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Fascinating facets of function and structure of amylolytic enzymes of glycoside hydrolase family 13

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Glycoside hydrolase family 13 currently comprises enzymes of 28 different specificities, 13 of which are represented by crystal structures. Ligand complex structures are reported for fewer specificities and typically only describe enzyme-sugar interactions for part of the binding area and for α-1,4-linked compounds. Molecular modeling can fill this lack of knowledge and is also supporting the idea that longer substrates apply several binding modes. The double displacement mechanism leading to retention of the substrate anomeric configuration allows production of oligosaccharides by transglycosylation. This is demonstrated using α-amylase 1 isozyme (AMY1) and limit dextrinase from barley. Moreover, the mechanism motivated site-directed mutagenesis of the catalytic nucleophile in an attempt to convert AMY1 into a glycosynthase. Despite correlation of specificity with short sequence motifs in $\beta \rightarrow \alpha$ loops of the catalytic $(\beta/\alpha)_8$-barrel, rational design to alter specificity is not straightforward and the motifs mainly serve to identify target regions for engineering. Here single and dual subsite mutants in AMY1, produced using various mutagenesis strategies, confer changes in i) substrate preference, ii) oligosaccharide product profiles, and iii) degree of multiple attack. Certain hydrolases and transglycosylases have extra N- and C-terminal domains, which mostly are not assigned a function. *Aspergillus niger* glucoamylase, however, has linker-connected catalytic and starch-binding domains, and served to investigate intramolecular domain communication in starch-hydrolases. Subse-

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quently fusion of the *A. niger* starch-binding domain with barley AMY1 enhanced the binding affinity and rate of granule hydrolysis, which may be an advantage e.g. in brewing. The presence of proteinaceous inhibitors has been reported for very few GH13 members and generally involves isozyme and species discrimination. Interaction with such naturally-occurring inhibitors has particular relevance in nutrition and for plant defense against pathogens. The sensitivity of barley α-amylase for the endogenous α-amylase/subtilisin inhibitor has been controlled through structure-based mutagenesis.

Key words: barley α-amylase, bond-type specificity, subsite engineering, degree of multiple attack, N-terminal domains, starch binding domain, protein inhibitors.

Introduction

Studies on starch-degrading and related enzymes include the very first report on an enzyme-catalysed reaction published almost two centuries ago. Today amylolytic enzymes are categorised in 7 glycoside hydrolase families based on sequence similarities (Henrissat, 1991; Henrissat & Bairoch, 1993; `http://afmb.cnrs-mrs.fr/~cazy/CAZY/index.html`). Family 13 is largest both in sequence entries, which exceed 700, and enzyme specificities, currently amounting to 28. Recent reviews describe the relationship between structure and function for enzymes in family 13 (Janecek, 1997, 2000; MacGregor et al., 2001), from which selected enzymes served as glycoside hydrolase prototypes. Likewise recently-described facets of other glycoside hydrolases have been applied to GH13 members. These include i) attempts to create a glycosynthase (Mackenzie et al., 1998; Ly & Withers, 1999; Rydberg et al., 1999), ii) the stereo-specific lateral protonation in the catalytic mechanism (Heightman & Vasella, 1999), iii) molecular recognition of ligands and analogues and contribution of specific substrate groups to activity (Bundle & Young, 1992; Sierks & Svensson, 1992; Lemieux et al., 1996), iv) intramolecular domain-domain interactions (Sigurskjold et al., 1998; Christensen et al., 1999; Payre et al., 1999), and v) attack on solid substrates and role of carbohydrate-binding modules, CBMs (Coutinho & Henrissat, 1999; Southall et al., 1999; Giardina et al., 2001).

In this lecture, following a presentation of the α-amylase family with emphasis on features of the architecture and catalytic mechanism (MacGregor et al., 2001), the focus changes to specificity design and engineering of barley α-amylase 1 (AMY1) involving site-directed as well as irrational and semi-rational mutagenesis procedures. This addresses the array of 10 consecutive subsites each accommodating a substrate glucose residue and composing binding areas extending on either side of the site of catalysis (Ajandouz et al., 1992). The work is based on i) insight into structure and function of various family members, ii) barley AMY2 and AMY2/acarbose crystal structures (Kadziola et al., 1994, 1998), modeled AMY2/maltodecaose and AMY2/maltododecaose complexes (André & Tran, 1999; André et al., 1999), iii) established heterologous expression (Søgaard & Svensson, 1990; Søgaard et al., 1993a; Juge et al., 1996, 1998), iv) different mutagenesis strategies, and v) different activity assays to monitor changes in AMY1 properties (Matsui & Svensson, 1997; Gottschalk et al., 2001; Mori et al., 2001). Dual subsite mutants get special attention. Subsequently, focus is on the multidomain architecture of amylolytic enzymes and techniques and tools (Sigurskjold et al., 1998; Christensen et al., 1999; Payre et al., 1999; Sauer et al., 2001) found useful in describing the communication between the catalytic and the starch-binding domains of glucoamylase from *Aspergillus niger,* which are connected by a long, highly *O*-glycosylated linker. This modular structure is then explored for enhancing the action of AMY1 by fusion with the glucoamylase starch-binding domain (Juge et al., 2002). Finally, some approaching issues will be briefly dealt with including proteinaceous inhibitors, represented by the AMY2/BASI (barley α-amylase/subtilisin inhibitor) complex (Mundy et al., 1983; Abe et al., 1993; Vallée et al., 1998; Rodenburg et al., 2000), and the use of proteome analysis techniques to monitor the fate of barley AMY2 during seed germination (Østergaard et al., 2000). At the end selected problems, questions, and prospects will be listed.

Architectural themes in the α-amylase family

Members of glycoside hydrolase clan H (GH-H)

