**Analysis of Surface Binding Sites (SBS) within GH62, GH13, and GH77**

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Abstract: Certain interactions between carbohydrate active enzymes and polysaccharides involve surface binding sites (SBS) situated on catalytic domains outside of the active site. We recently undertook to develop a toolbox for SBS identification and characterization. In affinity gel electrophoresis (AGE) SBS containing proteins are migrating slower in native polyacrylamide electrophoresis gels cast with polysaccharide *versus* without polysaccharide. Amylolytic enzymes from GH13 and GH77 and xylanases from GH10 and GH11 are the best studied GH families with respect to SBS, presenting about half of the reported SBSs. In GH13 SBSs have been seen in 17 subfamilies including SBSs with highly diverse functions in the same enzyme. Circumstantial evidence is provided for an SBS in the GH77 MalQ from *Escherichia coli*, the bacterial orthologue of *Arabidopsis* DPE2 involved in starch metabolism. Furthermore, *Aspergillus nidulans* α-L-arabinofuranosidase AnAbf62A-m2,3 of GH62 that has very high activity on wheat arabinoxylan (WAX) shows an unusually strongly retarded migration by WAX during AGE analysis. Using a recent GH62 crystal structure as template, Trp2 and Tyr46 in an AnAbf62A-m2,3 model are proposed to form an SBS situated about 30 Å from the catalytic site. Compared to wild-type, W23A/Y44A AnAbf62A-m2,3 retained 45% activity on WAX and was less retarded in AGE by WAX as well as by barley β-glucan and birchwood xylan, which are neither hydrolysed nor inhibiting activity towards WAX. The presence of a functional SBS agrees with W23A/Y44A AnAbf62A-m2,3 retaining only 3–25% activity for arabinosyl-oligosaccharides (AXOS) of DP 3–5 possibly reflecting allosteric activation of wild-type through SBS occupation by AXOS.

Key words: affinity gel electrophoresis, surface plasmon resonance, arabinofuranosidase, α-amylase, arabinoxylan, starch

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**INTRODUCTION**

Interactions between carbohydrate-active enzymes and polysaccharide substrates or polysaccharides residing in complex biological matrices, such as plant cell walls are characterized by the interacting macromolecules being much larger than the enzyme and often of very diverse structural organization. As a step to facilitate intimate and appropriately persisting contact the enzymes have evolved a number of handles. Best known and by far the most widely occurring are carbohydrate binding modules (CBMs). CBM polysaccharide specificity can be the same as that of the enzyme catalytic domain or different from this, *e.g.*, for a non-substrate polysaccharide component in plant biomass. Examples of the first case are starch binding domains (SBDs) attached to the starch-converting enzymes glucoamylase, cyclodextrin glucanotransferase or glucan water dikinase, while an example of the second case can be a cellulose binding domain attached to a xylanase. In particular during the past decade, however, a growing number of
studies reported about surface binding sites (SBSs), also called secondary binding sites, in carbohydrate-active enzyme that interact with polysaccharides. SBSs are characteristically situated at a distance of the active site on the surface of the enzyme catalytic domain or of a domain intimately associated with the catalytic domain. SBSs are able to engage in specific binding providing for example a polysaccharide anchoring function comparable to that of CBMs. Recently, SBSs have been the topic of reviews and a book chapter. It is noted that SBSs and CBMs appear also to play complimentary roles as some enzymes contain both these types of structures. Even though the majority of SBSs have been identified in glycoside hydrolases, they are also reported in glucan phosphatases, starch, and glycogen synthases as well as plant α-glucan phosphorylase PHS2 and from early on in glycogen phosphorylases.

FUNCTIONAL ROLES PLAYED BY SBSSS

To summarize the type of roles played by SBSs in carbohydrate-active enzymes, classically SBSs can target enzymes to their polysaccharide substrate, guide the substrate polysaccharide chain into the active site and probably be involved in disentangling of supramolecular structures by isolating separate constituting polysaccharide chains for access by the enzyme active site. These various mechanisms may apply to water-insoluble as well as water-soluble or solubilised (dissolved) polysaccharides. By effectively localizing the enzyme to a polysaccharide target and directing the polysaccharide chain to the active site, SBSs can facilitate processive action, also referred to as multiple attack, which stands for consecutive catalytic events carried out in a single enzyme substrate encounter, hence augmenting the efficacy of product formation which is important once an enzyme is functionally advantageous. In fact a large number of enzymes interacting with different polysaccharides can share architectural features of their SBSs, whereas several enzymes interacting with xylan apply distinctly different modes of binding (Casper Wilkins, unpublished work). Characteristic features of SBSs include adjacent carbohydrate residues stacking onto a couple of aromatic rings which may be from neighboring residues in the protein sequence. Other SBSs look like clamps or coin slots constructed by two aromatic rings and yet others also appear as clamps and undergo conspicuous conformational changes by grasping the sugar ligand as a “pair of tongs”.

