Analysis of surface binding sites (SBS) in carbohydrate active enzymes with focus on glycoside hydrolase families 13 and 77 – A mini-review

Darrell COCKBURN[1], Casper WILKENS[1], Christian RUZANSKI[2], Susan ANDERSEN[1], Jonas WILLUM NIELSEN[3], Alison SMITH[2], Martin WILLEMOËS[3], Maher ABOU HACHEM[1] & Birte SVENSSON[1,*]

[1.] *Enzyme and Protein Chemistry, Department of Systems Biology, Technical University of Denmark, DK 2800 Kgs. Lyngby, Denmark*

[2.] *John Innes Center, Norwich, England*

[3.] *Department of Biology, University of Copenhagen, Denmark*

dcoc@bio.dtu.dk (Darrell Cockburn)

cwil@bio.dtu.dk (Casper Wilkens)

christian.ruzanski@carlsberglab.dk (Christian Ruzanski)

sjes@bio.dtu.dk (Susan Andersen)

jonaswillum@gmail.com (Jonas Willum Nielsen)

alison.smith@jic.ac.uk (Alison Smith)

willemoes@bio.ku.dk (Martin Willemoës)

maha@bio.dtu.dk (Maher Abou Hachem)

bis@bio.dtu.dk (Birte Svensson)

Running Title: Surface Binding Sites in GH13 and GH77

Abstract: Surface binding sites (SBSs) interact with carbohydrates outside of the enzyme active site. They are mostly situated on catalytic domains and are distinct from carbohydrate

binding modules (CBMs). SBSs are found in a variety of enzymes and often seen in crystal structures. Notably about half of the > 45 enzymes (in 17 GH and two GT families) with an identified SBS are from GH13 and a few from GH77, both belonging to clan GH-H of carbohydrate active enzymes. The many enzymes of GH13 with SBSs provide an opportunity to analyse their distribution within this very large diverse family. SBS containing enzymes in GH13 are spread among 15 subfamilies (two were not assigned a subfamily). Comparison of these SBSs (some enzymes have more than one) reveals a complex evolutionary history with evidence of conservation of key residues between some SBSs, divergence for others with residues changing or convergence where SBSs arise at varying structural locations. An array of investigations of the two SBSs in barley α-amylase demonstrated they play different functional roles in binding and degradation of polysaccharides. At least one GH77 enzyme is known to contain an SBS and there are likely more. *Escherichia coli* MalQ is an α-1,4-glucanotransferase of GH77 and binding studies using surface plasmon resonance (SPR) indicated similar affinity for β-cyclodextrin for a homolog DPE2 from *Arabidopsis thaliana* although MalQ lacks CBMs and DPE2 has two CBM20s. SPR showed that MalQ binds malto-oligosaccharides of >DP4 at a second site in competition with β-cyclodextrin yielding a stoichiometry >1.

Key words: secondary binding sites; carbohydrate binding modules; GH13 subfamilies; crystal structures; surface plasmon resonance; affinity gel electrophoresis; amylopectin hydrolysis kinetics

Introduction

Polysaccharides degraded or modified by carbohydrate active enzymes are often binding to the enzymes *via* dedicated carbohydrate binding sites that are situated outside of the active

site area. Such additional binding sites can be found on carbohydrate binding modules (CBMs) that are independent domains or they can be present in the form of so-called surface binding sites (SBSs) that are exposed on the surface of catalytic domains or on a module intimately associated with this domain. SBSs have been described to carry out a suite of functions especially needed for enzymatic reactions with biological macromolecules and supramolecular structures as found in *e.g.* plant cell walls, chitin, and starch granules. This review focuses on SBSs engaged in enzyme catalyzed reactions involving prominent naturally occurring α-glucans, *i.e.* the starch components amylose and amylopectin, glycogen and related polysaccharides and oligosaccharides. SBSs play essential roles in a number of amylolytic enzymes, particularly in the degradation of starch granules, which can be of a variety of sizes, shapes and amylopectin : amylose ratios depending on the botanical sources. Gene manipulations have enabled production of biosynthetically engineered starches, *e.g.* with characteristic high or low contents, respectively, of amylose and amylopectin, which has allowed the study of the importance of these properties of starch granules in enzyme degradation, including binding at SBSs (Cockburn et al. in preparation).

As of today SBSs have been identified in more than 45 enzymes from 17 glycoside hydrolase (GH) and two glycosyl transferase (GT) families classified in the database of Carbohydrate Active enzymes CAZy (http://www.cazy.org/; Cantarel et al. 2009) as well as in α-glucan phosphatases (for a review see Gentry et al. 2009). The vast majority of the known SBSs have been observed by structural analysis of enzyme ligand complexes primarily by X-ray crystallography, but also in a couple of cases by using NMR spectroscopy (Ludwiczek et al. 2007; for reviews see Cuyvers et al. 2012 and Cockburn & Svensson 2013). Surprisingly, inspection of the CAZy database (Cantarel et al. 2009) revealed that a larger proportion of SBS containing glycoside hydrolases also possess a CBM as compared to the frequency of

CBM occurrence in all glycoside hydrolases in the database (Cockburn & Svensson 2013). It was suggested therefore that rather than performing the same roles, SBSs and CBMs that co-exist in selected enzymes have complementary functions.

The increasing awareness of SBSs has in recent years driven the establishing of a set of analytical tools applicable in their identification and characterization. A straightforward strategy has basis in SBSs observed in three-dimensional structures subsequently subjected to mutational analysis and comparison of functional properties of mutant and wild-type enzyme forms. Confounding effects of carbohydrate interaction with active sites may be eliminated by blocking these by aid of mechanism based covalent inhibitors or other tight-binding specific inhibitors. Noticeably binding analysis by using NMR is able to resolve contributions from interaction at the active site and at SBSs, respectively. However, in the case of α-glucan active enzymes these latter approaches have not been implemented, whereas they were applied for two xylanases (Ludwiczek et al. 2007; Cuyvers et al. 2012).

