The starch binding domain of glucoamylase from *Aspergillus niger*: overview of its structure, function, and role in raw-starch hydrolysis

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A carbohydrate-binding module is defined as a contiguous amino-acid sequence within a carbohydrate-active enzyme, with a discrete fold having carbohydrate-binding activity. Glucoamylase 1 from *Aspergillus niger* contains a C-terminal starch-binding domain (SBD), which is connected to the catalytic domain via a semi-rigid linker. Over the last 20 years, a combination of techniques (mutagenesis, nuclear magnetic resonance spectroscopy, differential scanning calorimetry, isothermal titration calorimetry, ultraviolet difference spectroscopy, atomic force microscopy, protein engineering) have been used to investigate the structure-function relationships of this particular domain. This review focuses on recent findings on the structure, role in ligand binding, cooperation in the hydrolysis of granular starch, and engineering of the *A. niger* glucoamylase SBD.

Key words: starch-binding domain, *A. niger*, glucoamylase, barley α-amylase, atomic force microscopy, raw-starch, fusion.

Introduction

The starch-binding domain (SBD) is a functional domain which can bind raw-starch and thus enhances the ability of the enzyme to digest granular starch at the catalytic site. The SBD is present in a number of amylolytic enzymes of the glycoside hydrolase families (SVENSSON et al., 1989; JANECEK & SEVCIK, 1999). In family 13 it is present in almost all CGTases, in a few α-amylases, and in maltotetraose-forming exo-amylases. Moreover it is found in most fun-
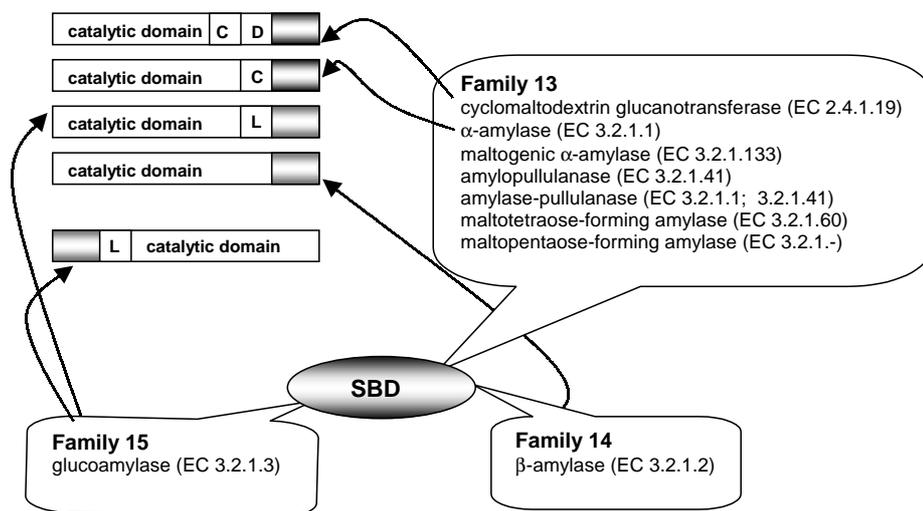
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Fig. 1. Occurrence of the starch-binding domain (SBD) within the glycoside hydrolase families 13, 14, and 15.

gal glucoamylases of family 15 and in some β-amylases of family 14. The SBD always occurs at the C-terminal end of the protein, apart from the glucoamylase from *Rhizopus oryzae.* The SBD can be connected directly to the catalytic domain (e.g. β-amylase), or located after domain C of α-amylases, after domain D of CGTases, or following a typically long *O*-glycosylated linker region as in fungal glucoamylases (Fig. 1). According to a recent classification based on primary structure similarities, SBD belongs to the carbohydrate-binding module family 20 (`http://afmb.cnrs-mrs.fr/~cazy/CAZY/index.html`).

