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All three surface tryptophans in Type IIa cellulose binding domains play a pivotal role in binding both soluble and insoluble ligands

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Abstract The three surface tryptophans of the Type IIa cellulose binding domain of *Pseudomonas fluorescens* subsp. *cellulosa* xylanase A (CBD_{XYLA}) were independently mutated to alanine, to create the mutants W13A, W49A and W66A. The three mutant proteins were purified, and their capacity to bind to a variety of ligands was determined. The mutant proteins have native-like structures but exhibited much weaker affinity for crystalline and amorphous cellulose and for cellohexaose than the wild type. These data indicate that all three tryptophans are important for binding to cellulose, and support a model in which the three tryptophans form an aromatic strip on the surface of the protein that binds to a single cellulose.

Key words: Cellulose binding domain; Tryptophan mutant; Xylanase A; *Pseudomonas fluorescens*

1. Introduction

The β-1,4-glycosidic bonds in cellulose and xylan, the most abundant polysaccharides in the biosphere, are hydrolysed by cellulases and xylanases, respectively. The molecular architectures of these enzymes are complex. In general, they have a modular structure consisting of discrete structural and functional domains [1,2]. Many cellulases and xylanases contain a catalytic domain and one or more non-catalytic cellulose binding domains (CBDs; [1,2]). Several studies have shown that CBDs potentiate the catalytic activity of cellulases against crystalline substrates, and xylanases against cellulose/xylan complexes; however, these domains do not potentiate the activity of glycosyl hydrolases against soluble substrates [3–8]. The mechanism by which CBDs potentiate plant cell wall hydrolase activity is unclear. Din et al. [4,5] have shown that CBDs from *Cellulomonas* enzymes can disrupt the structure of cellulose microfibrils, and can increase the activity of cellulases in trans, suggesting that these domains increase the accessibility of the substrate to enzyme attack by disrupting the surface of cellulose microfibrils. In contrast, Bolam et al. [8] have shown that the CBDs from *Pseudomonas* cellulases and xylanases do not appear to disrupt the structure of cellulose microfibrils or the integrity of the plant cell wall, and do not potentiate cellulase or xylanase activity in trans. Thus, it has been suggested that the *Pseudomonas* CBDs potentiate plant cell wall hydrolases by increasing enzyme-substrate proximity.

In bacteria, the majority of CBDs is highly conserved, and has been classified as Type II domains [1]. Recently, the three dimensional structure of the Type II CBD from *Cellulomonas fimi* Cex (CBD_{Cex}) has been solved by nuclear magnetic resonance (NMR) [9]. The ligand binding region of CBD_{Cex} was suggested to contain W54 and W72, which are adjacent and exposed on the surface, and possibly W17 which is also surface-exposed, but separated from W54 and W72. Site-directed mutagenesis studies of a similar Type II CBD, from *C. fimi* CenA, showed that the equivalent residues to CBD_{Cex} W72 and W17 play an important role in the interaction of the domain with crystalline cellulose [10]. In contrast, Poole et al. [11] showed, in a qualitative assay, that when W66A or W66F (the equivalent residue to W72 in CBD_{Cex}) mutants of the Type II CBD from *Pseudomonas fluorescens* subsp. *cellulosa* xylanase A (CBD_{XYLA}) were fused to alkaline phosphatase, without a linker sequence separating the two proteins, the fusion polypeptide retained significant affinity for crystalline cellulose (Avicel). This led to the suggestion that W66 does not play an important role in cellulose binding in CBD_{XYLA}, and that the function of conserved tryptophan residues in Type IIa CBDs varies in different proteins. However, the data of Poole et al. must be viewed with some caution as the assays were only qualitative, and the effect on the interaction of the CBD with cellulose, of fusing the CBD_{XYLA} to a heterologous protein is unknown.

In addition to studies focusing on the interaction of CBDs with insoluble ligands, the binding of these proteins to oligosaccharides has also been investigated. Thus, NMR analysis of cellohexaose titrations of CBD_{Cex} showed that the ligand had a significant effect on the chemical shift of W72 and W54, but only a very small effect on the chemical shift of W17 [9]. The authors concluded that W54 and W72 play a central role in binding cellohexaose, while W17 might be less important for cellohexaose binding but could participate in the binding of longer β-1,4-glucans in cellulose [9]. The titration of CBD_{XYLA} with cellohexaose produced similar results; the chemical shifts of two tryptophan residues were significantly affected on binding of the oligosaccharide, while the chemical shift of a third tryptophan was only very slightly affected by the ligand [8].