SBSs are reported to display a variety of distinct potential functional roles; i) substrate targeting; ii) guiding substrate into the active site; iii) disrupting substrate structure; iv) enhancing processivity; v) allosteric regulation; vi) passing on reaction products; vii) attachment to cell walls; viii) substrate specificity control; and ix) as pharmaceutical chaperones (Guce et al. 2010; Cuyvers et al. 2012; Nielsen et al. 2012; Cockburn & Svensson 2013). Examples on most of these roles have been found for SBSs in different enzymes active on α-glucans. So far evidence of SBSs has not been given for amylolytic or related transglycosylating enzymes from other families than GH13 and GH77, which together with GH70 constitute clan GH-H. There is no report, however of SBSs in GH70 enzymes (glucan and dextran sucrases) (Leemhuis et al. 2013), although the related glycosidase amylosucrase of GH13_4 has two SBSs (Albenne et al. 2004) and the reactions catalysed by enzymes in

GH70 are anticipated to benefit from participation of SBSs.

Occurrence of surface binding sites in α-glucan active enzymes

The existence of secondary binding sites was first described for porcine pancreatic α-amylase almost five decades ago by Loyter & Schramm (1966), who investigated the interaction with macromolecular limit dextrins of glycogen by using light scattering, analytical ultracentrifugation and electron microscopy and also determined a binding stoichiometry of two for maltotriose by aid of equilibrium dialysis. Also early on, a binding site 25 Å from the active site was reported for rabbit muscle glycogen phosphorylase (Fletterick et al. 1976). Later, differential chemical modification of barley α-amylase 2 (AMY2) in complex with β-cyclodextrin, identified a carbohydrate binding site outside of the active site that contained two adjacent tryptophanyl residues (Gibson & Svensson 1987). This SBS was confirmed in the crystal structure of AMY2 in complex with the pseudotetrasaccharide inhibitor acarbose that was accommodated at both the active site and on the "double tryptophan" site situated on the side of the catalytic $(\beta/\alpha)_8$ barrel domain (Kadziola et al. 1998). A second SBS was occupied by different oligosaccharides on the C-terminal five-strand anti-parallel β-sheet domain in the crystal structure of the AMY1 isozyme that has approximately 80% sequence similarity to AMY2 (Robert et al. 2003, 2005) (Figure 1A). The two sites were named "starch granule binding site" and "a pair of sugar tongs" and have more recently been referred to as SBS1 and SBS2, respectively.

GH13 today contains more than 16,000 sequences in CAZy (Cantarel et al. 2009) and was in 2006 divided into subfamilies represented by clustering in a phylogenetic tree (Stam et al. 2006). Currently, SBSs have been found in 15 of the 36 GH13 subfamilies (1-11, 14, 24, 31, 36) and in two GH13 members with no subfamily assignment (Table 1). Several of these

enzymes, similarly to the barley α-amylase of GH13_6, have more than one SBS. Human salivary α-amylase of GH12_24 thus was shown to have three SBSs (Ragunath et al. 2008) (Figure 1B) and *Neisseria polysaccharea* amylosucrase in GH13_4 has two SBSs (Albenne et al. 2004) (Figure 1C). Five and seven SBSs were reported in maltooligosyltrehalose trehalohydrolase from *Deinococcus radiodurans* (Timmins et al. 2005) and *Escherichia coli* branching enzyme (Fawaz et al. 2013).

With respect to GH77 an SBS was seen in the crystal structure of the amylomaltase from *Thermus aquaticus* in complex with acarbose (Przylas et al 2000). Circumstantial evidence has also been obtained for the presence of an SBS in the GH77 member *E. coli* amylomaltase MalQ that is able to restore an essentially wild-type phenotype when expressed in mutant Arabidopsis plants lacking the 4-glucanotransferase DPE2 (Ruzanski et al. 2013).

In addition α-glucan active enzymes containing an SBS include starch and glycogen synthases (Baskaran et al. 2011; Diaz et al. 2011; Cuesta-Seijo et al. 2013), phosphatases (Gentry et al. 2009) and glycogen phosphorylases (Fletterick et al. 1976; Pinotsis et al. 2003), which will not be addressed in the present review.

Methodologies relevant for detection, identification and characterization of SBS

SBS are most readily identified in three-dimensional structures and functional properties are then described typically by aid of site-directed mutagenesis. Despite the possibility for binding interactions with polysaccharides at both active sites as well as SBSs, it turned out that retardation affinity gel electrophoresis (AGE) is a relatively simple and informative method when it comes to screening for potential SBS containing enzymes. AGE consists in comparison of the migration of the enzyme under study in native gel electrophoresis in the presence and in the absence of the polysaccharide(s) to be tested for binding. AGE can be

replaced by pull-down or co-precipitation experiments in case of insoluble polysaccharides. In AGE the enzyme migration will be retarded by polysaccharide interactions. Other options include carbohydrate array binding analysis and sequence comparison with closely related SBS containing proteins, though as discussed above, within GH13 SBSs are diverse in both their composition and structural location. An overview of these various procedures has been published recently (Cockburn & Svensson 2013). However, based on a positive indication of the presence of an SBS in the form of retarded migration in AGE, it is still not known where one or perhaps more SBSs are found in the structure of the enzyme. Molecular modeling or an available crystal structure can be very helpful in identifying amino acid side chains belonging to a putative SBS. The next step is then site-directed mutagenesis of the residue(s) in question followed by AGE and other binding studies, *e.g.* surface plasmon resonance (SPR) analysis for the mutant forms of the enzyme. When an SBS has been confirmed, quantitative studies of its binding affinity and ligand specificity can be performed by SPR or isothermal titration calorimetry (ITC) to determine dissociation constants and binding thermodynamic parameters. Typically these analyses are conducted with oligosaccharide analogues resembling the targeted polysaccharide. It is also possible, however, to gain insight into binding of soluble polysaccharides by using SPR (Diemer et al. 2012).