The SBD of *A. niger* glucoamylase

Most fungal glucoamylases (GAs) contain an SBD and in the glucoamylase GA-I from *Aspergillus niger,* the SBD (approx. 100 amino acids) is connected to the catalytic domain via a 70-amino-acids-long highly *O*-glycosylated linker (SVENS-SON et al., 1983). SBD increases the activity towards starch granules of this GA by around 100-fold (SVENSSON et al., 1982). Free SBD can be obtained either by proteolytic digestion of the entire GA-I (BELSHAW & WILLIAMSON, 1990) or by engineering and expression of the gene fragment encoding the SBD in *A. niger* (LE GAL-COËFFET et al., 1995). The SBD of this particular enzyme has been extensively studied over the past 20 years and a lot is known about its structure (SORIMACHI et al., 1996, 1997) and its role in binding to granular starch (SVENSSON et al., 1982; BELSHAW &
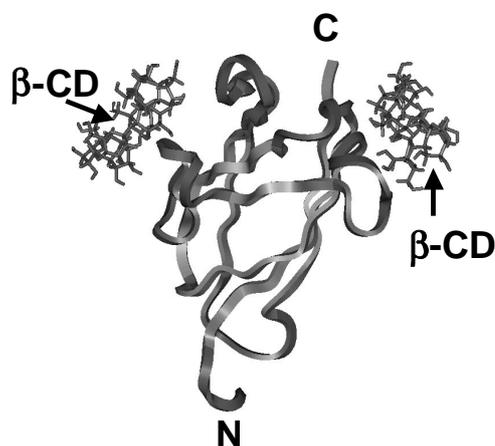

Fig. 2. Three-dimensional structure of *A. niger* glucoamylase SBD complexed with the starch mimic β-cylclodextrin (β-CD) at the two binding sites. The **C**- and **N**-termini are indicated (Figure courtesy of J. SAUER).

WILLIAMSON, 1991, 1993; LE GAL-COËFFET et al., 1995; WILLIAMSON et al., 1997; SOUTHALL et al., 1999; SAUER et al., 2000).

Structure of the *A. niger* glucoamylase SBD

The three-dimensional structure of the isolated recombinant SBD has been determined in solution by NMR either in a free state (SORIMACHI et al., 1996) or in a complex with β-cyclodextrin, a small
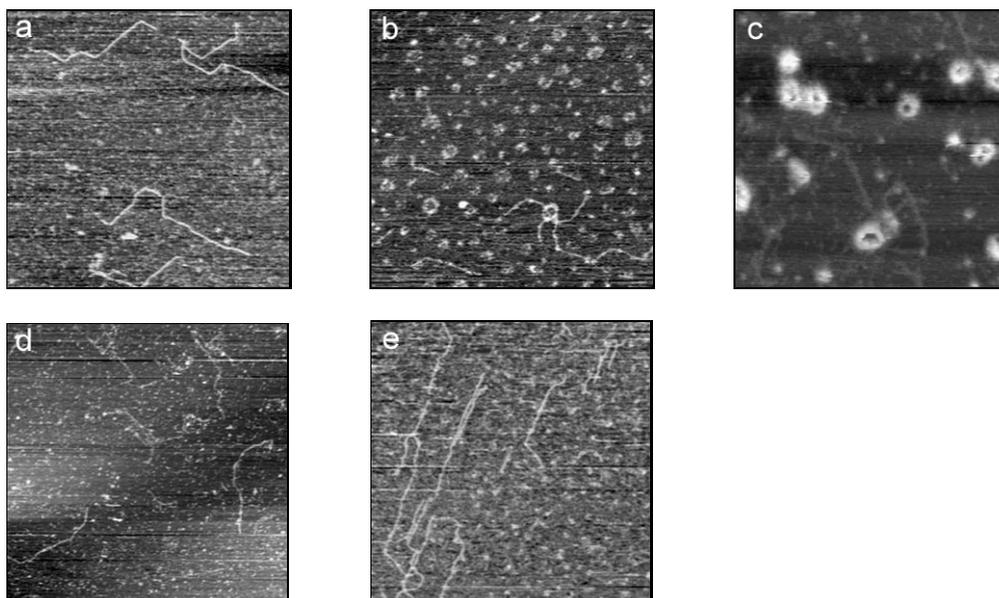
Fig. 3. AFM images of (a) amylose after treatment with iodine and Tween-20, drop deposited from dilute solution at room temperature; scan size 1200x1200 nm. Note that discrete and extended amylose chains are seen, as well as one branched amylose chain, (b) the structures formed after incubation of amylose/iodine/Tween 20 solution with wild-type SBD; scan size 1200×1200 nm, (c) entire inactive mutant GA-I; scan size 800×800 nm, (d) SBD mutant W590K; scan size 2000×2000 nm, and (e) SBD mutant W563K; scan size 1200×1200 nm. (Reproduced from GIARDINA et al., 2001 with permission).