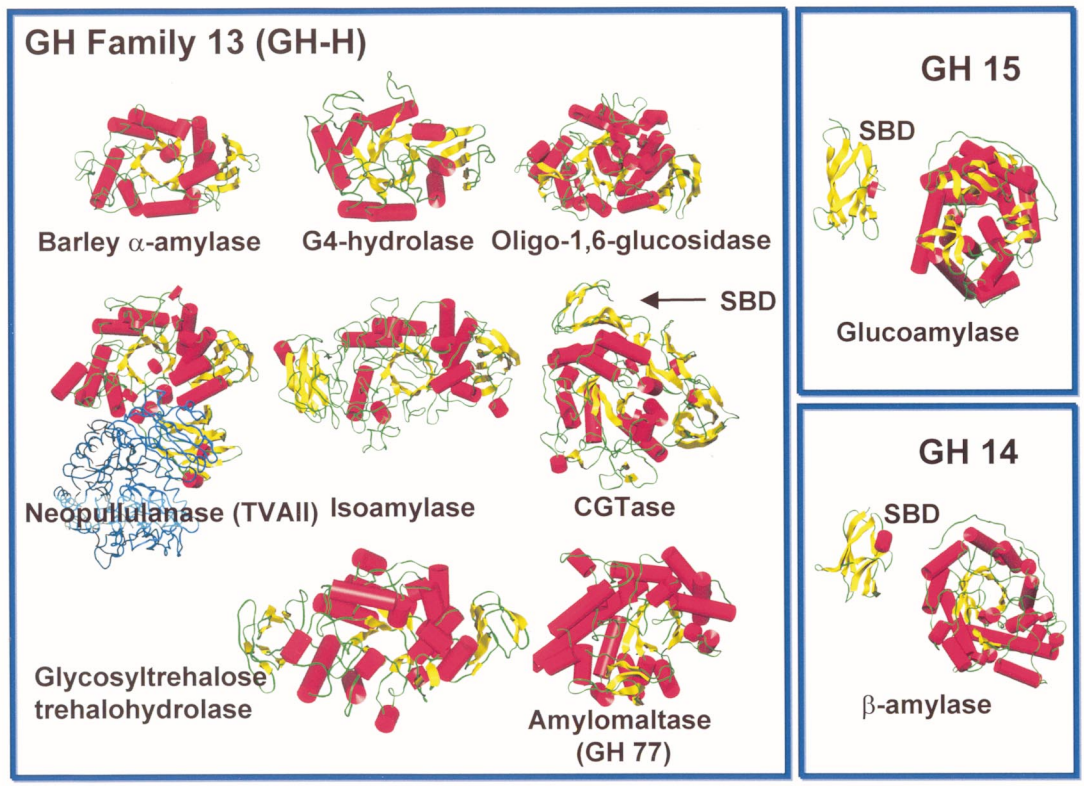

Fig. 1. Overview of the three-dimensional structures of selected endo- and exo-acting, α-1,4- and α-1,6-specific members of the α-amylase family (GH13 and GH77) containing domains A, B, and C (top row), extra N-terminal or C-terminal domains (center row), and with exceptional features of the $(\beta/\alpha)_8$-domain or without domain C (bottom row). Examples of glucoamylases (GH15) and β-amylases (GH14) are included.

(`http://afmb.cnrs-mrs.fr/~cazy/CAZY/index.html`), i.e., families 13, 70, and 77 share a catalytic $(\beta/\alpha)_8$-barrel domain, in case of GH70 in circularly permuted form (MacGregor et al., 1996, 2001). Currently crystal structures (Fig. 1) are reported for 15 specificities from GH13 and GH77; a few of these, however, are very closely related (Park et al., 2000), thus reducing the number to truly 13 different ones. The prototype structure comprises an N-terminal $(\beta/\alpha)_8$-barrel (domain A) having a rather long segment (domain B) connecting β-strand and α-helix 3, and a C-terminal antiparallel β-sheet fold (domain C). This type includes hydrolases and transglycosidases, endo- and exo-acting, as well as α-1,4- and α-1,6-bond-specific enzymes. Extra N- or C-terminal domains (Jespersen et al., 1991) are recognized in some members, e.g. in the dimeric cyclodextrinase [closely related to, if not in the same enzyme class as, neopullulanase, maltogenic amylase, and TVAII (*Thermoactinomyces vulgaris* α-amylase II)], in debranching and branching enzymes, and in cyclodextrin glycosyltransferase. Together these enzymes also represent hydrolases and transglycosidases as well as both α-1,4- and α-1,6-bond-type specificity. Larger structural diversity is found in glycosyltrehalose trehalohydrolase (Feese et al., 2000) and amylomaltase; the latter belongs to GH77 and characteristically lacks domain C (Przylas et al., 2000). In addition structures are available for two important exo-acting, inverting starch hydrolases, β-amylase (GH14) and glucoamylase (GH15) (Fig. 1). Recently, the structure of 4-α-glucanotransferase from a hyperthermophilic archaeon *Thermococcus litoralis*, a member of GH57 that includes several specificities found in GH-H, was determined at 2.8 Å resolution (Imamura et al., 2001). Because Glu123, however, was labelled by trapping a covalent enzyme-substrate intermediate in the absence of acceptor (Imamura et al., 2001), GH57 seems not to belong to clan GH-H in

GLYCOSIDE HYDROLASE FAMILIES 13, 70, 77
(http://afmb.cnrs-mrs.fr/~cazy/CAZY/index.html**)**

Enzyme class	*Enzyme name*	*EC number*	*N**
Hydrolases			
	α-Amylase (Family 13) #	**3.2.1.1**	**145**
	Oligo-1,6-glucosidase #	**3.2.1.10**	**6**
	α-Glucosidase	**3.2.1.20**	**19**
	Pullulanase +#	**3.2.1.41**	**13**
	Amylopullulanase +	**3.2.1.1/41**	**6**
	Cyclodextrinase + #	**3.2.1.54**	**6**
	Maltotetraohydrolase #	**3.2.1.60**	**3**
	Isoamylase + #	**3.2.1.68**	**6**
	Dextran glucosidase	**3.2.1.70**	**3**
	Trehalose-6-phosphate hydrolase	**3.2.1.93**	**2**
	Maltohexaohydrolase	**3.2.1.98**	**3**
	Maltotriohydrolase	**3.2.1.116**	**3**
	Maltogenic amylase #	**3.2.1.133**	**6**
	Neopullulanase + #	**3.2.1.135**	**8**
	Glycosyltrehalose trehalohydrolase + #	**3.2.1.141**	**6**
	Maltopentaosehydrolase	**3.2.1.-**	**2**
Transferases			
	Amylosucrase + #	**2.4.1.4**	**1**
	Glucosyltransferase (Family 70) +	**2.4.1.5**	**13**
	Sucrose phosphorylase	**2.4.1.7**	**6**
	Glucan branching enzyme + #	**2.4.1.18**	**25**
	Cyclodextrin glucosyltransferase #	**2.4.1.19**	**18**
	4-α-glucanotransferase (Family 77) #	**2.4.1.25**	**9**
	Glycogen debranching enzyme +	**2.4.1.25/3.2.1.33**	**3**
	Alternansucrase (Family 70) +	**2.4.1.140**	**1**
	Maltosyltransferase + #	**2.4.1.-**	**1**
	Acarbose-modifying glycosyltransferase	**2.4.1.-**	**(1)**
Isomerases			
	Maltooligosyltrehalose synthase #	**5.4.99.15**	**8**
	Trehalose synthase	**5.4.99.16**	**5**
	Isomaltulose synthase (sucrose isomerase)	**5.4.99.11**	**1**

 # = X-ray structure known; + = has extra N-terminal domain(s)

***N = number of sequences used for specificity motif definition**

Fig. 2. Specificities reported in GH-H (up-date from MacGregor et al., 2001).

which the catalytic nucleophile is an aspartic acid (Uitdehaag et al., 1999; MacGregor et al., 2001).