Case stories of functional roles of SBSs in GH13_6 and GH77

Barley α-amylase isozymes (AMY1 and AMY2) of subfamily 13_6 (Stam et al. 2006) are among the first carbohydrate active enzymes to have an SBS identified and the most thoroughly investigated (Gibson & Svensson 1987; Kadziola et al. 1998; Robert et al. 2003, 2005; Bozonnet et al. 2007; Nielsen et al. 2008, 2009, 2012). While an SBS (SBS1, Figure 1A) was first discovered in AMY2 (Gibson & Svensson 1987; Kadziola et al. 1998), most

characterizations of functional properties of SBS1 and SBS2 were carried out with the AMY1 isozyme for two reasons; i) the yields of recombinant AMY1 produced by *Pichia pastoris* are about 60 fold higher than of AMY2 (Juge et al. 1996) and ii) preliminary work indicated that binding to SBS2 is weaker in AMY2 (Seo et al. 2008, 2010) in agreement with SBS2 also not been occupied in the crystal structure (Kadziola et al. 1998). Actually this functional difference between the two isozymes may have biological relevance for their individual roles in the seed during grain filling and germination, but this was so far not been further investigated.

SBS2 binds the starch mimic β-cyclodextrin with about 20 fold higher affinity than SBS1 (Nielsen et al. 2009). Furthermore, the binding of starch granules at SBS2 in AMY2 □ or actually the variant AMY2 A42P that has AMY2 wild-type properties but higher production yield in *P. pastoris* (Fukuda et al. 2005) – was slightly weaker than to SBS2 in AMY1 and K_D increased five fold (to 16 mg/ml) for the SBS2 mutant Y378A compared to the AMY2 A42P parent form (Seo et al. 2008, 2010).

In the mobilization of starch granules the two sites SBS1 and SBS2 seems to possess distinct functional roles, SBS1 being the most important. At SBS1 the central interaction involves Trp278 and Trp279, while at SBS2 the key residue is Tyr380. The Y380A SBS2 mutant lost about 10 fold (K_D = 1.4 mg/ml) in affinity compared to wild-type AMY1 and retained less than half the activity to release soluble reducing sugars from starch granules, noticeably these effects were more prominent for single or double SBS1 alanine mutants of Trp278 and Trp279. Complete loss of the affinity for barley starch granules (K_D > 100 mg/ml) required both SBSs to be modified as in the triple mutant W278A/W279A/Y380A that retained only 0.2% of the wild-type hydrolytic activity towards barley starch granules (Nielsen et al. 2009). Both affinity and rate of hydrolysis could be increased roughly 10 fold if a starch binding

domain of the CBM20 family from *Aspergillus niger* glucoamylase was fused C-terminally to AMY1 (Juge et al. 2006). While this CBM20 has been put in connection with facilitated enzyme access to the α-glucan chains in the granular starch due to a disruption of the double helical conformation adopted by α-glucan chains mediated by interaction with the two sites on the CBM20, a similar facilitated access can be proposed to arise by double helical chain disentangling as a consequence of binding interactions with both SBS1 and SBS2 (Southall et al. 1999; Nielsen et al. 2009). Although different in chemical structure the spatial orientation of SBS1 and SBS2 in the AMY1 appears reminiscent to that of the two sites in the CBM20 (Figure 2).

Polysaccharide modifying enzymes are commonly acting in a processive fashion, which takes advantage of the productive encounter being made with the macromolecular and often insoluble substrate by enabling relocation in the active site of the polysaccharide chain after the first cleavage to execute a second and possibly subsequent cleavages. In the case of starch degrading enzymes this is commonly referred to as a multiple attack mechanism (Robyt & French 1967). For AMY1 a degree of multiple attack of 1.9 was determined for the action on amylose of DP 440 (Kramhøft et al. 2005), reflecting each encounter to comprise two extra hydrolytic events following the initial attack. Mutation at SBS1 or SBS2 reduced the degree of multiple attack to values in the range 1.1 – 1.6, the strongest effect being found with the SBS2 Y380A mutant (Nielsen et al. 2009).

In several cases it has been proposed that SBSs can participate in allosteric regulation of the enzyme activity although it is not necessarily polysaccharide binding to the SBS but rather oligosaccharide products, inhibitors or other effectors that may elicit the regulation. In the case of AMY1 and AMY2 it was early on suggested based on an advanced kinetics analysis of acarbose inhibition of the hydrolysis of short, medium length and macromolecular linear

substrates, respectively, that an SBS plays an allosteric role in the sense that substrate binding was noticed to happen i) at a so-called starch granule binding site, ii) that binding at the active site was required for the regulatory SBS to be functional and iii) that binding at that SBS was required for maximal activity (Oudjeriouat et al. 2003). This obviously supports that cross-talk exists between the SBS (probably SBS2) and the active site but it should be emphasized that the interpretation relies on a model defined by results obtained in kinetics experiments combined with the knowledge about the presence of SBSs in the structure of barley α-amylase.