cyclic mimic of starch (SORIMACHI et al., 1997). The SBD structure consists of 8 β-strands arranged essentially in an antiparallel fashion, forming an open-sided β-barrel organised into two β-sheets, in accordance with the previously-reported secondary structure (JACKS et al., 1995). The two substrate- binding sites of SBD are located on opposite sides of the domain away from the linker attachment point (Fig. 2). Previous studies using differential scanning calorimetry showed that the thermal unfolding of the structure of SBD is reversible (WILLIAMSON et al., 1992; CHRISTENSEN et al., 1999).

The role of *A. niger* glucoamylase SBD in binding

The residues involved in the binding sites have been identified using NMR, site-directed mutagenesis, and UV difference spectroscopy (WILLIAMSON et al., 1997). Two tryptophan residues have been shown to be essential for binding, one at each site, i.e., W590 in site 1, and W563 in site 2. The binding affinity towards β-cyclodextrin has been determined using single mutants of each binding site, W563K and W590K. Binding site 2 has a markedly higher affinity ($K_d = 6.4$ μM) for this ligand than site 1 ($K_d = 28$ μM). Site 1 might be involved in initial recognition of the substrate while site 2 is involved in tighter binding, preparing the substrate for catalysis, as previously suggested for the homologous binding domain of a cyclodextrin glycosyltransferase (PENNINGA et al., 1996). This is also in accordance with the structural data obtained from the three-dimensional structures of both free and ligand-bound SBD, which show that site 1 is a compact and exposed binding site undergoing virtually no structural change on binding, whereas site 2 spans a much larger area, does undergo conformational changes upon ligand binding, and is proposed to lock the SBD into place (SORIMACHI et al., 1997). Binding site 2 of the SBD is capable of recognizing a range of orientations of starch strand and the two starch strands are bound to the SBD in an unexpected perpendicular orientation, suggesting that the structural change allows this site to interact with a starch strand in a non-optimal orientation, and subsequently to re-orient the strand (SOUTHALL et al., 1999).
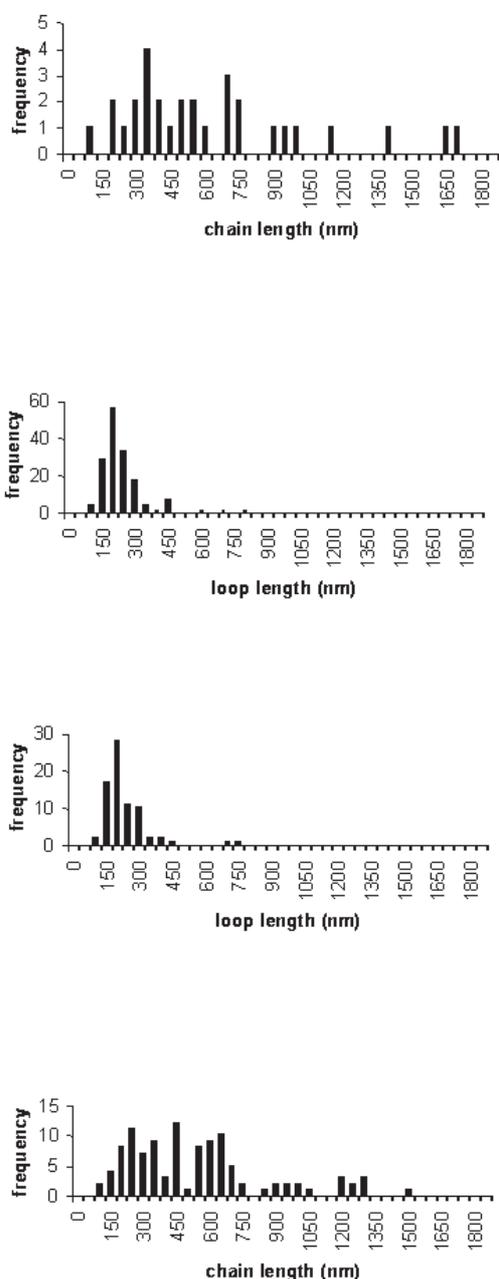
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Fig. 4. Histograms of contour or perimeter length of complexes for (a) amylose/iodine/Tween 20 complex alone, (b) the complex formed between amylose and wild-type SBD, (c) the complex formed between amylose and the inactive mutant E400Q of glucoamylase GA-I, and (d) the complex formed between amylose and the mutant SBD W563K. (Reproduced from Giardina et al., 2001 with permission.)

