The starch-binding domain from glucoamylase disrupts the structure of starch

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Abstract **The full-length glucoamylase from *Aspergillus niger*, G1, consists of an N-terminal catalytic domain followed by a semi-rigid linker (which together constitute the G2 form) and a C-terminal starch-binding domain (SBD). G1 and G2 both liberate glucose from insoluble corn starch, although G2 has a rate 80 times slower than G1. Following pre-incubation of the starch with SBD, the activity of G1 is uniformly reduced with increasing concentrations of SBD because of competition for binding sites. However, increasing concentrations of SBD produce an initial increase in the catalytic rate of G2, followed by a decrease at higher SBD concentrations. The results show that SBD has two functions: it binds to the starch, but it also disrupts the surface, thereby enhancing the amylolytic rate.**
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Key words: Starch-binding domain; Glucoamylase; *Aspergillus niger*

1. Introduction

The glycosidic bonds in the polysaccharides starch, cellulose and xylan are hydrolysed by amylases, cellulases and xylanases, respectively. In general these enzymes have a modular structure, consisting of a catalytic domain, and at least one non-catalytic domain, whose function is generally described as that of a polysaccharide-binding domain (PBD), respectively starch-binding domains (SBDs), cellulose-binding domains (CBDs) and xylan-binding domains [1–3]. The PBDs generally bind selectively to the polysaccharide substrate for the enzyme, although some xylanases have binding domains that recognise cellulose rather than xylan [4]. Enzymes from which the PBDs have been removed have unchanged hydrolytic rates against soluble substrates, but dramatically slower rates against insoluble substrates: thus, it has been reported that a form of glucoamylase lacking the SBD is some 25 times slower against insoluble starch than the full-length G1 [5]. PBDs achieve this functional effect by binding to the polysaccharide and therefore increasing the substrate concentration at the active site of the catalytic domain. However, there has been considerable debate as to whether PBDs also accelerate hydrolysis in other ways. Din et al. [6,7] have shown that CBDs from *Cellulomonas* enzymes added in trans (i.e. addition of extra exogenous CBD not covalently attached to the catalytic domain) can disrupt the structure of cellulose microfibrils and hence increase the activity of cellulases by making the substrate more accessible to the enzyme. However, it has

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been shown that CBDs from *Pseudomonas* cellulases do not disrupt the structure of cellulose fibrils or plant cell walls, and do not potentiate cellulase or xylanase activity in trans [4]. This suggests that CBDs may have different roles depending on the enzyme context.

The structure of the starch-binding domain of *Aspergillus niger* glucoamylase ('SBD') has been determined, both free in solution [8] and bound to β-cyclodextrin, a cyclic analogue of the starch double helix [9]. The structure was used to construct a model of the complex with starch, which is shown in Fig. 1. SBD binds two molecules of cyclodextrin, with dissociation constants of 28 μM and 6.4 μM at sites 1 and 2 respectively [11]. Significantly, the orientation of the two starch helices in the two sites is almost mutually perpendicular. This is of interest because the starch helices in starch granules are normally roughly parallel. Therefore it was proposed [9,11] that the SBD may function *either* to twist starch strands apart and therefore expose more substrate to the catalytic domain, *or* to localise the SBD to non-parallel (and thus presumably more open and therefore more easily hydrolysed) regions of the starch granule. The aim of the work described here was to test these suggestions by measuring whether SBD, added in trans, has an effect on the activity of glucoamylase. If SBD disrupts starch structure, then SBD added in trans should accelerate the hydrolytic rate, whereas if it merely localises the enzyme to disordered regions of the starch, then it should decrease the hydrolytic rate by occupying binding sites on the starch. In either case, high concentrations of SBD should decrease the hydrolytic rate as SBD will cover the starch surface and therefore reduce access to the enzyme.

2. Materials and methods

G1 and G2 were obtained from crude glucoamylase (Sigma) by chromatographic methods, as described [12]. SBD was expressed in *A. niger* with a pIGF fusion vector and purified as described [13]. Other chemicals were obtained from Sigma. For optimal reproducibility of the assay, insoluble corn starch was prepared as a stock suspension in 5 mM sodium acetate, pH 4.0, various amounts of SBD were added, and the suspension was incubated for 30 min at 37°C in a 2 ml round-bottomed plastic Eppendorf tube (BDH) (the standard 1.5 ml plastic tubes proved less suitable because starch tended to settle in their more pointed tips). SBD alone had no amylolytic activity (data not shown). G1 or G2 was added to a total volume of 1 ml and the reaction was carried out at 37°C in a vertical rotary mixer (the more standard shakers gave less reproducible results, because they allowed the starch to settle). For assays with G1, 0.5–5 mg of starch was incubated with 3–5 μg G1 for 20 min. For assays with G2, 1 mg of starch was incubated with 10 μg G2 for 2 h. After the reaction, the suspensions were cooled on ice, centrifuged at 13 000 rpm for 30 s, and filtered through a 0.45 μm filter (Spartan, Aldrich). 0.8 ml of the filtered solution was assayed for the presence of