Although the data described above suggest that the binding of cellohexaose to CBD_{Cex} and CBD_{XYLA} is broadly similar, it is unclear whether the tryptophan residue, whose chemical shift is only slightly affected by the oligosaccharide, is playing

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an important role in binding cellohexaose. In addition, it remains to be established whether the data from the W66A CBD_{XYLA}-alkaline phosphatase fusion protein [11] provided a true picture of the importance of W66 in the binding of the CBD_{XYLA} to insoluble cellulose. To resolve these questions we have substituted alanine for each of the tryptophan residues in CBD_{XYLA}, which correspond to the surface tryptophans in CBD_{Cex} (the CBD_{XYLA} residues W13, W49, and W66 correspond to the CBD_{Cex} residues W17, W54 and W72, respectively) and have investigated the capacity of the mutant proteins, expressed as discrete entities, to bind to various ligands. The data show that all three tryptophan residues play an important role in binding both cellohexaose and insoluble cellulose. Thus, although the chemical shift of W13 (equivalent to W17 in CBD_{Cex}) is largely unaffected by CBD_{XYLA} binding to cellohexaose, this residue does play an important role in the interaction between the protein domain and the oligosaccharide.

2. Materials and methods

2.1. Bacteria, vectors and culture media

Escherichia coli BL21 (DL3) containing pLysS, obtained from Novagen, was used as the host for expressing CBD_{XYLA}. The region of the *Pseudomonas* xylanase A gene (*xynA*; [8]) encoding CBD_{XYLA} was cloned into *Nde*I/*Bam*HI-restricted pET16b (Novagen) and the recombinant plasmids generated were transformed into BL21 (DE3) pLysS to express the CBD. The conditions used to culture the recombinant strains and the subsequent purification of the CBD were described previously [8]. Purified native and mutant forms of CBD_{XYLA} were homogeneous as judged by SDS-PAGE [12].

2.2. Construction of CBD_{XYLA} mutants

Mutants of the CBD_{XYLA} were generated by the transformer method (Clonetec). The target DNA molecule was recombinant pET16b containing the region of *xynA* [8] encoding CBD_{XYLA}. The selection primer was designed to destroy the *Sca*I restriction site in the bla^+ gene of pET16b. The mutagenic primers used to generate the appropriate mutations in *xynA* were as follows: W13A, 5′-ATTACCAACGAA**GC**GAACACGGGCTAT-3′; W49A, 5′-CTCAGTAGTAGC**G-C**GAATGCCAACGTG-3′; W66A, 5′-TCAAACCTGAGC**GC**GAATGGCAATATC-3′. The nucleotides in bold are the mutations introduced into the target DNA sequence. The region of *xynA* encoding the mutant forms of CBD_{XYLA} cloned into pET16b was sequenced using an ABI 373 DNA sequenator, to ensure that only the desired mutations were incorporated into the *Pseudomonas* sequence.

2.3. Biochemical analysis of CBD_{XYLA}

The capacity of native and mutant forms of CBD_{XYLA} to bind to insoluble cellulose was determined by performing binding isotherms using amorphous cellulose (acid swollen cellulose) and crystalline cellulose (bacterial microcrystalline cellulose; BMCC) as the ligands. Details of how these binding isotherms were conducted are described previously [8]. The capacity of the different forms of the CBD_{XYLA} to bind to cellohexaose was determined from 1D NMR spectra of the CBDs titrated with increasing concentrations of cellohexaose, as described previously [8].

3. Results and discussion

3.1. Interaction of CBD_{XYLA} mutants with insoluble cellulose

To investigate the importance of W13, W49 and W66 in the capacity of CBD_{XYLA} to bind to different forms of cellulose, the following three mutants of the protein domain were constructed: W13A, W49A and W66A. Native and mutant forms of the CBD were purified as described previously [8], and the capacity of the four proteins to bind to insoluble forms of cellulose was investigated. The data, presented in Table 1, show that all three mutants exhibit very low affinities for both amorphous and crystalline cellulose compared to the native protein. These data are consistent with the study of Din et al. [10], which showed that the equivalent residues to W13 and W66, in *C. fimi* CenA, also played a critical role in the capacity of the domain to bind to crystalline cellulose. The results are also in agreement with the location of the equivalent three residues in the ligand binding site of CBD_{Cex}; the three tryptophan side chains are co-planar, and are likely to form strong hydrophobic interactions with the glucose pyranose rings in the cellulose molecules situated in the site [9].