METHODS FOR IDENTIFICATION AND CHARACTERIZATION OF SBSSS

By far the largest number of SBSs have been identified in crystal structures, which were solved either after crystallization or soaking of preformed crystals with substrates, products, inhibitors or substrate mimics. NMR has also been to identify and characterize an SBS in a xylanase. To validate the possible importance of the sites with observed capacity to bind oligosaccharides, mutational analysis represents a straightforward approach that consists in site-directed mutagenesis of the recombinant protein followed by characterization of functional and stability properties of the variants. It has been sought to uncover characteristic structural elements of SBSs for the purpose of mining structural databases. However, no robust tool has yet been developed possibly reflecting a wide structural diversity of SBSs (Darrell Cockburn, unpublished work). It was also noticed that a larger number of enzymes interacting with different α-glucans can share architectural features of their SBSs, whereas several enzymes interacting with xylan apply distinctly different modes of binding (Casper Wilkins, unpublished work). Characteristic features of SBSs include adjacent carbohydrate residues stacking onto a couple of aromatic rings which may be from neighboring residues in the protein sequence. Other SBSs look like clamps or coin slots constructed by two aromatic rings and yet others also appear as clamps and undergo conspicuous conformational changes by grasping the sugar ligand as a “pair of tongs”.

Despite the hurdles encountered with developing useful prediction tools for identifying SBS candidates, insight available on the structural characteristics of SBSs has been exploited in attempts to introduce improvements of SBSs by protein engineering. A few relatively successful cases have been reported on such structure-guided SBS engineering as well as a case of in vitro evolution in different xylanases. However, for rational introduction of novel functionally advantageous SBSs one shall probably consider in silico protein engineering procedures involving computational structural design.

Identification of novel SBSs by simply screening protein crystals with oligosaccharides (e.g., via soaking experiments) for occupation on the surface at a distance of the active site seems very attractive. However, such an approach is practical only for those working with crystallography groups and is likely to result in false positives and false negatives due to interactions of carbohydrates in protein crystals that are not having a functional foundation. Another rather simple way to test an enzyme for the presence of SBSs is by affinity gel electrophoresis (AGE) in which native PAGE in gels cast with soluble polysaccharide causes slower migration of an interacting protein versus its migration in a control without polysaccharide. (Wilkens et al., in preparation). We conducted screens using AGE for about 50
enzymes and 20 polysaccharides. An excerpt of our data is given in Fig. 1 (Darrell Cockburn and Casper Wilkens, unpublished work).

AGE can also be used to estimate the binding constant, $K_d$, for the enzyme complex formation from a series of experiments giving migration distances measured relative to a reference protein and applying the equation $\frac{1}{R_{mi}} = \frac{1}{R_{mo}} \times \{1 + (c/K_D)\}$ in plots of $\frac{1}{R_{mi}}$ versus the polysaccharide concentration ($c$), where $R_{mi}$ and $R_{mo}$ are mobilities of the protein relative to reference protein in the presence and absence of polysaccharide.48)

SBSs have also been characterized for oligosaccharide binding and specificity by aid of surface plasmon resonance (SPR) and isothermal titration calorimetry (ITC) analyses.8,12,14,15,20,25-27,32,49,50) Combination of SPR and ITC as well as AGE with a mutational analysis is usually very powerful for elucidation and characterization of the roles of key functional residues in SBS. This strategy has been employed also to investigate individual SBSs in the same enzyme and discriminate interactions at SBSs from those occurring at the active site, which is generally an important shortcoming of screening for SBSs using AGE and SPR. Even though active site mutagenesis may counter this complication, it can still be problematic to efficiently eliminate binding along the entire length of the substrate binding area at the active site and only few suitable reactive carbohydrate derivatives or analogues of substrates or products are available for mechanism-based covalent modification at the catalytic site or have a level of affinity suitable for discriminating between the active site and the SBSs in question.25-27) Finally direct observation of the binding onto insoluble substrates, e.g., starch granules can be pursued combining confocal laser scanning microscopy (CLSM) and mutational analysis of SBSs.32)