The binding of SBD to individual amylose chains was recently monitored in detail using Atomic Force Microscopy (Giardina et al., 2001). When the amylose chain is incubated with wild-type SBD, either free or within a catalytically inactive glucoamylase GA-I mutant, the amylose chain, which is normally seen as linear in the AFM images, undergoes a conformational change, which is then lost when one of the binding sites is abolished using mutants of SBD (W563K or W590K) (Fig. 3). Similarly, comparisons of the histograms representing the perimeter length of the individual amylose chains clearly show two sets of patterns corresponding either to the amylose on its own or upon incubation with one of the mutants, and the amylose upon incubation with wild-type SBD, repectively (Fig. 4). Moreover these data show that in the latter case the perimeter length is one half of that observed for the amylose chain on its own, suggesting that binding of the SBD induces a 2-turn helix complex structure of the amylose chain and that both binding sites have to be functional in order to allow this conformational change (Fig. 5). Furthermore, as demonstrated earlier for the binding of β-cyclodextrin, site 2 has higher affinity towards the soluble linear substrate maltoheptaose ($K_d = 0.95$ μM) than site 1 ($K_d = 16.8$ μM), which supports the hypothesis of a different functional role of these two sites in binding (Giardina et al., 2001). However, previous characterization of the thermodynamics of ligand binding to the SBD using isothermal titration calorimetry suggested that the 2 sites bind independently and with similar affinity in the isolated SBD (Sigurskjold et al., 1994).

The role of *A. niger* glucoamylase SBD in starch hydrolysis

The function and structure of this particular SBD has been thoroughly investigated, but the dynamics of the cooperation between the catalytic and binding domain in the enzyme remain to be determined. It is commonly suggested that the role of the SBD is to attach onto granular starch, thereby increasing the local concentration of the substrate at the active site of the enzyme and dramatically increasing the rate of reaction. It is not clear whether the SBD has other functions: for example, it might force starch strands apart as seen for cellulases (Din et al., 1994) or target the enzyme to noncrystalline, thus more readily hydrolyzable, areas of starch. However, it has recently been demonstrated that isolated SBD acting on starch granules together with the glucoamylase catalytic do-
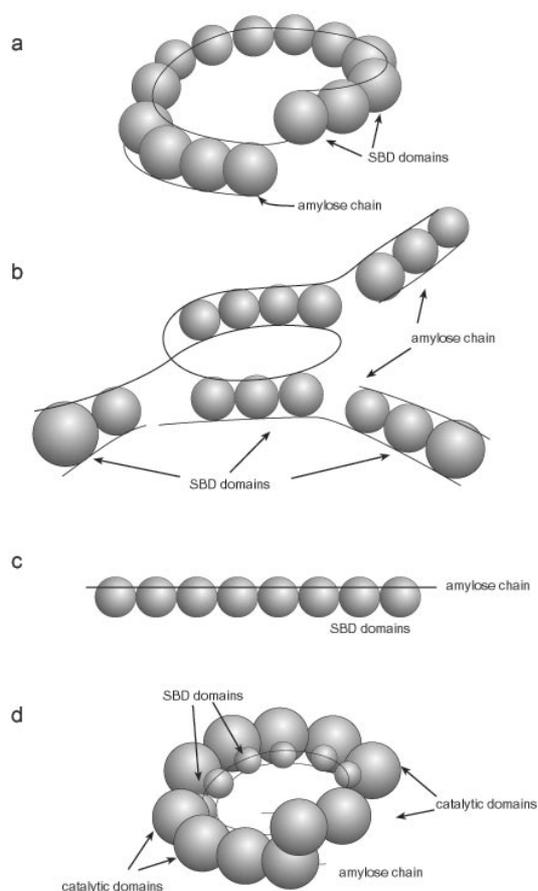
Fig. 5. Schematic models of SBD binding to amylose. (a) Macrocyclic structure formed by the binding of wild-type SBD to amylose. (b) Interconnected loop structures formed by binding of SBD to amylose by showing extended chains radiating from loop structures. (c) Linear amylose-SBD complex formed by mutants with one binding site. (d) Macrocyclic structure formed on binding of intact, inactive GA-I molecules to amylose. The models are schematic and are not intended to accurately represent the relative sizes of the proteins and the diameter of the amylose chain, or the relative numbers of proteins bound per chain. (Reproduced from GIARDINA et al., 2001 with permission.)