While there is no evidence for SBS being involved in passing on reaction products of AMY1 nor in attachment to cell walls or in serving as a chaperone site for example in folding and/or conformational stabilization (*i.e.,* functional roles vi), vii) and ix) listed above), evidence was presented in a recent publication for SBS2 to be essential in the hydrolysis of amylopectin (Nielsen et al. 2012). The progress curve for hydrolysis of amylopectin can be described to follow a biexponential model that can be resolved in a component with high maximal rate and high affinity and another component with a slower maximal rate and about 10 fold weaker affinity. In the presence of increasing concentrations of β-cyclodextrin known to bind to SBS2 with a low K_D, essentially only the high maximal rate component is affected and a K_i for β-cyclodextrin was determined to 0.2 mM which is comparable to the dissociation constant of SBS2 measured to 0.07□0.14 mM (Bozonnet et al. 2007; Nielsen et al. 2009), strongly suggesting that SBS2 contributes to the high maximal rate component of AMY1 catalysed hydrolysis of amylopectin. This finding was confirmed by analysis of the Y380A mutant, which showed the same reduction in the fast rate of hydrolysis as obtained in the presence of a saturating β-cyclodextrin concentration (Nielsen et al. 2012). It is proposed therefore that structural elements such as amylopectin branch points and/or the presence of multiple

neighbouring chains facilitate binding of AMY1 to the substrate.

One may wonder if it is possible to predict the presence and function of SBS1 and SBS2 in family GH13 or perhaps just in the subfamily GH13_6. When comparing 16 members of GH13_6 the AMY1 Trp278 and Trp279 were 75% and 70% conserved, respectively, while Tyr380 and His395 (also involved in SBS2) were 56% and 37% conserved, respectively. However, when SBS containing GH13 members from other subfamilies were compared with AMY1 none of these four key residues were conserved, illustrating that particular SBSs are only likely to be conserved within subfamilies or among closely related subfamilies of GH13 (Cockburn & Svensson 2013).

Previously, the GH77 amylomaltase from *Thermus aquaticus* was demonstrated to possess an SBS (Przylas et al. 2000), which was proposed to be involved in allosteric regulation of the activity by exerting an impact on the conformation of the active site. An attempt was made to improve the properties of the enzyme by use of random and saturation mutagenesis (Fujii et al. 2005, 2007). Amylomaltases catalyse four reactions; disproportionation, coupling (transglycosylation), hydrolysis and cyclisation (intra-molecular transglycosylation). Subtitution of Tyr54, situated in the SBS, was found to decrease the activity of the three former reactions while that of the cyclisation was increased. This was interpreted to show that binding to Tyr54 at the SBS was a prerequisite for acquiring optimal conformation of the active site. Since the cyclisation is a unimolecular reaction requiring substrate flexibility, the loss of binding to the SBS provided such flexibility, whereas for the other three reactions the accommodation of substrate to the intact SBS would be required to for achieving a conformational change connected with activation of the active site (Fujii et al. 2005, 2007).

GH77 enzymes have important roles in maltose metabolism. In plants an unusual multidomain protein with 4-glucanotransferase activity, DPE2, is believed to transfer glucosyl

moieties to a complex heteroglucan prior to their conversion to hexose phosphate *via* a cytosolic phosphorylase. The distantly related amylomaltase MalQ from *E. coli* is able to restore in Arabidopsis mutants lacking DPE2 the maltose metabolism required for starch-to-sucrose conversion in leaves during the night, although in bacteria the conversion of maltose to hexose phosphate does not require the heteroglucan acceptor (Ruzanski et al. 2013). The MalQ and the DPE2 enzymes show some interesting structural and functional differences. For instance DPE2 contains two CBM20s in tandem N-terminally of the catalytic domain, whereas MalQ has no CBMs. Still β-cyclodextrin binds to MalQ and DPE2 with very similar affinity, K_D being 250□350 μM as shown using SPR. However, for binding of maltooligosaccharides DPE2 has highest affinity for maltohexaose, whereas MalQ binds maltotriose and maltotetraose most strongly and its overall affinity is about 500 fold higher than that of DPE2 (Ruzanski et al. 2013). MalQ is not inhibited by β-cyclodextrin and when saturated by 250 μM maltopentaose it can still bind β-cyclodextrin with the usual affinity. Also solving binding curves according to two-site binding models shows two distinct K_d values for MalQ. The structure of MalQ or a closely related enzyme is unfortunately not known making the prediction of the SBS containing region impossible.

Perspectives

SBSs in polysaccharide converting enzymes play a variety of crucial roles as demonstrated in those cases which have been subject to further investigation, *e.g.* mutational analysis. The near future challenges include establishing rational procedures for identifying SBSs when three-dimensional models are unavailable and also for introducing functional SBSs to achieve gained functionalities. Crystallography can be explored as a way to get SBS hits by exposing the protein either under co-crystallisation conditions or by soaking a pre-made crystal to a

range of oligosaccharides to disclose surface areas with affinity for carbohydrates. It may also lead to novel insight to create a structural database of non-active site oligosaccharide protein complexes. Obviously aromatic residues are often part of SBSs, but one may imagine that there exists additional unifying structural properties for certain types of SBS complexes.

Acknowledgements

This work is supported by project and instrument grants from the Danish Council for Independent Research | Natural Sciences (FNU), an HC Ørsted post-doctoral fellowship from the Technical University of Denmark (to DC) and a joint PhD stipend from the Technical University of Denmark and from the FNU grant (to CW).

References

Abe, A., Yoshida H., Tonozuka T., Sakano Y. & Kamitori S. 2005. Complexes of *Thermoactinomyces vulgaris* R-47 α-amylase 1 and pullulan model oligosaccharides provide new insight into the mechanism for recognizing substrates with α-(1,6) glycosidic linkages. FEBS J. **272:** 6145–6153.

Albenne C., Skov L.K., Mirza O., Gajhede M., Feller G., D'Amico S., André G., Potocki-Véronèse G., van der Veen B.A., Monsan P. & Remaud-Simeon M. 2004. Molecular basis of the amylose-like polymer formation catalyzed by *Neisseria polysaccharea* amylosucrase. J. Biol. Chem. **279:** 726–734.