glucose using the assay of Lloyd and Whelan [14]: glucose oxidase and horseradish peroxidase are used at 37°C in the presence of O-dianisidine dihydrochloride to generate a colour which is detected at 525 nm. Reagents were made up as required and stored in the dark. All reactions were carried out in the dark and compared to glucose standards.

3. Results

The intact enzyme G1 was found to digest starch approximately 80 times faster than G2, which lacks the SBD, in agreement with previous results [5]. When SBD was added to the starch under a wide range of G1/SBD ratios, it was only possible to observe a reduction in the hydrolytic rate of G1 with increasing amounts of SBD (Fig. 2).

However, when the effect of SBD in trans was tested using G2, it was found that low concentrations of SBD gave an increase in hydrolytic rate. At higher concentrations of SBD, it produced a reduction in rate (Fig. 3). The maximum increase in rate was just over 50%, and both the increase in rate and the subsequent decrease at higher SBD concentration were statistically highly significant using the Student's t-test.

4. Discussion

The primary function of SBD is to bind to starch. Therefore one would expect that as increasing concentrations of SBD are added to starch, it will compete with the enzyme for binding sites on starch and reduce the hydrolytic rate. Even if SBD has any secondary function to increase the rate by other means, at some point the competition for binding sites will overcome any rate enhancement, and the enzyme rate will decrease. G1 and SBD use identical protein domains
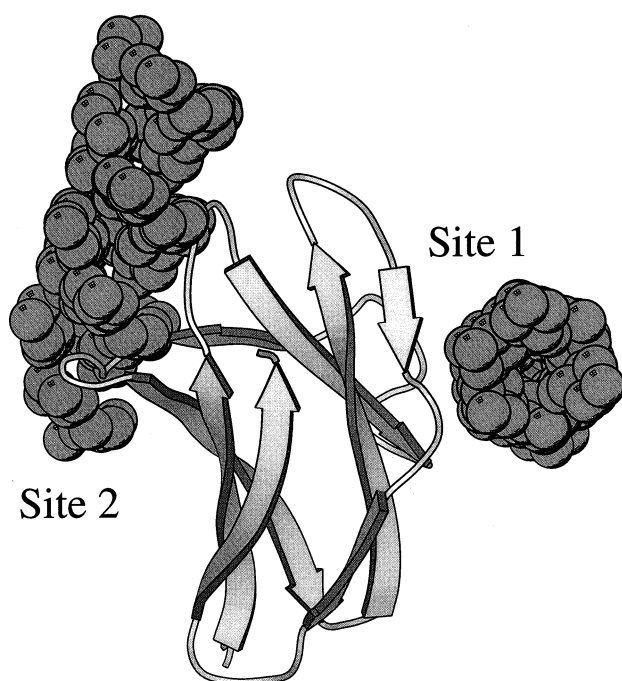

Fig. 1. A model of the structure of the complex between the *Aspergillus niger* glucoamylase starch-binding domain and two molecules of double helical starch, based on the solution structure of the complex of SBD with cyclodextrin [9]. The SBD is shown as a ribbon representation, and the starch is shown as a grey space-filling model. Drawn using MOLSCRIPT [10].
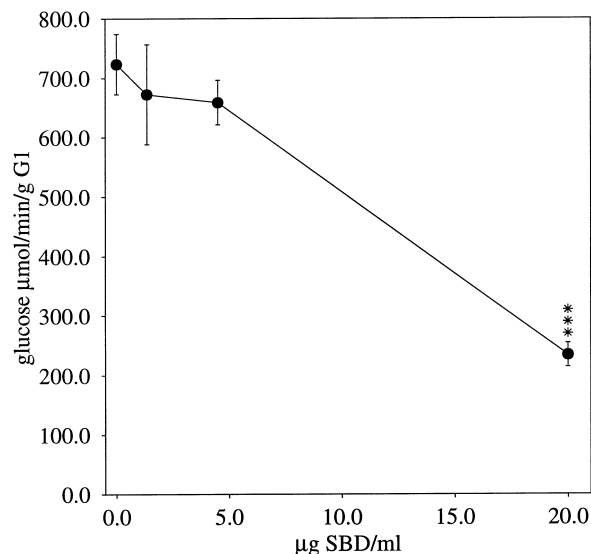

Fig. 2. Amount of glucose liberated from corn starch by glucoamylase G1 following pre-incubation of the starch with SBD. The error bars show the standard deviations of the results, and the asterisks show the significance of the difference from the rate at zero SBD concentration (*** denotes $P < 0.001$).

to attach to starch, and it is therefore not surprising that the effect of SBD is merely to decrease the hydrolytic rate of G1.