Mostly no role was assigned to the additional N- or C-terminal domains, two prominent exceptions being i) the starch-binding domain (SBD, classified as CBM20; `http://afmb.cnrs-mrs.fr/~cazy/CAZY/index.html`) that occurs in GH13 members of varying specificity, GH14, and GH15, and ii) the N-terminal domain in cyclodextrinase involved in dimerization and regulation of multisubstrate specificity (Kim et al., 2001). In Figure 2 the 28 specificities reported in GH-H are listed, indicating enzymes with a crystal structure and those with the N-terminal domain(s) that is (are) common in transglycosidases and hydrolases able to act on or near α-1,6 linkages (Svensson et al., 2002).

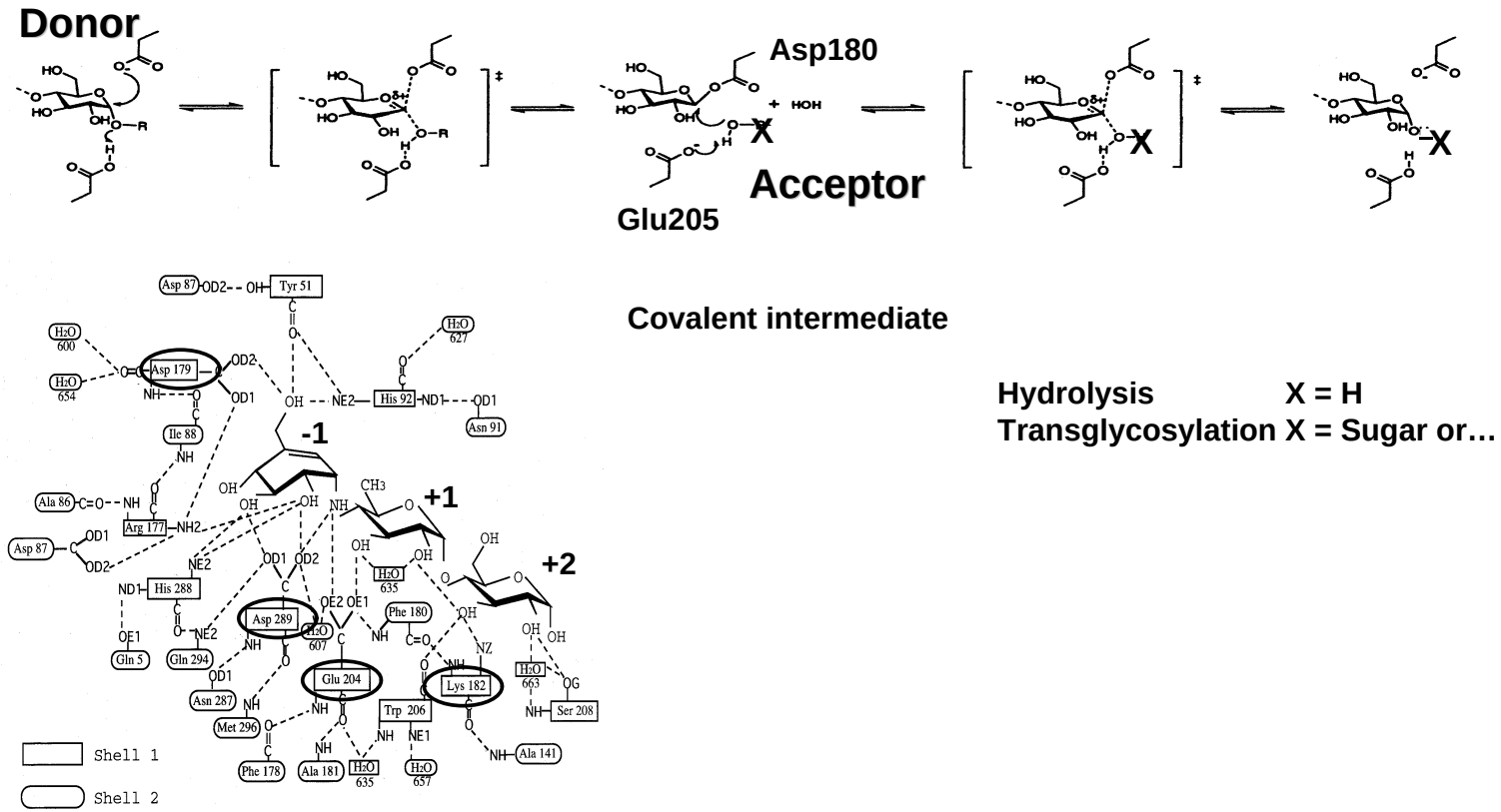

Fig. 3. Double-displacement mechanism used by enzymes of the α-amylase family (see for example Ly & Withers, 1999; Uitdehaag et al., 1999). The AMY1 numbers of the catalytic acid/base (Glu205) and catalytic nucleophile (Asp180) are indicated. The hydrogen bond network for AMY2 is shown at the lower left (Kadziola et al., 1998). Note that the Asp180Ala/Gly nucleophile mutants do not function as a glycosynthase. (The equivalent residue in AMY2 is Asp179.)

Mechanistic facets in the α-amylase family

The double displacement mechanism of retaining glycoside hydrolases is compatible with catalysis of both hydrolytic and transglycosylation reactions (Fig. 3). In fact, guided by sequence similarities and insight into structure/function relationships an α-amylase was recently engineered to act as a cyclodextrin transglycosylase (Beier et al., 2000). Most natural enzymes are principally either transglycosylases or hydrolases (Fig. 2), which to varying degree also catalyse "the other" reaction. This has been exploited in transglycosylation reactions designed to produce novel oligosaccharides. For both barley α-amylase and limit dextrinase it was possible to promote transglycosylation by using α-maltosyl fluoride (2 mM) as donor (Figs 3,4) with excess of appropriate acceptors (40 mM) to form high yields of e.g. the linear tetrasaccharide 4'-maltosyl-cellobiose using AMY1 and the pentasaccharide 6"-maltosyl-isopanose using limit dextrinase. If, however, a transglycosylation product is also a reasonable substrate, it accumulates only transiently, as in the case of formation of 6"-maltosyl-maltotriose by limit dextrinase (Fig. 4).

Originally for a retaining β-glucosidase and later for different enzymes acting on β-glycosidic linkages, Withers and collaborators took ingenious advantage of the double displacement mechanism to create so-called glycosynthases from mutants at the catalytic nucleophile (Mackenzie et al., 1998; Ly & Withers, 1999). When a reasonably reactive sugar derivative of the "wrong" anomeric configuration, typically an α-fluoride, was added to such a mutant enzyme, the substrate could bind at the active site in an orientation suited for catalysis of attack by an acceptor molecule (Fig. 3). The resulting product was not degraded because the mutant enzyme was unable to catalyze hydrolysis, but it might participate in additional "rounds" of glycosynthase reaction. This approach was attempted for barley AMY1 by mutation of the catalytic nucleophile in Asp180Gly/Ala. These AMY1 mutants showed 10^5–10^6 times reduced wild-type activity, but did not catalyse transglycosylation reactions with β-maltosyl fluoride nor did they undergo chemical nucleophile rescue by azide ions described to substitute for the lost nucleophile catalyst in β-glycosidase mutants (Ly & Withers, 1999). It is not understood why a glycosynthase reaction failed with these AMY1 mutants. Also for the corresponding mutant of human pancreatic α-amylase a glycosynthase reaction was not reported (Rydberg et al., 1999). It may be an inherent property of all GH13 or even all α-glycoside hydrolases. Trials with more nucleophile mutants of GH13 and related enzymes are needed to help find an explanation.