Baskaran S., Chikwana V.M., Contreras C.J., Davis K.D., Wilson W.A., Depaoli-Roach A.A., Roach P.J. & Hurley T.D. 2011. Multiple glycogen binding sites in eukaryotic glycogen synthase are required for high catalytic efficiency toward glycogen. J. Biol. Chem. **286:** 33999–34006.

Bozonnet S., Jensen M.T., Nielsen M.M., Aghajari N., Jensen M.H., Kramhøft B., Willemoës

M., Tranier S., Haser R. & Svensson B. 2007. The 'pair of sugar tongs' on the non-catalytic domain C of barley α-amylase participates in substrate binding and activity. FEBS J. **274:** 5055–5067.

Caner S., Nguyen N., Aguda A., Zhang R., Pan Y.T., Withers S.G. & Brayer G. 2013. The structure of *Mycobacterium smegmatis* trehalose synthase reveals an unusual active site configuration and acarbose-binding-mode. Glycobiology **23:** 1075–1083.

Cantarel B.L., Coutinho P.M., Rancurel C., Bernard T., Lombard V. & Henrissat B. 2009. The Carbohydrate-Active enZymes database (CAZy): an expert resource for glycogenomics. Nucleic Acids Res. **37:** D233–D238.

Chaen K., Noguchi J., Omori T., Kakuta Y. & Kimura M. 2012. Crystal structure of the rice branching enzyme I (BEI) in complex with maltopentaose. Biochem. Biophys. Res. Commun. **424:** 508–511.

Cockburn D. & Svensson B. 2013. Surface binding sites in carbohydrate active enzymes: an emerging picture of structural and functional diversity, pp 204–221. In: Rauter P. & Lindhorst T. (eds), Carbohydrate Chemistry vol. 39, Royal Society of Chemistry, Cambridge, United Kingdom.

Cuesta-Seijo J.A., Nielsen M.M., Marri L., Tanaka H., Beeren S.R. & Palcic M.M. 2013. Structure of starch synthase I from barley: insight into regulatory mechanisms of starch synthase activity. Acta Crystallogr. **69:** D1013–D1025.

Cuyvers S., Dornez E., Abou Hachem M., Svensson B., Horhorn M., Chory J., Delcour J.A. & Courtin C.M. 2012. Isothermal titration calorimetry and surface plasmon resonance allow quantifying substrate binding to different binding sites of *Bacillus subtilis* xylanase. Anal. Biochem. **420:** 90–92.

Cuyvers S., Dornez E., Delcour J.A. & Courtin C.M. 2012. Occurrence and functional

significance of secondary carbohydrate binding sites in glycoside hydrolases. Crit. Rev. Biotechnol. **32:** 93–107.

Dauter Z., Dauter M., Brzozowski A.M., Christensen S., Borchert T.V., Beier L., Wilson K.S. & Davies G.J. 1999. X-ray structure of Novamyl, the five-domain "maltogenic" α-amylase from *Bacillus stearothermophilus*: maltose and acarbose complexes at 1.7 A resolution. Biochemistry **38:** 8385–8392.

Díaz A., Martínez-Pons C., Fita I., Ferrer J.C. & Guinovart J.J. 2011. Processivity and subcellular localization of glycogen synthase depend on a non-catalytic high affinity glycogen-binding site. J. Biol. Chem. **286:** 18505–18514.

Diemer S.K., Svensson B., Babol L.N., Cockburn D., Grijpstra P., Dijkhuizen L., Folkenberg D.M., Garrigues C. & Ipsen R.H. 2012. Binding interactions between α-glucans from *Lactobacillus reuteri* and milk proteins characterized by surface plasmon resonance. Food Biophys. **7:** 220□226.

Fawaz R., Feng L., Hovde S., Mort A. & Geiger J.H. 2013. Structure, function and specificity of branching enzyme. ALAMY_5, Programme and abstracts, page 27

Fletterick R.J., Sygusch J., Semple H. & Madsen N.B. 1976. Structure of glycogen phosphorylase at 3.0-A-resolution and its ligand binding sites at 6-A. J. Biol. Chem. **251:** 6142–6146.

Fujii K, Minagawa H., Terada Y., Takaha T., Kuriki T., Shimada J. & Kaneko H. 2005. Use of random mutageneses to improve the properties of *Thermus aquaticus* amylomaltase for efficient production of cycloamyloses. Appl. Microenv. Microbiol. **71:** 5823–5827.

Fujii K, Minagawa H., Terada Y., Takaha T., Kuriki T., Shimada J. & Kaneko H. 2007. Function of second glucan binding site including tyrosines 54 and 101 in *Thermus*

aquaticus amylomaltase. J. Biosci. Bioengng. **103:** 167–173.

Guce A.I., Clark N.E., Salgado E.N., Ivanen D.R., Kulminskaya A.A., Brumer H. & Garman S.C. 2010. Catalytic mechanism of human α-galactosidase. J. Biol. Chem. **285:** 3625–2632.

Gentry M.S., Dixon J.E. & Worby C.A. 2009. Lafora disease: insights into neurodegeneration from plant metabolism. Trends Biochem. Sci. **34:** 628–639.

Juge N., Andersen J.S., Tull D., Roepstorff P. & Svensson B. 1996. Overexpression, purification, and characterization of recombinant barley α-amylases 1 and 2 secreted by the methylotrophic yeast *Pichia pastoris*. Prot. Express. Purif. **8:** 204–214.