Interestingly, the results presented above contradict a previous study by Poole et al. [11] which showed, in a qualitative assay, that when W66A or W66F mutants of CBD_{XYLA} were fused to alkaline phosphatase, without a linker sequence separating the two proteins, the fusion polypeptide retained significant affinity for crystalline cellulose (Avicel). This led to the suggestion that W66 does not play an important role in cellulose binding by CBD_{XYLA}. In view of the positioning of this tryptophan residue in CBD_{Cex}, the loss of binding observed when it is mutated in CBD_{CenA} or CBD_{XYLA} (expressed as discrete domains), and the high degree of conservation of this aromatic amino acid in Type II CBDs, we suggest that W66 does play a critical role in the binding of CBD_{XYLA} to insoluble cellulose. The previous study of Poole et al. [11] in which the CBDs were fused to alkaline phosphatase did not reflect the true cellulose-binding capacity of the mutated CBDs. It is apparent, therefore, that when analysing the structural basis for the ligand binding of CBDs, these domains should be studied either as discrete entities, or as components of the original protein, but not when fused to a heterologous polypeptide.

3.2. Identification of W13, W49 and W66 in the NMR spectrum of CBD_{XYLA}

Previously, we showed that when CBD_{XYLA} binds to cellohexaose, there is a significant change in the chemical shifts of two tryptophans (at 10.13 ppm) and a small change in the chemical shift of a third tryptophan (at 10.00 ppm) [8]. To assign these three tryptophans, the NMR spectra of the native and mutant proteins were compared (Fig. 1). Most signals in the spectra of the mutant proteins are in the same place as in the native protein, demonstrating that the mutant proteins are folded correctly and have no significant conformational differences from the native protein. The data in Fig. 1 show that the tryptophan signal at 10.00 ppm in the native protein is not present in the W13A mutant protein, and can therefore be assigned to W13. A tryptophan signal at 10.39 ppm is present

Table 1
Affinity of native and mutant forms of CBD_{XYLA} for insoluble cellulose

CBD_{XYLA}	Binding affinities (K_a)[a]	
	Amorphous cellulose[b]	Crystalline cellulose[c]
Native	1.87	2.02
W13A	0.11	0.12
W49A	0.10	0.12
W66A	0.05	0.07

[a] K_a values were determined from binding isotherms essentially as previously described [8]. The affinities are expressed as 10^6 M^{-1}.
[b] Amorphous cellulose is generated from acid swollen Avicel.
[c] Crystalline cellulose is bacterial microcrystalline cellulose.