main showed a synergistic effect on the degradation of the insoluble substrate (SOUTHALL et al., 1999). It is thus suggested that SBD not only binds onto raw-starch but also disrupts α-glucan interchain binding at the surface of the starch granule. This behaviour will further facilitate the hy-

drolysis of amylose and amylopectin chains by the exo-acting catalytic domain that specifically releases glucose from non-reducing ends of the polymer chains (SOUTHALL et al., 1999). This is also in agreement with structural and AFM data that shows that amylose chains can bind to the SBD in a configuration that differs from that present in the starch double helix, implying that the SBD does more than merely locate the catalytic domain onto the starch granule (SORIMACHI et al., 1997). The SBD thus seems to have several simultaneous functions, which all contribute to the acceleration of hydrolysis of raw-starch.

In order to probe further the role of the *A. niger* glucoamylase SBD with regards to starch hydrolysis, a molecular approach has been used to introduce the SBD at the C-terminal end of an amylolytic enzyme. The barley α-amylase, like the majority of α-amylases, does not possess a well-defined separate SBD (JANECEK & SEVCIK, 1999; MACGREGOR et al., 2001). It was therefore our aim to investigate whether a modular fusion protein created between this enzyme and the SBD would exhibit positive cooperation between the catalytic and binding domains, resulting in an increase of the ability of AMY1 to bind to and hydrolyse raw-starch. Previously, a fusion of the *A. niger* glucoamylase SBD to β-galactosidase showed that SBD can confer starch-binding capacity to a fusion protein (CHEN et al., 1991a, 1991b), but there is no report on the effect of this SBD on a starch-degrading enzyme. Exchanging the domain E of *Bacillus macerans* CGTase by the *A. niger* SBD resulted in a drastic loss of activity of the fusion protein towards soluble starch (CHANG et al., 1998), probably due to improper folding of the protein.

The three-dimensional structure of the barley α-amylase AMY1 isozyme has recently been solved (ROBERT et al., 2002) and the structure is very similar to that of AMY2 (KADZIOLA et al., 1994, 1998), apart from the presence of an extra binding site at the C-terminal domain of the protein. Recently the three-dimensional structure of a maltotetraose-forming amylase from *Pseudomonas stutzeri* has been solved and the fold of the domain C was very similar to that of barley α-amylase (MORISHITA et al., 1997). However, in contrast to the barley enzyme, this enzyme possesses a functional SBD connected to the C-terminal of domain C via a short linker. This SBD, however, was not arranged in an ordered manner in the crystalline state (MEZAKI et al., 2001). Based on the above findings we assumed that barley AMY1 could accommodate the *A. niger* SBD

Table 1. Enzymatic Activity and Starch Binding Capacity of AMY1 and AMY1-SBD Fusion