**SURFACE BINDING SITES IN GH13**

The most thoroughly investigated SBSs in GH13 belong to barley $\alpha$-amylase 1 (AMY1).5,7,8,32,33,35,37,45,49) This very large GH13 family was first subdivided into 34 subfamilies51) and today 17 of the more than 40 subfamilies in GH13 have been found to contain an SBS.26,27) In the past we published work on the discrimination between the two SBSs of AMY1 (SBS1 and SBS2). SBS1 has a prominent role in adsorption onto starch granules, while SBS2 was involved in interaction with single $\alpha$-glucan chains and associated with the fast and high-affinity component in the kinetics of amylopectin hydrolysis.8,33) Recently, we thus extended characterization of SBS1 and SBS2 in AMY1 to include interaction with starch granules of different botanical origin and type (i.e., normal, waxy, high-amylose) as well as the retardation by amylose, amylopectin, pullulan, and glycogen in AGE and distinction of interactions of maltooligosaccharides at the active site.32) The binding to starch granules was determined using the Langmuir adsorption isotherm and the modified Langmuir adsorption isotherm:

$$B = \frac{B_{\text{max}} [S]}{K_d + [S]} \quad B = \frac{B_{\text{max}} [S]}{K_d + [S] + \frac{[S]^2}{K_s}}$$

$B$ is the fraction of bound enzyme, $[S]$ the concentration of starch granules, $B_{\text{max}}$ the maximum bound fraction, $K_d$ the dissociation constant, and $K_s$ the substrate inhibition constant. While $K_d$ for normal corn starch was 0.24 mg/mL, the affinity was 50% higher for waxy and only 60% for high-amylose corn starch granules compared to normal ones.25) When analyzing the SBS2 Y380A mutant, its affinity for all three types of starch granules was approximately 10% of that of wild-type, however, compared to wild-type AMY1 for the...
SBS1 mutant W278A/W279A the affinity was 50, 75, and 30 fold reduced for normal, waxy and high-amylose corn starch granules, respectively. This indicated both the higher affinity for the waxy, i.e., “all amylopectin” starch granules and the lower affinity for the high-amylose granules as well as the larger importance of SBS1 versus SBS2 in binding onto starch granules. Secondly it underscored the preference for amylopectin binding by SBS2 over SBS1. The binding data were complemented by CLSM analyses of the SBS1, SBS2 and double SBS1/SBS2 mutants, of which the latter failed to bind to the granule surface. However, at damaged areas of the granules, e.g., cracks interrupting surface integrity, even the double SBS1/SBS2 mutant showed intense binding to the cracks suggesting that in this case the active site was able to interact with these newly exposed distinctly different structural elements of the granule.32)

When analyzing the same SBS mutants and wild-type AMY1 in AGE for interaction with amylopectin, glycogen, amylose, and pullulan in solution (Fig. 2) retardation of wild-type was largest by glycogen followed by pullulan, amylopectin and amylose, decreasing in this order. The SBSs mutants again migrated illustrating the greater role of SBS1 in interaction with the α-glucans. A much more prominent effect, however, resulted with the SBS1/SBS2 double mutant W279A/Y380A, indeed the two SBSs in AMY1 are acting in synergy. SPR analysis of the different SBS1, SBS2, and SBS1/SBS2 mutants in combination with the binding of β-cyclodextrin, that has 20 fold higher affinity for SBS2 than SBS1 in wild-type AMY1,8) indicated that binding to maltoheptaose primarily occurred at SBS2 with $K_d$ around 1.2 mM, while $K_m$ for the active site was 1.1 mM, and binding to SBS1 was concluded to be much weaker than to SBS2 and the active site.32)

SURFACE BINDING SITES IN GH77

The disproportionating enzymes of GH77 belong to the same clan GH-H of glycoside hydrolases as members of GH13, also called the α-amylase family. The clan GH-H enzymes share similar substrates and protein structural and domain organization including catalytic residues and catalytic site geometry. An enigmatic GH77 enzyme DPE2 involved in starch metabolism in plants has a counterpart in MalQ from Escherichia coli that is able to complement an Arabidopsis mutant plant lacking the DPE2 protein via a dpe2 gene knock out.50) MalQ does not contain a starch binding domain (SBD), while DPE2 has two SBDs of the CBM20 family N-terminally to the catalytic domain. Both MalQ and DPE2 wild-type bind the small starch mimic β-cyclodextrin with a sub-millimolar $K_d$ as measured by SPR. Since β-cyclodextrin is not expected to be able to bind at the active site, this raised the question as to whether MalQ has an SBS that serves the same role as the CBM20(s) in DPE2.50) However, comparing the binding of maltooligosaccharides of DP 3–7 to DPE2, MalQ and a variant with the proximal DPE2 CBM20 fused to the N-terminus of MalQ, showed the characteristic almost three orders of magnitude larger affinity of MalQ compared to DPE2 being essentially preserved in the CBM20-MalQ protein chimera. The DPE2 has a preference for maltohexaose as opposed to MalQ and CBM20-MalQ both showing increasing affinity with decreasing DP of maltooligosaccharides. Thus in spite of MalQ being capable of complementing for the physiological loss of DPE2 in dpe2 knock out plants, the two enzymes display distinctly different substrate preference and range of affinity.50)