Juge N., Nøhr J., Le Gal-Coëffet M.-F., Kramhøft B., Furniss C.S.M., Planchot V., Archer D.B., Williamson G. & Svensson B. 2006. The activity of barley α-amylase on starch granules is enhanced by fusion of a starch binding domain from *Aspergillus niger* glucoamylase. Biochim. Biophys. Acta **1764:** 275 ▪ 284.

Koropatkin N.M. & Smith T.J. 2010. SusG: a unique cell-membrane-associated α-amylase from a prominent human gut symbiont targets complex starch molecules. *Structure* **18:** 200–215.

Kramhøft B., Bak-Jensen K.S., Mori H., Juge N., Nøhr J. & Svensson B. 2005. Involvement of individual subsites and secondary substrate binding sites in multiple attack on amylose by barley α-amylase. Biochemistry **44:** 1824–1832.

Leemhuis H., Pijning T., Dobruchowska J.M.. Leeuwen S.S., Kralj S., Dijkstra B.W. & Dijkhuizen L. 2013. Three-dimensional structures, reactions, mechanism, α-glucan analysis and their implications in biotechnology and food applications. J. Biotechnol. **163:** 250–272.

Linden A., Mayans O., Meyer-Klaucke W., Antranikian G. & Wilmanns M. 2003. Differential regulation of a hyperthermophilic α-amylase with a novel (Ca,Zn) two-metal center by zinc. J. Biol. Chem. **278:** 9875–9884.

Ludwiczek M.L., Heller M., Kantner T. & McIntosh L.P. 2007. A secondary xylan-binding site enhances the catalytic activity of a single-domain family 11 glycoside hydrolase. J. Mol. Biol. **373:** 337–354.

Lyhne-Iversen L., Hobley T.J., Kaasgaard S.G. & Harris P. 2006 Structure of *Bacillus halmapalus* α-amylase crystallized with and without the substrate analogue acarbose and maltose. Acta Crystallogr. Sect. F **62:** 849□854.

Nielsen J.W., Kramhøft B., Bozonnet S., Abou Hachem M., Stipp S.L.S., Svensson B. & Willemoës M. 2012. Degradation of the starch components amylopectin and amylose by barley α-amylase 1: Role of surface binding site 2. Arch. Biochem. Biophys. **528:** 1–6.

Nielsen M.M., Bozonnet S., Seo E.-S., Mótyán J.A., Andersen J.M., Dilokpimol A., Abou Hachem M., Gyémánt G., Naested H., Kandra L., Sigurskjold B.W. & Svensson B. 2009. Two secondary carbohydrate binding sites on the surface of barley α-amylase 1 have distinct functions and display synergy in hydrolysis of starch granules. Biochemistry **48:** 7686–7697.

Nielsen M.M., Seo E.-S., Bozonnet S., Aghajari N., Robert X., Haser R. & Svensson B. 2008. Multi-site substrate binding and interplay in barley α-amylase 1. FEBS Lett. **582:** 2567–2571.

Oudjeriouat N., Moreau Y., Santimone M., Svensson B., Marchis-Mouren G. & Desseaux V. 2003. Mechanism of barley α-amylase. Inhibition of amylose, maltodextrin and maltoheptaose hydrolysis by acarbose and cyclodextrin. Eur. J. Biochem. **270:**

3871–3879.

Pinotsis N., Leonidas D.D., Chrysina E.D., Oikonomakos N.G. & Mavridis I.M. 2003. The binding of β- and γ-cyclodextrins to glycogen phosphorylase b: Kinetic and crystallographic studies. Protein Sci. **12:** 1914–1924.

Przylas I., Terada Y., Fujii K., Takaha T., Saenger W. & Strater, N. 2000. X-ray structure of acarbose bound to amylomaltase from *Thermus aquaticus* - Implications for the synthesis of large cyclic glucans. Eur. J. Biochem. **267:** 6903–6913.

Qian M.X., Haser R. & Payan F. 1995. Carbohydrate-binding sites in a pancreatic α-amylase-substrate complex derived from X-ray structure analysis at 2.1 Å resolution. Protein Sci. **4:** 747–755.

Ragunath C., Manuel S.G., Venkataraman V., Sait H.B.R., Kasinathan C. & Ramasubbu N. 2008. Probing the role of aromatic residues at the secondary saccharide-binding sites of human salivary α-amylase in substrate hydrolysis and bacterial binding, J. Mol. Biol. **384:** 1232–1248.

Robert X., Haser R., Mori H., Svensson B. & Aghajari N. 2005. Oligosaccharide binding to barley α-amylase 1. J. Biol. Chem. **280:** 32968–32978.

Robert X., Haser R., Gottschalk T., Ratajczek F., Driguez H., Svensson B. & Aghajari N. 2003. The structure of barley α-amylase isozyme 1 reveals a novel role of domain C in substrate recognition and binding. A pair of sugar tongs. Structure **11:** 973–984.

Robyt J.F. & French D. 1967. Multiple attack hypothesis of α-amylase action: action of porcine pancreatic, human salivary, and *Aspergillus oryzae* α-amylases. Arch. Biochem. Biophys. **122:** 8–16.

Ruzanski C., Smirnova J., Rejzek M., Cockburn D., Pedersen H.L., Pike M., Willats W.G.T., Svensson B., Steup M., Smith A.M. & Field R.A. 2013 A simple bacterial

glucanotransferase can complement Arabidopsis mutants defective in cytosolic maltose metabolism. J. Biol. Chem., in press.

Seo E.-S., Andersen J.M., Nielsen M.M., Vester-Christensen M.B., Christiansen C., Jensen J.M., Mótyán J.A., Glaring M.A., Blennow A., Kandra L., Gyémánt G., Janeček Š., Haser R., Aghajari N., Abou Hachem M. & Svensson B. 2010. New insight into structure/function relationships in plant α-amylase family GH13 members. J. Appl. Glycosci. **57:** 157–162.