By contrast, G2 can only bind to starch *via* its catalytic domain, and it is therefore easier for SBD to demonstrate its secondary function of enhancing the hydrolytic rate. The observation that SBD increases the hydrolytic rate demonstrates unambiguously that SBD does disrupt the surface of starch. This conclusion is consistent with measured binding constants. The individual binding sites have dissociation constants of 28 μM and 6.4 μM (as measured at 25°C using mutants in which only one site remains intact), while the wild-type SBD has a dissociation constant (at 4°C) of 12.7 μM [12]. Cooperative binding to a pre-formed binding site on starch should occur with a dissociation constant considerably stronger than either of the two individual binding constants, whereas if the SBD uses some of its binding energy to disrupt the starch structure, then the overall binding can be of the same order as the individual dissociation constants, as observed here. Another possible explanation for the relatively weak overall binding is that the SBD binds to two rigid strands that are in a sub-optimal orientation; however, this would not explain the rate enhancement seen here.

The rate enhancement seen here could also be explained by a quite different mechanism, namely that SBD interacts with the catalytic domain, and induces a conformational change which increases the activity of the enzyme. However, several lines of evidence suggest that this cannot be the explanation: (i) the presence of SBD has no effect on the activity of the catalytic domain against soluble substrates [15], (ii) thermodynamic studies have shown no measurable interaction between the two domains, and no change in the melting temperature of the catalytic domain in the presence of the SBD [16], and (iii) NMR studies of the full-length protein and of different domains showed that the two domains have independent mobility [17].

The structure of the domain (Fig. 1) offers an obvious explanation for this disruptive function: the domain has two
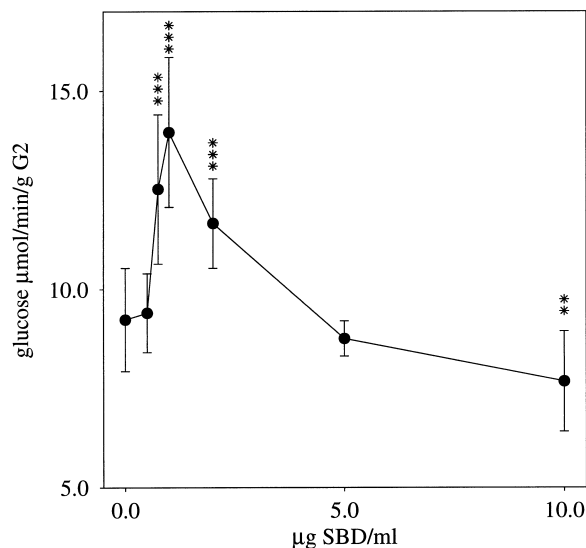
Fig. 3. Amount of glucose liberated from corn starch by glucoamylase G2 following pre-incubation of the starch with SBD. The error bars show the standard deviations of the results (using roughly 10 measurements at each concentration: ** denotes $P < 0.01$).

independent binding sites that bind starch strands in an approximately perpendicular orientation. The structure of one of the two binding sites (site 2) alters substantially on binding to cyclodextrin [9], and we suggest that the structural change allows the site to interact with a starch strand in a non-optimal orientation, and subsequently to re-orient the strand. The structural flexibility of this site is suggested to be functionally important. It has previously been suggested for a homologous binding domain from cyclodextrin glycosyltransferase that the two sites have differentiated functions, in that one (equivalent to site 1) acts as the main recognition site, while the other is more involved in preparing the substrate for catalysis [18].

Starch is much more readily hydrolysed than crystalline cellulose. One may therefore anticipate that if starch-binding domains have found it advantageous to evolve disruptive functions, similar evolutionary pressures must exist for cellulose-binding domains. To date, none of the CBD structures has two binding sites, and so they cannot use an analogous method for disruption. It has been suggested [19] that CBDs may use hydrogen-bonding residues flanking the binding site

to achieve similar results, but so far there is no evidence to support this suggestion.

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