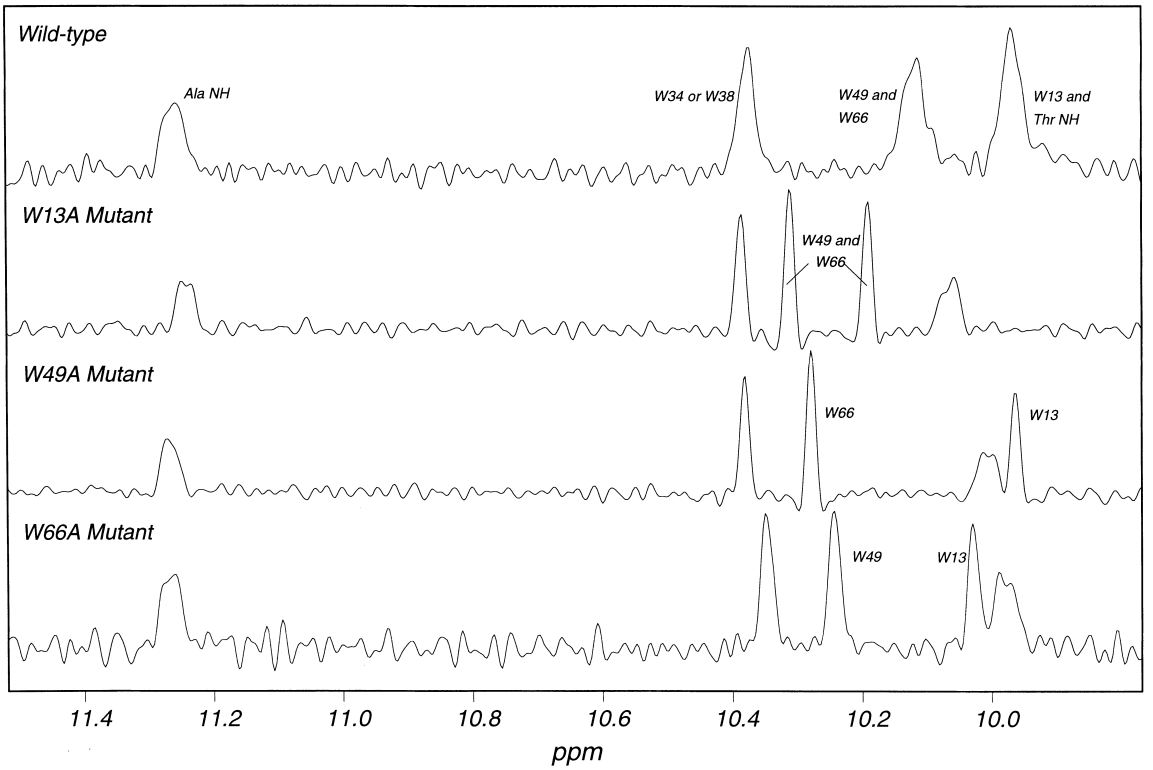

Fig. 1. NMR spectra of native and mutant forms of CBD_{XYLA}. Proteins were all approximately 0.7 mM in 50 mM sodium phosphate buffer, pH 7.0, 30°C. The spectra were acquired on a Bruker DRX 500 spectrometer and ^1H shifts were referenced to TSP as an internal standard at 0 ppm. The spectra show the low-field region of the NMR spectrum: the other regions of the spectra are almost identical to each other. Spectra of native protein at different temperatures show that there are two tryptophan signals at 10.13 ppm; 2D total correlated spectroscopy (TOCSY) demonstrated that the signals at 11.27 and 10.00 ppm are from alanine and threonine backbone amide protons, respectively.

in native and mutant proteins, and is assigned to either W34 or W38. The two overlapping signals at 10.13 ppm are shifted slightly in W13A, and one is missing in both W49A and W66A. Therefore the signals at 10.13 ppm have been assigned to W49 and W66. The changes in chemical shift seen in the mutants are consistent with the close proximity of W49 and W66 in the NMR structure of CBD_{Cex}, which means that removal of one tryptophan affects the signals from the other. A similar argument applies to changes affecting W13, which is close to W49.

These assignments show that the signals undergoing a large chemical shift change on titration of native protein with cellohexaose are from W49 and W66, and the signal undergoing a smaller chemical shift change is from W13. The results (and those of the binding studies presented above) are consistent with those of Xu et al. [9] on CBD_{Cex}, and suggest that the structures of the Type IIa CBD_{XYLA} and CBD_{Cex} are highly conserved and do not change significantly on addition of cellulooligosaccharides, and that the location and function of W13, W49 and W66 in CBD_{XYLA} are equivalent to those of W17, W54 and W72, respectively, in CBD_{Cex}.

3.3. *Importance of W13 in the binding of CBD_{XYLA} to cellohexaose*

Although the data described above clearly implicate W13, W49 and W66 in binding CBD_{XYLA} to insoluble cellulose, the very small change in the chemical shift of W13 (and its equivalent W17 in CBD_{Cex}) calls into question the importance of this residue in binding soluble cellulooligosaccharides. For this reason, Xu et al. [9] suggested that it may rather be involved in the binding of longer β-1,4-glucans (or possibly parallel cellulose chains in cellulose fibrils). To investigate the importance of W13 in the binding of CBD to cellohexaose, native and mutant CBD_{XYLA} were titrated with cellohexaose, and the NMR spectra of the proteins were recorded. The results (Fig. 2) show that, when any one of the three surface tryptophans is mutated to alanine, the chemical shift changes of the remaining two are much smaller than they are in the native protein. These results imply that all three tryptophans are required for good binding to cellohexaose, and that the binding site encompasses all three tryptophans (or the remaining two when one is mutated out). This conclusion is sterically plausible, since in CBD_{Cex} W17 and W72 are about 30 Å apart, which is the same as the separation between the terminal saccharide units of cellohexaose [13]. Furthermore, the conclusion is in agreement with the model presented by Tormo et al. [13], which proposes that the three surface exposed tryptophan residues in CBDs act as a planar binding strip for a single cellulooligosaccharide chain.

Several NMR studies have shown that there is no direct relationship between the magnitude of chemical shift changes and binding strength. This study shows that W13 appears to be as important as W49 or W66 to cellohexaose or cellulose binding to CBD_{XYLA}, even though its chemical shift changes are much smaller. The chemical shift of Trp NH∋ can be affected by neighbouring aromatic rings, peptide bonds or hydrogen bonds, and is highly sensitive to small changes in local geometry, in a way that is not easily predictable [14], implying that the magnitude of a chemical shift change should be interpreted with caution.