Enzyme				Substrate		
	DP17 amylose	Cl-pNPG7	Soluble starch		Starch granules	
	k_{cat}/K_M s^{-1} mg^{-1}ml	k_{cat}/K_M s^{-1} mg^{-1}ml	activity U $nmol^{-1}$	[1]activity U $nmol^{-1}$	[2]initial rate U $nmol^{-1}$	[3]$K_{d(app.)}$ mg ml^{-1}
AMY1-SBD*	610	57	0.27	0.034	0.7	0.13
AMY1*	415	40.4	0.14	0.015	0.07	0.67

Kinetic constants were determined at 37 °C in 20 mM NaOAc, 1 mM CaCl$_2$, pH 5.5 (DP17 amylose, soluble potato starch) and 30 °C, 20 mM Mes, pH 6.8, 5 mM CaCl$_2$ (Cl-pNPG7). Activity on barley starch granules (0.05%) was determined at 37 °C in 20 mM NaOAc, 1 mM CaCl$_2$, 0.05% BSA, pH 5.5 at [1]30-50 nM enzyme or [2]2 nM enzyme, and adsorption onto barley starch granules was determined in the same buffer at 4 °C and [3]10 nM enzyme using different starch concentrations.
* produced in *A. niger*.

at the C-terminal end of the protein and that the fusion protein would fold and retain the individual functional properties of AMY1 as well as of SBD.

Construction of a gene encoding a C-terminal fusion protein of *A. niger* SBD (amino acids 471-616), i.e. including residues 471-508 of the highly *O*-glycosylated linker, with barley α-amylase 1 was conducted by overlapping PCR. Secretion to the supernatant in *A. niger* cultures was directed by the AMY1 signal sequence, and the fusion protein was found to be correctly N-terminally processed. Furthermore the fusion protein showed apparent molecular weight (75 kDa), amino-acid composition, and carbohydrate content (23%) in agreement with the theoretical values based on the fusion amino-acid sequence. This suggests a proper folding and processing of the fusion protein, including a natural glycosylation pattern of the linker region. Compared to recombinant AMY1 likewise produced in *A. niger* (JUGE et al., 1998), the specific activity of AMY1-SBD was essentially unaltered towards a maltooligosaccharide substrate (Tab. 1). The K_d for barley starch granules for AMY1-SBD was 0.13 mg/mL compared to K_d = 0.8 mg/mL for AMY1, showing that increased affinity was achieved by the SBD-addition. Similarly, a two-fold increase in specific activity was obtained towards barley starch granules and towards soluble potato starch, and at lower enzyme concentration, the initial rate of starch-granule hydrolysis was ten-fold higher for the fusion protein compared to AMY1 (Tab. 1). In addition, the degree of multiple attack on amylose DP440, determined as described earlier (KRAMHOFT & SVENSSON, 1998), increased significantly from 2 for AMY1 to 3 for AMY1-SBD, possibly as a consequence of the extra two binding sites in SBD. In conclusion, the fusion of SBD to AMY1 led to the production of an active and conformationally stable modular enzyme, which reacted with enhanced enzymatic activity towards starch substrates.

Conclusion and perspectives

The *A. niger* glucoamylase SBD has been thoroughly described, especially with regards to its role in ligand binding. SBD appears to have several simultaneous functions, such as attaching the enzyme to its polymeric substrate, allowing the catalytic domain to access large areas of the starch granule surface, and also disrupting the starch surface to enhance hydrolysis. The major questions to address concerning basic knowledge include (i) insight into the co-operation between SBD and the catalytic domain and (ii) the three-dimensional structure determination of this particular SBD within an intact enzyme. The data obtained on the functions of *A. niger* GA-I SBD in raw-starch binding have important implications for our understanding of other carbohydrate-binding domains of the glycoside hydrolase family.

Protein engineering has been used to address the specific role of SBD, demonstrating that recombinant SBD remains a functional domain either free or fused to the catalytic domain of an amylolytic enzyme, increasing its ability to bind to and hydrolyse raw-starch. A similar approach can be used in the future to develop engineered enzymes with improved properties towards enzymatic conversion of starch into glucose for industrial application.

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