Seo E.-S., Christiansen C., Abou Hachem M., Nielsen M.M., Fukuda K., Bozonnet S., Blennow A., Aghajari N., Haser R. & Svensson B. 2008. An enzyme family reunion – similarities, differences and eccentricities in actions on α-glucans. Biologia **63:** 967–979.

Southall S.M., Simpson P.J., Gilbert H.J., Williamson G. & Williamson M.P. 1999. The starch-binding domain from glucoamylase disrupts the structure of starch. FEBS Lett. **447:** 58–60.

Stam M.R., Danchin E.G.J., Rancurel C., Coutinho P.M. & Henrissat B. 2006. Dividing the large glycoside hydrolase family 13 into subfamilies: towards improved functional annotations of α-amylase-related proteins. Prot. Eng. Des. Sel. **19:** 555–562.

Syson K., Stevenson C.E.M., Rashid A., Leiba J., Tuukkanen A., Svergun D.I., Molle V., Lawson D. & Bornemann S. 2013. Binding, catalysis and regulation of the anti-TB target GH13_3 GlgE. ALAMY _5, Programme and abstracts, page 22

Tan T.-C., Mijts B.N., Swaminathan K., Patel B.K.C. & Divne C. 2008. Crystal structure of the polyextremophilic α-amylase AmyB from *Halothermothrix orenii*: details of a productive enzyme-substrate complex and an N domain with a role in binding raw starch. J. Mol. Biol. **378:** 850–868.

Timmins J., Leiros H.-K.S., Leonard G., Leiros, I. & McSweeney S. 2005. Crystal structure of maltooligosyltrehalose trehalohydrolase from *Deinococcus radiodurans* in complex with disaccharides. J. Mol. Biol. **347:** 949–963.

Vujicic-Žagar A. & Dijkstra B.W. 2006. Monoclinic crystal form of *Aspergillus niger* α-amylase in complex with maltose at 1.8 Å resolution. Acta Crystallogr. Sect. F **62:** 716–721.

Footnotes

*corresponding author

Abbreviations:

AGE: affinity gel electrophoresis; AMY1 and AMY2: Barley α-amylase isozymes 1 and 2; CBM: carbohydrate binding module; GH: glycoside hydrolase family; GT: glycosyl transferase family; SBS: surface binding site; SPR: surface plasmon resonance

Table and Figure legends

Table 1: SBSs in GH13 (the "α-amylase family")

Figure 1. SBSs in family GH13 (the "α-amylase family"). A. Barley α-amylase 1 (GH13_6). B. Human salivary α-amylase 1 (GH13_24). C. *Neiserria polysaccharea* amylosucrase (GH13_4)

Figure 2. Comparison of orientation of SBS1 and SBS2 in a barley α-amylase 1 catalytic

nucleophile mutant D180A in complex with maltoheptaose and the two binding sites of the CBM20 in glucoamylase from *Aspergillus niger* in complex with β-cyclodextrin

