1 h of incubation, the enzyme was added to a final concentration of 1–25 mM. The initial rate of enzymatic hydrolysis, v_0, was determined by monitoring the rate of generation of reducing sugar.23,69 Enzyme concentrations and reaction times were chosen such that less than 5% of the total substrate was hydrolyzed over the course of the experiment. Experimental rates measured at each given substrate concentration were non-linear least-squares fit to the standard Michaelis–Menten equation (8) to obtain apparent or relative K_M and k_cat values.

To investigate the dependence of hydrolysis rate on the chain length (or DP) and the percentage of hydrolyzable bonds hydrolyzed (extent of reaction), soluble xylan was fractionated via ultrafiltration to obtain longer polymers with a DP of ~180, as described above. Hydrolysis was
Reducing sugar assay

Reducing sugar assays were performed according as described. Samples were assayed in 1 ml of 20 mM 4-hydrobenzoylaldehyde, 0.5 M NaOH, 2 mM bismuth (III) citrate (final concentrations) and incubated at 60 °C for 5 min. After cooling to room temperature, samples were analyzed spectrophotometrically at 420 nm, with xylose as reference, to determine the initial concentrations were analyzed spectrophotometrically at 420 nm, with xylose as reference, to determine the initial concentrations of reducing sugar present before and after total hydrolysis, with xylotriose assumed to be the main product. The average chain length (DP) during the reaction as well as the extent of reaction, were calculated from the amount of reducing sugar ends generated relative to these initial and final points.

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Supplementary Data

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