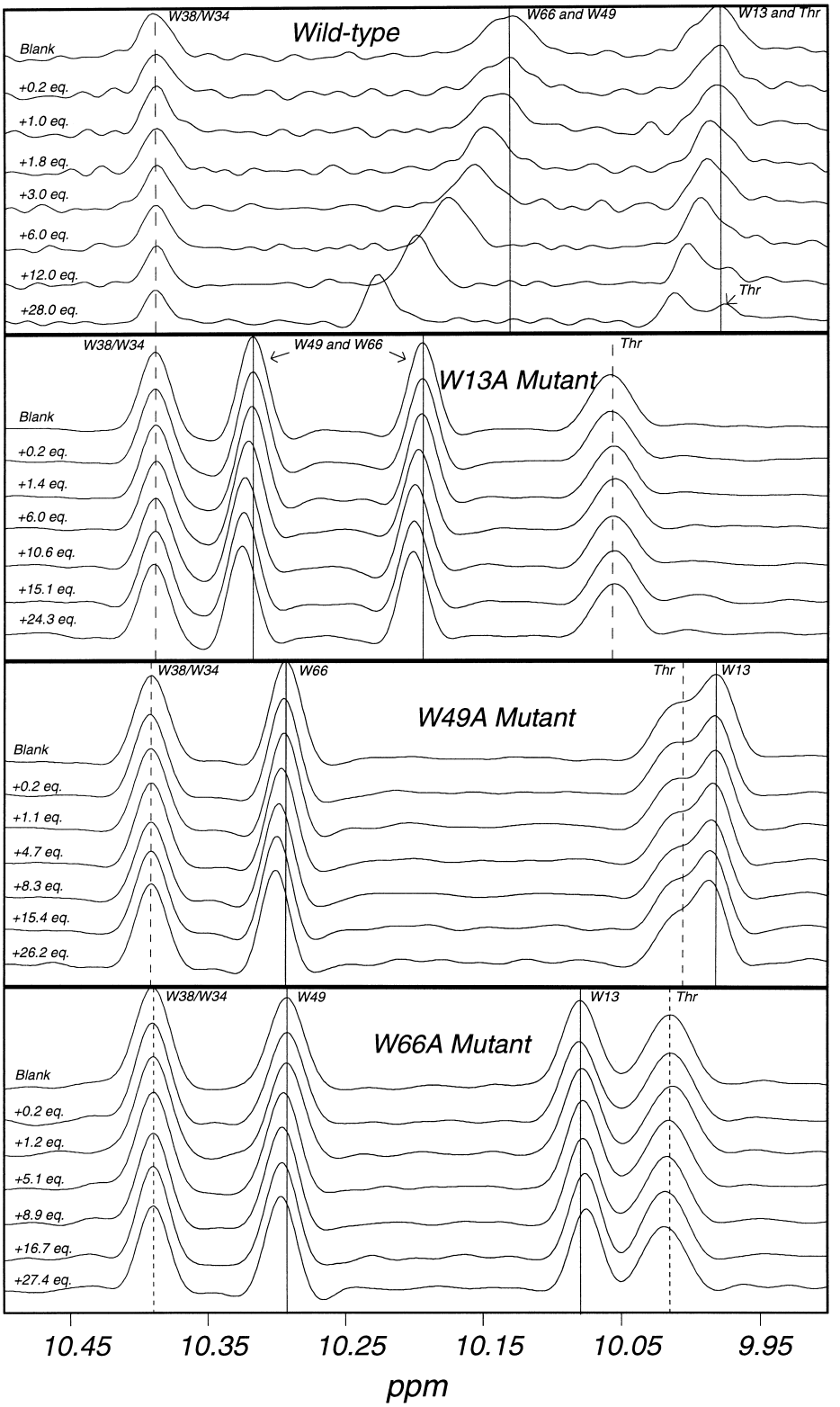

Fig. 2. Titrations of native and mutant forms of CBD_{XYLA} with cellohexaose. Spectra show the regions of the NMR spectra of wild-type, W13A, W49A and W66A (all ca. 0.7 mM) that contain the tryptophan NH? signals for W13, W49 and W66, with (from top to bottom) increasing amounts of cellohexaose, up to about 25 equivalents in each case. To guide the eye, vertical lines have been drawn through each signal. Dashed lines indicate signals that are not affected by the addition of cellohexaose, while solid lines indicate signals that move on addition of cellohexaose. Chemical shift changes in all three mutants are smaller than in the wild type.

To conclude, the data presented in this study have clarified the role of the tryptophan residues located on the surface of Type IIa CBDs. It is apparent that the pivotal role of these residues in the binding of CBDs to both crystalline cellulose

and soluble oligosaccharides, is conserved in a range of Type IIa CBDs. There is no evidence to suggest that the function of these aromatic amino acids varies depending on the origin of the Type IIa domain, as suggested previously [1,8,11].

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