Figure
Click here to download Figure: FIgure 1 Cockburn et al ALAMY_5.pdf

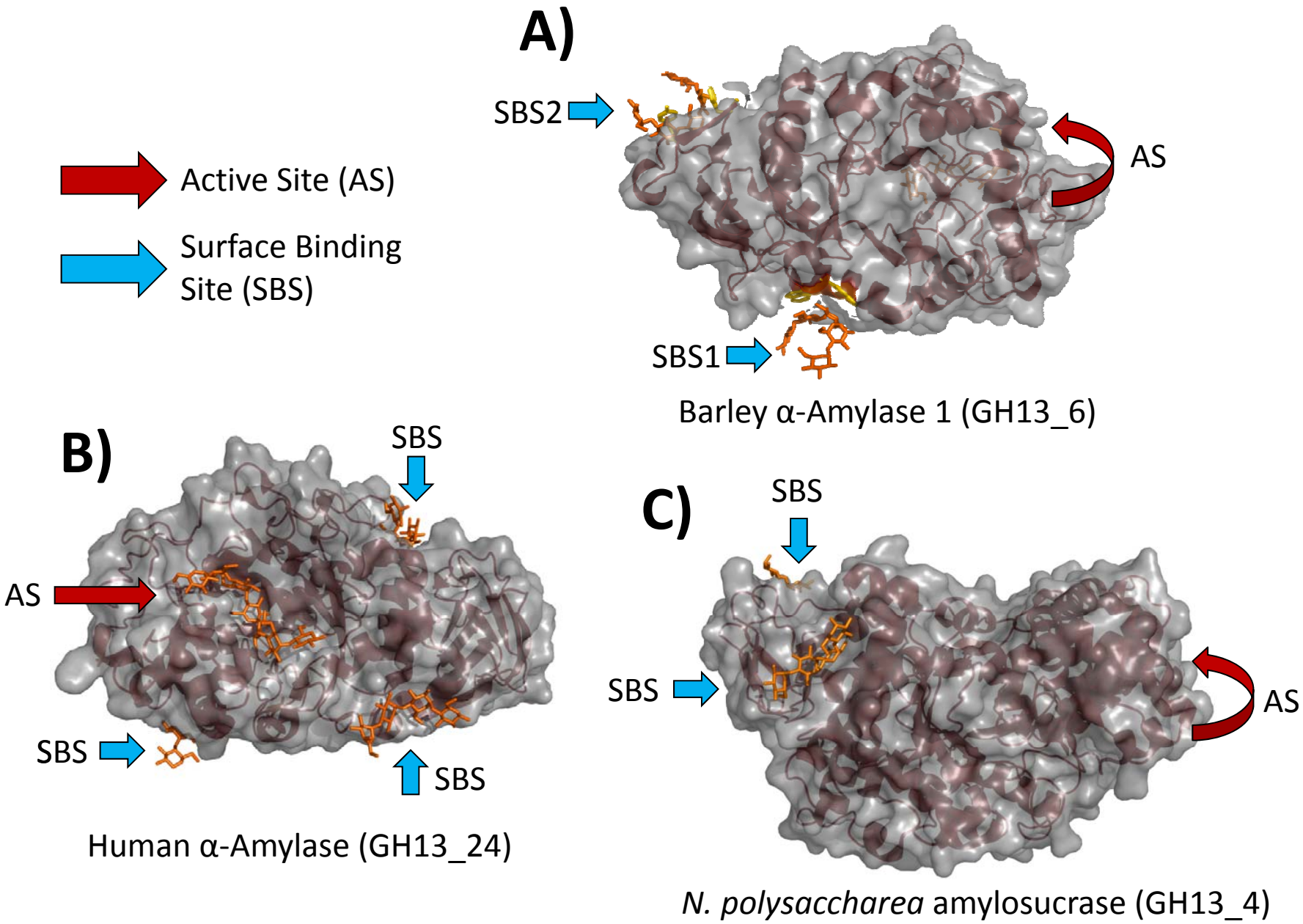
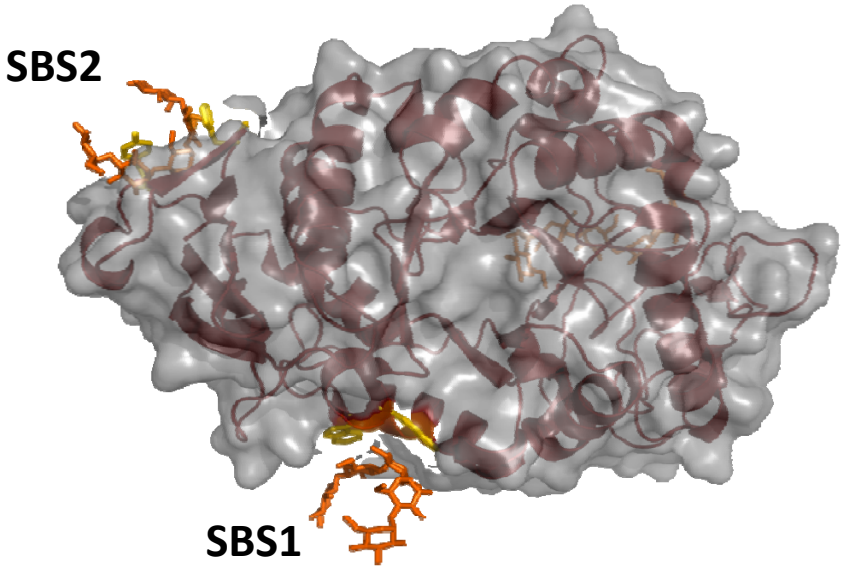
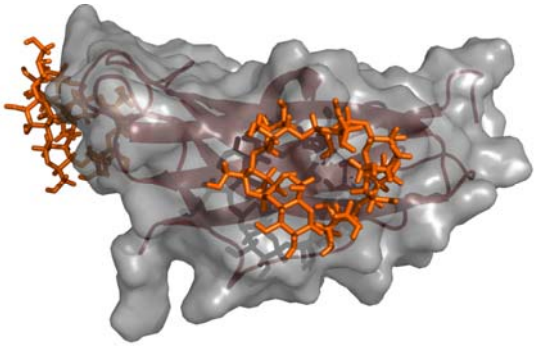


Figure
Click here to download Figure: Figure 2 Cockburn et al ALAMY_5.pdf



Inactive D180A AMY1/maltoheptaose



Starch binding domain (CBM20)

Table
Click here to download Table: Table 1 Cockburn et al ALAMY_5 Final.pdf

Subfamily	Enzyme	Number of SBSs	Literature
GH13_1	*Aspergillus oryzae* α-amylase	one	Vujicic-Zagar & Dijkstra 2006
GH13_2	*Geobacillus stearothermophilus* maltogenic α-amylase	one	Dauter et al. 1999
GH13_3	*Streptomyces coelicolor* GlgE; (1→4)-α-D-glucan:phosphate α-D-maltosyltransferase	one	Syson et al. 2013
GH13_4	*Neisseria polysaccharea* amylosucrase	two	Albenne et al. 2004
GH13_5	*Bacillus halmapalus* α-amylase	one	Lyhne-Iversen et al. 2006
GH13_6	Barley AMY1 and AMY2 α-amylase	two and one	Robert et al. 2005 Kadziola et al. 1998
GH13_7	*Pyrococcus woesei* α-amylase	three	Linden et al. 2003
GH13_8	Rice branching enzyme	three	Chaen et al. 2012
GH13_9	*E. coli* branching enzyme	seven	Fawaz et al. 2013
GH13_10	*Deinococcus radiodurans* maltooligosyltrehalose trehalohydrolase	five	Timmins et al. 2005
GH13_11	*Chlamydomonas* isoamylase	two	Sim et al. (personal commun.)
GH13_14	*Bacillus subtilis* pullulanase	one	2E9B (unpublished)
GH13_24	Human salivary and pig pancreatic α-amylase	three	Ragunath et al. 2008 Qian et al. 1995
GH13_31	*Mycobacterium smegmatis* trehalose synthase	one	Caner et al. 2013
GH13_36	*Thermoactinomyces vulgaris* α-amylase	three	Abe et al. 2005
n.a.	*Bacteroides thetaiotamicron* SusG α-amylase	two	Koropatkin & Schmidt 2010
n.a.	*Halothermothrix orenii* α-amylase	three	Tan et al. 2008

n.a. = not assigned