Molecular recognition of substrate in the α-amylase family

The contribution of individual sugar OH groups to activity has been thoroughly investigated for the inverting, exo-acting starch-hydrolase glucoamylase (GH15) by using synthetic deoxygenated analogues of maltose and isomaltose (Bock & Pedersen, 1987; Sierks & Svensson, 1992; Sierks et al., 1992; Frandsen et al., 1996; Lemieux et al., 1996). For the vast majority of GH13 members, however, it will be extremely difficult to synthesise useful deoxygenated analogues and interpret their effects, as the minimum substrates are mostly larger in size, and substrates in addition employ several binding modes. Only one enzyme category from GH13, namely the α-glucosidases, meets the same requirements as glucoamylase for a simple substrate structure and a single productive binding mode.

In contrast to glucoamylase, removal of one of any of the four OH groups on the non-reducing ring of isomaltose caused a major loss in transition-state stabilisation for two GH13 yeast α-glucosidases and a yeast oligo-1,6-glucosidase, indicating that all of these OH groups interact with charged groups in the enzyme (Frandsen et al., 2002). Glucoamylase had only OH-4' and -6' as key polar groups in the non-reducing ring of both maltosides (Bock & Pedersen, 1987; Sierks & Svensson, 1992; Sierks et al., 1992) and isomaltosides (Frandsen et al., 1996; Lemieux et al., 1996). Similarly, a retaining α-glucosidase of GH31 from barley malt also required only OH-4' and -6' from this ring (Frandsen et al., 2000). The present GH13 yeast oligo-1,6-glucosidase required, in contrast to the two GH13 yeast α-glucosidases, the 2 and 3-OH groups of the reducing-sugar ring, which afforded significant, albeit weaker, stabilization and therefore presumably participate in neutral hydrogen bonds with the enzyme (Frandsen et al., 2002). Remarkably, glucoamylase (Palcic et al., 1993) and α-glucosidase (Frandsen et al., 2002) furthermore preferred the *R*- and the *S*-diastereoisomer of methyl 6-alkyl isomaltosides, respectively, for which glucoamylase discriminated at step(s) related to the reversible binding (K_m) and α-glucosidase at subsequent steps in the mechanism associated with k_{cat}

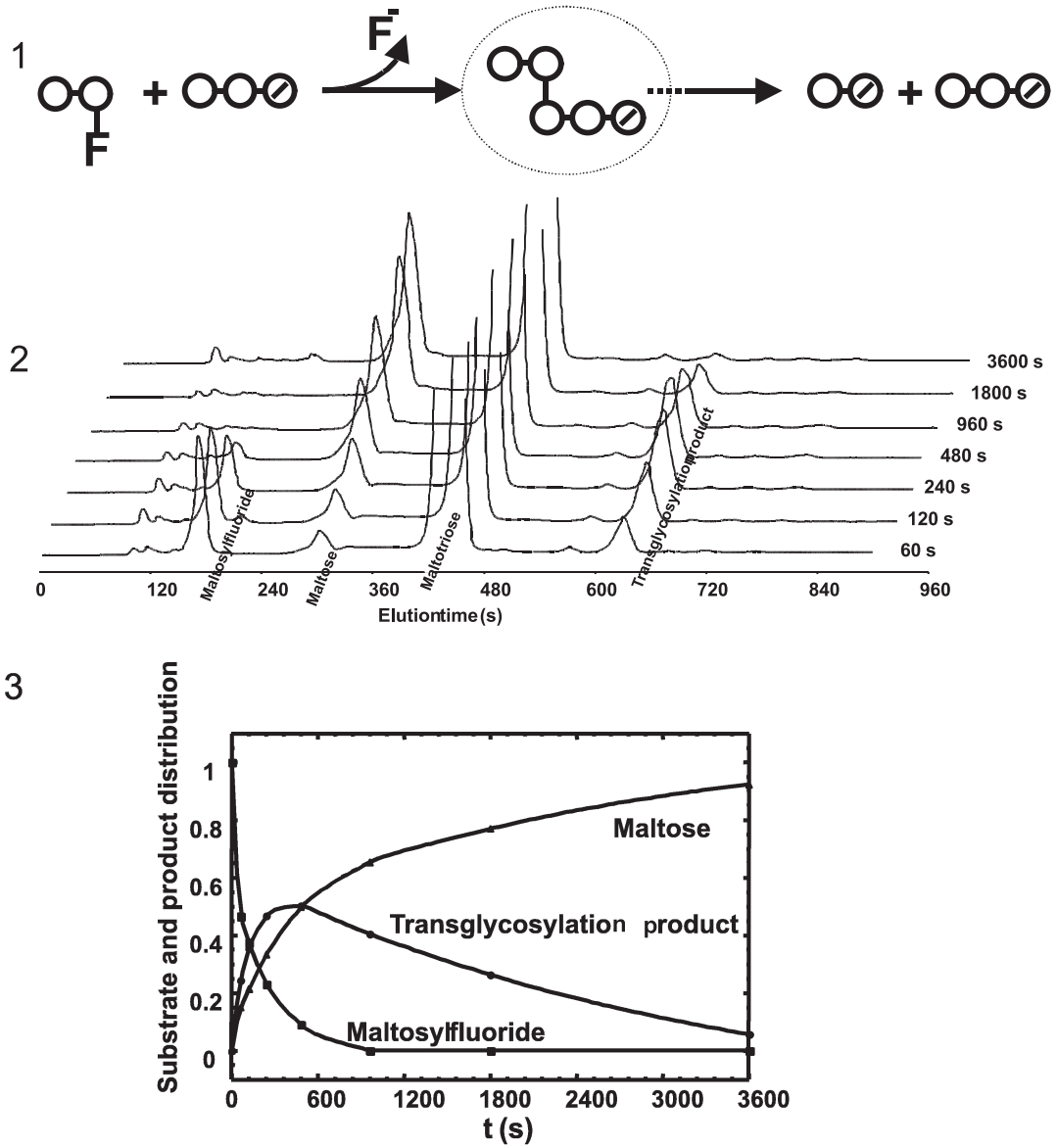

Fig. 4. Transglycosylation catalysed by barley limit dextrinase using α-maltosyl fluoride as donor and maltotriose as acceptor (1). The Dionex chromatograms (2) and the time course of the relative contents of substrates and products (3) are included.