Previously, the amylomaltase from Thermus aquaticus was found to have an SBS in the crystal structure.39) Mutational analysis of Tyr$^{34}$ situated at the SBS suggested that a resulting loss of binding at this site was accompanied by favoured flexibility for substrate enzyme interactions at the active site compatible with the unimolecular cyclization reaction producing large cyclodextrins. By contrast allosteric regulation through occupation of the SBS was needed for the disproportionation, hydrolysis, and coupling (transglycosylation) reactions also catalysed by this enzyme and this
was negatively influenced by mutation of the SBS.38)

SURFACE BINDING SITES IN GH62

α-L-Arabinofuranosidases (ABFs) are found in five different GH families.1) Recently we cloned and produced recombinantly an annotated enzyme from Aspergillus nidulans.52) AnAbf62A-m2,3 belonging to the small GH62 family that only contains ABFs and found that the enzyme exhibited exceptionally high activity towards WAX. AnAbf62A-m2,3 was exceptionally strongly retarded in AGE by WAX (Fig. 1) (Wilkens et al., in preparation), suggesting the presence of extra binding sites, since the enzyme has no CBM as deduced from analysis of the sequence (Casper Wilkens, unpublished observation). Lack of any crystal structure from the GH62 family left us at that time with a working model obtained using several GH43 ABF structures as template as GH62 and GH43 constitute clan GH-F.1) We also demonstrated the inverting mechanism by the release of β-arabinose from arabinoxylo-oligosaccharides (AXOS; Fig. 3) monitored by using NMR in addition showing that 1,3-linked arabinose was released about three times faster from the backbone xylose residue versus 1,2-linked arabinose (Wilkens et al., in preparation). In 2014 five crystal structures were published of GH62 enzymes from various fungi and bacteria.53–56) In a model of AnAbf62A-m2,3 made using the crystal structure of SthAbf62A from Streptomyces thermoviolacius55) (PDB ID 4O8O) as template, our attention was now drawn to Trp23 and Tyr44 in AnAbf62A-m2,3 situated near each other and adjacent to the extended putative substrate binding cleft. The candidate SBS was located at a distance of about 30 Å from the catalytic site (Fig. 4), where the AXOS decorating arabinose residue is accommodated in a narrow pocket at subsite −1 and cleaved off from the backbone xylose residue situated at subsite +1. The double mutant W23A/Y44A was much less retarded in AGE by WAX, but still retained 45% activity of wild-type on this polysaccharide substrate. Notably, two other cell wall polysaccharides barley β-glucan and birchwood xylan retarded AnAbf62A-m2,3 in AGE even though these are neither substrates nor inhibit the hydrolysis of WAX. The retardation in AGE by these two polysaccharides was affected by the mutation similarly to the effect on retardation by WAX suggesting that their binding interactions occur at an SBS that accommodate these different polysaccharides (Wilkens et al., in preparation). Surprisingly, the W23A/Y44A mutant lost 65–96% of the activity of wild-type towards AXOS substrates (Fig. 3). This larger reduction than found with WAX hinted at that AXOS binding, which was lost in the W23A/Y44A mutant, in wild-type elicits allosteric activation, reminiscent to roles played by oligosaccharide substrate SBS interactions observed for other enzymes.11,33,37–39) It was not possible to

![Fig. 4. Close-up of the putative surface binding site containing Trp23 and Tyr44 of AnAbf62A-m2,3 from Aspergillus nidulans modelled using SthAbf62A from Streptomyces thermoviolacius55) (PDB ID 4O8O) as template.](https://doi.org/10.1016/j.enzmictec.2020.10.015)

Fig. 3. Arabinoxylo-oligosaccharides (AXOS) preparations AX2(I), AX3 (I), and AX4 (I) of DP 3–5 used for characterization of the activity of GH62 α-L-arabinofuranosidase AnAbg62A-m2,3 from Aspergillus nidulans wild-type and double mutant W23A/Y44A at the putative surface binding site (SBS) (see Fig. 4).
predict based on structures and sequence comparisons if a similar functional site will be found in other GH62 enzymes. However, with regard to the two subgroups defined by phylogenetic analysis of GH62, Trp is conserved in 75% of all family members, but not strictly within subgroups 1 or 2, while Tyr is only present in 8% of the GH62 members, all from subgroup 2.

CONCLUSION AND PROSPECTS

Even though SBSs have been identified in rather few among the very large number of carbohydrate-active enzymes they do represent a functional feature confined to a well defined non-catalytic structural site. Cases are reported where SBSs have been proven to be essential for function and there is a need to establish a comprehensive database of SBSs and develop predictive search tools for structural databases. It is relevant to provide the scientific community with a knowledge base on the one hand delivering access to insights on SBSs and on the other generating a starting point for rational engineering to achieve desired properties by introduction of functional SBSs.

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