(Fig. 5). Charged conserved residues, i.e. two histidines, an arginine, and two aspartates, are known to interact with glucose at subsite -1 in GH13 regardless of enzyme specificity and hence these are readily identified in the sequence. There is no such conservation, however, related to subsite $+1$ interactions where residues from a motif at $\beta \rightarrow \alpha$ 4, that contains specificity-denoting characteristics, are seen to interact with sugar ligands (MacGregor et al., 2001; see also Figure 3, lower left). The present findings on molecular recognition for α-glucosidases can help to guide modeling of complexes by adjusting hydrogen-bond interactions to comply with key polar groups and the preferred conformer for α-1,6 linked substrates.

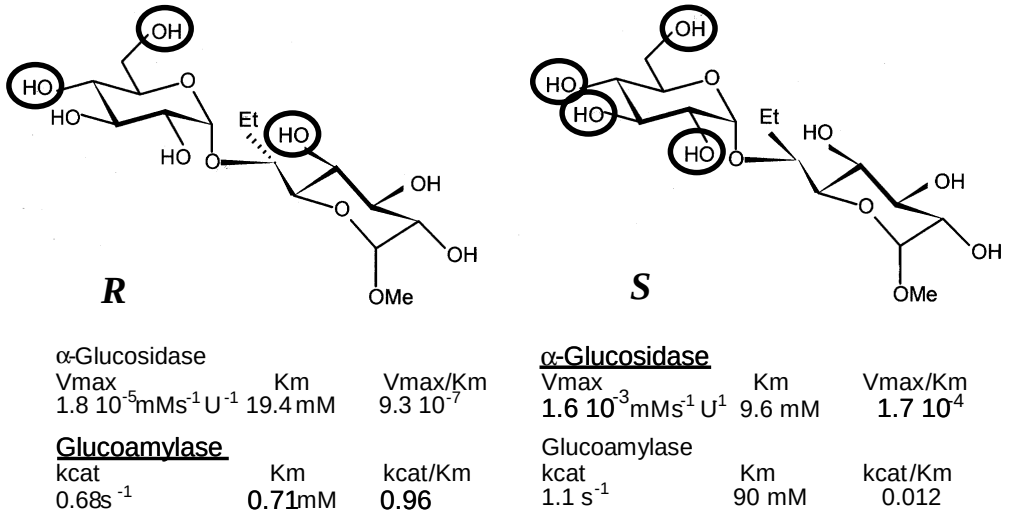

Fig. 5. Comparison of glucoamylase (GH15) and α-glucosidase (GH13) key polar groups in isomaltosides and kinetic parameters for hydrolysis of conformationally-biased substrate diastereoisomers (PALCIC et al., 1993; FRANDSEN et al., 2002). Underlining indicates the preferred kinetics.

Subsite mutagenesis in barley AMY1

The $\beta \rightarrow \alpha$ connecting segments of the catalytic $(\beta/\alpha)_8$-barrel in GH-H create the substrate-binding subsites and the catalytic site. The vast majority of GH-H enzymes have a long $\beta \rightarrow \alpha$ 3 segment (referred to as domain B) containing different secondary structure elements (JANECEK et al., 1997). It starts with a consensus sequence motif containing essential residues which, together with β-strands 4, 5, and 7 and their four immediate extensions, reflect enzyme specificity (JANECEK 1997, 2000; MACGREGOR et al., 2001). These four segments are thus particularly important in certain substrate-binding subsites. We explored a series of AMY1 mutants across the 10 subsites, -6 through $+4$ which are illustrated using a modeled AMY2/maltododecaose complex (Fig. 6; ANDRÉ & TRAN, 1999). The AMY1 structure has only recently been solved (ROBERT et al., 2002) and is not yet available.

Following initial analysis of structure/function relationships by mutation in AMY1 of the three catalytic acids and two transition-state-stabilizing conserved histidines all belonging to the four sequence motifs at $\beta \rightarrow \alpha$ segments 3, 4, 5, and 7 (SØGAARD et al., 1993b), a tripeptide in the motif situated at β-strand 4 was subjected to random mutagenesis (MATSUI & SVENSSON, 1997). Together these studies established the roles of two conserved histidines in transition-state stabilization and resulted in mutant enzymes of altered oligosaccharide-substrate-binding modes and higher activity than wild-type. Subsequently biased random mutagenesis was applied to $F^{286}VD$, a well-conserved tripeptide succeeded by a remarkably variable part of the $7^{th}\beta \rightarrow \alpha$ connecting segment. This tripeptide bridges the C-terminus of β-strand 7 and the N-terminus of a short 3_{10}-helix that carries the third catalytic acid and one of the transition-state-stabilizing histidines (GOTTSCHALK et al., 2001). The biased random mutation was designed to allow a total of 174 replacing sequences. Remarkably, in two of five reasonably active mutants glycine appeared at positions 287 and 288 where this residue occurs in only two known GH-H sequences. Compared to the parent enzyme C95A AMY1 (Tab. 1), used at that time to avoid inactivating glutathionylation of the Cys95 (SØGAARD et al., 1993a), C95A-F^{286} VG and C95A-F^{286}GG provided increased activity (k_{cat}/K_m) on Cl-PNPG$_7$ (2-chloro-4-nitrophenyl β-D-maltoheptaoside) combined with decreased activity toward insoluble Blue Starch. It turned out that this change in relative substrate specificity favoring the oligosaccharide over starch was rare in later subsite mutants. Moreover, the mutation in $F^{286}VD$ counteracted the low affinity for Cl-PNPG$_7$ and amylose DP17 of C95A AMY1 (Tab. 1; MATSUI & SVENSSON, 1997) involving a structural change near subsite -5 (Fig. 6). $F^{286}VD$ has no direct contact with substrate, but is situated near subsites $+1$ and $+2$ (Fig. 6).

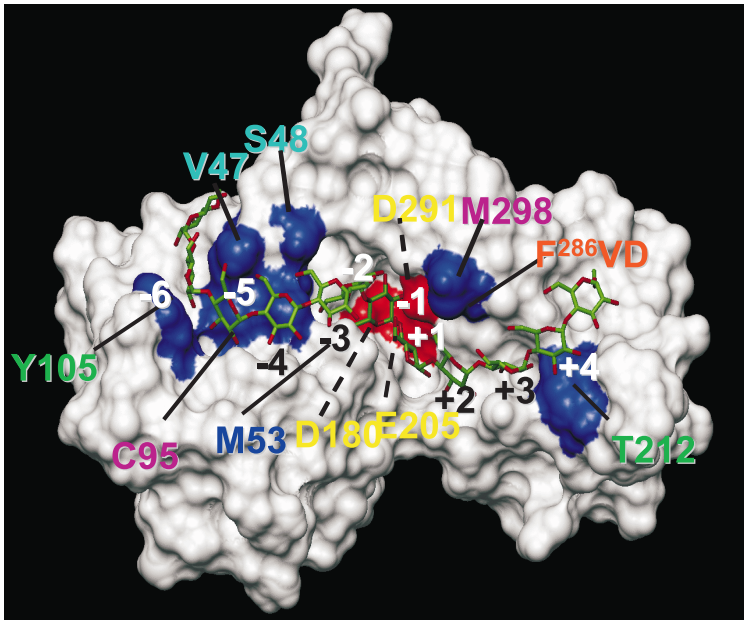

Fig. 6. Schematics of the location of AMY1 residues replaced in different subsite mutants (see text and Table 1) made using the AMY2/maltododecaose complex (modified from André & Tran, 1999). Catalytic acids are indicated by broken lines.

Based on the modeled structure of AMY2/maltododecaose (Fig. 6) residues involved in stacking at the extreme subsites −6 and +4 (Ajandouz et al., 1992) were mutated in e.g. Y105A and T212Y AMY1 to remove and introduce, respectively, aromatic stacking with sugar rings (Bak-Jensen, André, Paës, Tran & Svensson, in preparation). Surprisingly, while the former mutation drastically reduced activity toward oligosaccharides, activity for insoluble Blue Starch was enhanced (Tab. 1). In contrast, introduction of aromatic residues at subsite +4, here represented by T212Y, had no affect on activity toward the oligosaccharide, increased the activity for amylose DP17, and reduced activity for insoluble Blue Starch. The corresponding double mutant had intermediate activity for insoluble Blue Starch and Cl-PNPG$_7$, but inferior activity for amylose DP17 compared to both of the single mutants. It was concluded that stacking at the extreme ends of the binding cleft was disadvantageous for degradation of polymeric substrates, but favorable for action on oligosaccharides. However, for amylose DP17 that spans the binding cleft and interacts at both extreme end subsites, the double mutant, Y105A at subsite −6 dominated to cancel the highly-improved affinity in the single T212Y mutant at subsite +4 (Tab. 1).

As subsites +1 and +2 Met298 in AMY1 is within short distance of the OH-6 group of bound glucose residues and M298A/S/N were made in an attempt to facilitate substrate access, particularly the accommodation of branch chains in limit dextrins and amylopectin. These mutants showed wild-type-level activity towards insoluble Blue Starch and amylose DP17, but only 1–10% activity towards Cl-PNPG$_7$ (Mori et al., 2001). When combined with C95A at subsite −5 to give dual subsite mutants, these characteristics were accentuated, as activity toward insoluble Blue Starch was superior relative to wild-type, but reduced for amylose and the oligosaccharide to 15–30% and 0.4–1.2%, respectively (Tab. 1; Mori et al., 2001). When tested on a synthetic branched substrate 6'''-maltotriosyl maltohexaose, the mutants showed 5–15% activity compared to wild-type, which itself catalysed the release of only the glucose from the non-reducing end of the maltohexaose main chain at a very low rate of only 7% of that of maltotetraose hydrolysis (Mori et al., 2001).

In the dipeptide V^{47}S^{48} the side chains point toward subsites −5 and −3, respectively, and this dipeptide was an excellent candidate to explore the properties of variants involving several subsites by sampling all possible sequence combinations by saturation mutagenesis coupled with activity screening on starch plates (Mori, Andersen, Svensson, in preparation). Remarkably, although Val47, but not Ser48, is highly conserved in plant α-amylases, sequenced clones encoding active mutant enzymes varied little at position

Table 1. Enzymatic activities of subsite mutants of barley α-amylase 1.

AMY1	Cl-PNPG$_7$			Amylose DP17			Insoluble Blue Starch
	k_{cat} s^{-1}	K_m mM	k_{cat}/K_m s^{-1} mM^{-1}	k_{cat} s^{-1}	K_m mg ml^{-1}	k_{cat}/K_m s^{-1} mg^{-1} ml	U mg^{-1}
C95A-F^{286}VG[a]	58	3.5	17	47	0.70	67	1950
C95A-F^{286}GG[a]	32	1.1	29	48	0.70	69	375
Y105A[b]	<10	>10	–	146	2.4	61	3400
T212Y[b]	127	2.0	64	127	0.12	1058	1200
Y105A-T212Y[b]	31	6.0	5.2	78	2.3	34	1800
M298A[c]	34	3.0	11	348	0.66	527	3200
C95A-M298A[c]	n.d.	n.d.	1.3	373	2.9	129	5200
V47A[d]	40	5.5	7.3	94	1.5	63	1600
V47L-S48A[d]	80	11	7.3	370	4.0	93	4600
V47L-S48E[d]	n.d.	n.d.	0.7	75	12	6.3	2000
M53E[e]	n.d.	n.d.	0.4	206	6.6	31	3400
M53G[e]	4.0	11	0.4	65	7.1	9.2	1100
M53Y[e]	n.d.	n.d.	0.3	2.0	5.4	0.37	29
Wild-type[c]	122	1.1	111	248	0.52	477	2900
C95A[c]	258	20	13	351	2.5	140	5100

[a]Gottschalk et al., 2001. [b]Bak-Jensen, André, Paës, Tran & Svensson, in preparation. [c]Mori et al., 2001. [d]Mori, Andersen & Svensson, in preparation. [e]Mori et al. (2002).

48 compared to 47. Mutants selected for characterization showed large variation in activity (for three examples, see Table 1). While the predominant cleavage of 4-nitrophenyl maltoheptaoside (PNPG$_7$) by wild-type and subsite mutants resulted in formation of PNPG and G$_6$, indicating that productive binding includes interaction at the high-affinity subsite -6 (Ajandouz et al., 1992), several V^{47}S^{48} mutants, RD, KG, FS, and VY, produced substantial amounts of PNPG$_5$, PNPG$_3$, or PNPG$_2$ (not shown). These mutants also showed reduced activity for insoluble Blue Starch. The action pattern, however, of several other mutants e.g. LA was similar to that of AMY1 and these mutants had enhanced activity towards insoluble Blue Starch, but still had reduced activity towards amylose DP17 and Cl-PNPG$_7$ (Tab. 1).

Finally, Met53 at subsite $-2/-3$ is found only in plant α-amylases and a bacterial isoamylase, while many GH-H members have Asp, Gln or Trp at the equivalent position. The preceding Tyr52 is very highly conserved and stacks with substrate at subsite -1 as seen in several crystal structures (for examples, see Matsuura et al., 1984; Kadziola et al., 1998; Przylas et al., 2000). The two residues belong to a short sequence motif in $\beta \rightarrow \alpha$ loop 2. This loop interacts with the long $\beta \rightarrow \alpha$ loop 3 (domain B) to form part of the substrate glycon binding area. Both loops are short in the barley α-amylase compared to, for example, Taka-amylase (Matsuura et al., 1984; Kadziola et al., 1994). Three categories of Met53 mutants were obtained (Tab. 1; Mori et al., 2002). M53E represents those of high activity on insoluble Blue Starch and moderately reduced activity on amylose DP17; M53G represents those having moderately reduced activity also toward starch, and M53Y those of less than 1% activity toward starch and 0.1% toward amylose DP17 and Cl-PNPG$_7$. As for other subsite mutants the bonds cleaved in PNPG$_6$ and PNPG$_5$ reflected an apparent unfavorable glycon accommodation in the mutant compared to wild-type AMY1 (Fig. 7).

Mutational modification of oligosaccharide bond cleavage patterns

The various subsite mutants represent both altered substrate preferences (Tab. 1) and changes in the action patterns on oligosaccharide substrates. Some of the latter changes are already mentioned above. The major binding modes of the different mutants at subsites -6, -5, $-3/-2$, $+1/+2$, and $+4$ (Fig. 6) are summarized in Figure 7. The present mutants either maintained the wild-type binding mode or shifted to a larger coverage of the aglycon binding region. For PNPG$_7$ subsite -6 of high affinity essentially controlled the binding mode for both the mutant and wild-type enzymes. Typically 90–95% cleavage occurred to produce PNPG and G$_6$, but a few mutations (not

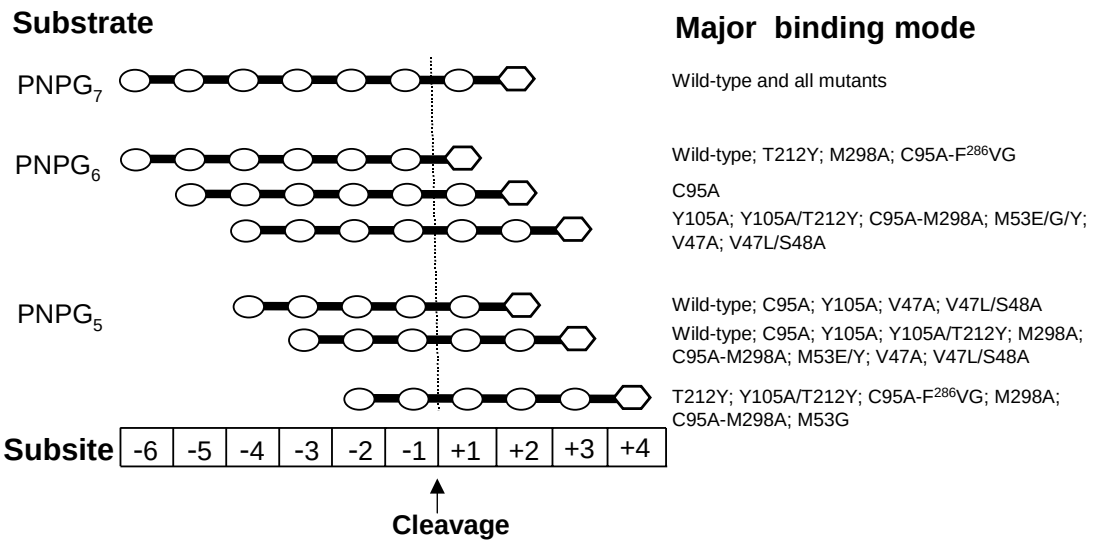

Fig. 7. Schematics of the major oligosaccharide-binding modes in different subsite mutants (see text and Table 1).

shown) caused substantial release of $PNPG_2$ and $PNPG_3$. With $PNPG_6$ the wild-type binding mode was kept only for T212Y, M298A – the two single mutants in the aglycon binding region – and C95A-F^{286}VG, which has no direct substrate contact but is located in the same region near subsite $+1/+2$. In contrast, mutation at glycon-binding subsites reduced interactions at this area shifting the cleaved bond toward the non-reducing end of the substrate (Fig. 7). Thus these structural changes were not overcome by the high affinity at subsite -6. Finally for $PNPG_5$ two wild-type binding modes were equally important producing 40–45% of $PNPG_2$ and $PNPG_3$. Mutations at the end of the binding cleft (Y105A, C95A, V47L, and V47L/S48A) had essentially no effect on this binding mode as $PNPG_5$ does not interact with subsite -6 in a productive complex. The other mutants showed a shift in the binding mode toward the aglycon-binding region (Fig. 7).

Manipulation of the degree of multiple attack

Using amylose of average DP440 as substrate (Kramhøft & Svensson, 1998) the degree of multiple attack (DMA) was determined for selected mutants. DMA indicates the number of substrate bonds hydrolysed subsequent to the initial cleavage without prior dissociation of the enzyme-substrate complex (Robyt & French, 1967). Whereas AMY1 has DMA = 2, values of 3.2 and 1.1 were determined for the mutants Y105A and M298S, respectively. Apparently loss in Y105A of substrate stacking at subsite -6 facilitates the processive mechanism of the enzyme-glycon complex at the active site, whereas mutation at subsite $+1/+2$ in M298S impedes contact between shorter parts at the reducing end of the substrate chain and the enzyme. Quantitative analysis of oligosaccharide products from amylose DP440 supported the DMA data as Y105A and M298S released higher and smaller amounts of maltooligosaccharides, respectively, than wild-type AMY1 (not shown).

The function of the starch-binding domain

Most GH-H members are multidomain proteins and some, including cyclodextrin glycosyltransferases, maltotetraose-forming amylase, and a small group of the α-amylases, contain a starch-binding domain (SBD, a member of CBM20; `http://afmb.cnrs-mrs.fr/~cazy/CAZY/index.html`). SBD has two binding sites seen in the structure of complexes of β-cyclodextrin and the isolated domain from *A. niger* glucoamylase (Sorimachi et al., 1997) and of maltose binding to the whole cyclodextrin glucosyltransferase protein (Lawson et al., 1994). In glucoamylase the interaction between the catalytic domain and SBD, which are connected by a long, highly *O*-glycosylated linker, was studied by using heterobifunctional inhibitors of varying length (Sigurskjold et al., 1998; Payre et al., 1999) and by genetically shortening the linker (Sauer et

al., 2001). The inhibitors consisted of acarbose, having picomolar affinity for the active site (SIGURSKJOLD et al., 1994), connected by a polyoxyethylene spacer to β-cyclodextrin. The results showed formation of a 1:1 complex for glucoamylase wild-type and linker variants indicating that one of the two SBD binding sites and the active site of the catalytic domain were near each other in the solution conformation. The second SBD site could still bind β-cyclodextrin with a 1:1 stoichiometry, however, with a minor entropy penalty for an engineered glucoamylase with shortened linker acting on the shorter form of the double-headed inhibitor without a spacer (SAUER et al., 2001). The thermodynamics of the binding of the bidentate inhibitor showed a large entropy penalty but essentially no loss in the enthalpy, as judged from the enthalpies determined for binding acarbose and β-cyclodextrin alone (SIGURSKJOLD et al., 1998). The linker was shortened to a certain extent without loss of enzyme function, albeit with some loss in conformational stability of the variants compared to wild-type glucoamylase (SAUER et al., 2001). Attempts, however, to introduce a very short linker present in a homologous fungal glucoamylase were unsuccessful, suggesting certain species-specific requirements for the linker structure (SAUER et al., 2001). A form of glucoamylase lacking SBD hydrolyzed granular starch at about 1% of the rate of the full-length form containing the C-terminal SBD (SVENSSON et al., 1982). This demonstrated the need for SBD in hydrolysis of natural substrates and motivated construction of a fusion between barley AMY1 and SBD from *A. niger* glucoamylase with the goal of enhancing the attack of α-amylase on starch granules and other solid starches. This fusion retained activity on amylose DP17 and Cl-PNPG$_7$, bound more tightly onto starch granules, and had about two-fold increased activity for both soluble and granular starch. Remarkably, when assaying at low enzyme concentration, the initial rate of granule hydrolysis catalysed by the AMY1-SBD fusion was 10 times higher than that of AMY1 and resulted also in more extensive degradation after prolonged incubation (for more details, see JUGE et al., 2002).

Proteinaceous inhibitors of GH13 enzymes

In GH13 only some few animal and plant α-amylases and the barley limit dextrinase (MACRI et al., 1993) are reported to be inhibited by proteins. These protein-protein interactions currently include five types for which the structure of the complex is known: porcine pancreatic α-amylase and Tendamistat from *Streptomyces tendae* (WIEGAND et al., 1995), porcine pancreatic α-amylase and αAI, a lectin-like inhibitor from *Phaseolus vulgaris* (BOMPARD-GILLES et al., 1996), barley α-amylase 2 and barley α-amylase/subtilisin inhibitor (BASI) (VALLÉE et al., 1998), yellow meal worm α-amylase and a bifunctional inhibitor from *Ragi* (Indian finger millet) (STROBL et al., 1998), and the same enzyme with a bound small inhibitor from *Amaranth* (PEREIRA et al., 1999). In three cases the catalytic acids in the enzyme and the inhibitor directly interact, i.e. the two complexes of porcine pancreas α-amylase and the yellow meal worm α-amylase/*Ragi* inhibitor complex. The inhibitor from *Amaranth* in contrast has contact through a water molecule and in AMY2/BASI, electrostatic networks via water molecules coordinated by a fully hydrated calcium ion at the protein interface make indirect contact between the three catalytic acids and side chains in BASI. Only αAI and the *Amaranth* inhibitor are described to exert substrate mimicry.

For AMY2/BASI the complex formation followed a simple two-step fast, tight binding mechanism as demonstrated using stopped-flow fluorescence spectroscopy (SIDENIUS et al., 1995). Furthermore, mutational analysis identified key groups in AMY2 for complex formation and suggested that a small number of mutations in AMY1 might render this isozyme sensitive to BASI (RODENBURG et al., 2000). Recently, expression and mutation of BASI extended this work. Thus while R128Q and D142N mutants in the enzyme increased K_i from 0.22 nM to 18 and 28 nM, respectively (RODENBURG et al., 2000), the BASI mutants S77A and K140N involving side chains that interact with R128 and D142, caused modest and dramatic reduction, respectively, of inhibitory activity. These and other BASI mutants are currently being examined. Surface plasmon resonance analysis indicates that k_{off} is generally more sensitive to mutation than k_{on}. Although the few mutations in the enzyme (RODENBURG et al., 2000) clearly confirmed the concept of protein-protein interactions being controlled by a few "hot spots", we seem to find an imperfect match between the effect of modifying each of two interacting groups of the protein partners. This may be due to the large interface comprising some ten conspicuous interacting groups (VALLÉE et al., 1998), some of which represent more than a single non-covalent contact but rather a small bonding network, or it may stem from adverse structural changes accompanying the individual mutations.

Approaching issues, some remaining problems, and prospects in GH-H

The era of post-genomics and the annotation of genes in entire genomes provide new ways to use bioinformatics (Henrissat et al., 2001) also on the GH-H clan. Some of the related experimental approaches include high-through-put technologies of advanced resolution and sensitivity which emphasize the complexity of the relation between protein chemistry and structural biology *in vivo*, *in vitro* and *in silico*. Although the proteomes present only snapshots to be compiled for studies of virtual organisms, the type of information gained using such techniques triggers new thinking also in well-established fields. For example 2D-gel electrophoretic patterns of samples prepared from seeds during germination showed, along with the *de novo* synthesized AMY2 forms (Østergaard et al., 2000), that a ladder of conspicuous immunoreactive fragments of AMY2 appeared early, even several days before the enzyme activity peaked. This apparent controlled proteolysis of specific AMY2 bonds reflected an efficient inactivation by degradation of different multiple AMY2 forms.

Central points remain less thoroughly understood in GH-H. Thus despite access to a large number of primary and rather many crystal structures, rational design of variants with desired properties e.g. substrate specificity, pH-activity dependence, or thermostability includes only a few described examples (Beier et al., 2000; Nielsen & Borchert, 2000). It seems, however, also from the present work on AMY1 from barley, that combination of rational and irrational mutagenesis approaches can lead to variants with new – albeit less predictable – properties in conjunction with high activity. This in the future should take advantage of coupling with *in vitro* evolution strategies. A special asset for rational engineering of an amylolytic enzyme is knowledge on the bound sugar ligand conformation and binding energies which could be obtained from an available or modeled complex structure, or evaluated through a molecular recognition approach. Furthermore, the potential of engineering calcium requirements or of introducing or removing calcium from the structures through engineering has been little addressed, although a commercial bacterial α-amylase variant of high activity at low concentration of calcium ions has been achieved (Hashida & Bisgård-Frantzen, 2000). In fact only some GH-H members require calcium ions and, while some crystal structures reveal a highly conserved calcium ion, as well as other calcium ions at varying positions, others contain no calcium ion at all.

The modular architecture of most amylolytic enzymes invites construction of fusion proteins or chimera. In both cases one may obtain a novel and advantageous combination of certain functionalities e.g. binding onto solid substrates, or manipulation of activity towards various categories of substrates, e.g. branched dextrins. Other applications could be in transglycosylation for production of novel oligo- or polysaccharides. Insight into these reactions at the structural level is limited. This also includes understanding of the rather large number of sugar-binding sites identified outside of the active site region. Questions thus remain on their role in substrate binding and catalysis and how these sites or extra domain(s) interact with the catalytic site or domain. This presumably has special relevance in degradation of insoluble substrates, but could also include interaction with proteinaceous inhibitors. This area is predicted to be opened up for discoveries, as only very few of the GH-H enzymes have been found to be sensitive to a protein inhibitor so far. Finally, even though a very detailed interpretation of substrate and enzyme conformational changes during individual steps of catalysis has been reported (Uitdehaag et al., 1999, 2001) we still do not know why nucleophile mutants of the two α-amylases, AMY1 and human pancreatic α-amylase, could not function in a glycosynthase reaction.

In conclusion, the knowledge of GH-H and GH57, which contains specificities related to GH-H, is rapidly growing. In particular, the increasing number of new crystal structures, including protein-inhibitor complexes, and emerging rational engineering of function, e.g. specificity and sugar recognition outside of the active site region, represent advances that in addition to developments in post-genomic bioinformatics will be changing the understanding of the biology of these enzymes.

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