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Interactions between Barley α-Amylases, Substrates, Inhibitors and Regulatory Proteins

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Abstract: Barley α-amylase binds sugars at two sites on the enzyme surface in addition to the active site. Crystallography and site-directed mutagenesis highlight the importance of aromatic residues at these surface sites as demonstrated by K_d values determined for β-cyclodextrin by surface plasmon resonance and for starch granules by adsorption analysis. Activity towards amylopectin and amylose follows two different kinetic models, degradation of amylopectin being composed of a fast and a slow component, perhaps reflecting attack on A and B chains, respectively, whereas amylose hydrolysis follows a simple Michaelian kinetics. β-cyclodextrin binding at surface sites inhibits only the fast reaction in amylopectin degradation. Site-directed mutagenesis and activity analysis, furthermore show that one of the surface binding sites as well as individual subsites in the active site cleft have distinct roles in the multiple attack on amylose. Although the two isozymes AMY1 and AMY2 share ligands for three structural calcium ions, they differ importantly in the effect of calcium on activity and stability, AMY1 having the higher affinity and the lower stability. The role of the individual calcium ions is studied by mutagenesis, crystallography and microcalorimetry. Further improvement of recombinant AMY2 production allows future direct mutational analysis in this isozyme. Specific proteinaceous inhibitors act on α-amylases of different origin. In the complex of barley α-amylase/subtilisin inhibitor (BASI) with AMY2, a fully hydrated calcium ion at the protein interface mediates contact between inhibitor residues and the enzyme catalytic groups in a manner that depends on calcium and which can be suppressed by site-directed mutagenesis of Glu168 in BASI. Finally certain inhibitors and enzymes are targets of the disulphide reductase thioredoxin h that attacks a specific disulphide bond in BASI and, remarkably, reduces two different disulphide bonds in the barley monomeric and dimeric amylase inhibitors that both belong to the CM-proteins and inhibit animal α-amylase.

Key words: glycoside hydrolase family13, secondary binding sites, surface plasmon resonance, calcium effects, regulatory proteins

α-Amylases belong to the glycoside hydrolase family 13 (GH13) that together with glycoside hydrolase families 70 and 77 constitute clan H of glycoside hydrolases (GH-H).[1)] GH-H contains approximately 30 different enzyme specificities acting in hydrolase and transglucosidase reactions on α-1,4 and / or α-1,6-glucan and α-glucoside substrates.[2)] The three-dimensional structures of α-amylases mostly contain three domains; domain A, a $(\beta/\alpha)_8$-barrel; domain B a small protruding loop situated between β-strand and α-helix 3 in the $(\beta/\alpha)_8$-barrel; and domain C, a C-terminal anti-parallel β-sheet composed of 5−10 strands. The substrate binding site is made from residues of domains A and B and the three acid residues involved in catalysis are situated at the C-terminal ends of β-strands 4, 5 and 7.[2)] These three catalytic residues are the only invariant residues in GH-H.[3)] A few new complexes between amylolytic enzymes and substrates or substrate analogues have been recently published.[4,5)] These structures provide novel insight in particular with respect to

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Abbreviations: AMY1 and AMY2, barley α-amylases 1 and 2; BASI, barley α-amylase/subtilisin inhibitor; β-CD, β-cyclodextrin; CM-protein family, family of chloroform-methanol soluble cereal seed proteins; DMA, degree of multiple attack; DP, degree of polymerization; GH13, glycoside hydrolase family 13; GH-H, glycoside hydrolase clan H; SBD, starch binding domain.

details at atomic resolution of the enzyme substrate interactions throughout long substrate binding clefts as well as on the binding of carbohydrate at secondary sites on enzyme surfaces.[4] One especially important functional property related to these recent complexes is the enzymatic activity on polysaccharide substrates. Information on the mode of action on these substrates is currently obtained from a combination of enzyme kinetics and substrate profile analysis. Special themes include the degradation of the polysaccharide by a multiple attack mechanism and the determination of the degree of multiple attack (DMA)[6] as well as subsite mapping of individual affinities of substrate binding subsites and relation with the fine structural details of the binding cleft (Kandra *et al*., submitted).

Mutational analysis has become a very powerful and widely utilized approach in the determination of functional roles of specific binding subsites[7–10] or secondary binding sites situated on the enzyme surface.[11] One objective, however, remains to combine the above types of information to advance toward a profound understanding of the interplay between amylolytic enzymes and macromolecular substrates. A separate facet includes the attack on insoluble substrates such as starch granules isolated from natural sources or other forms of insoluble starch or amylose. Finally, in the interaction with polysaccharides certain enzymes benefit from auxiliary carbohydrate binding modules that have particularly high affinity and specificity for starch and related sugars. The characterisation of the interaction of such modules with polysaccharide substrates and their exploitation in tailor-made enzyme fusion proteins with novel functionalities currently presents a highly efficient tool for enhancing the action of α-amylases. Although barley α-amylase, in addition to the active site, has two surface carbohydrate binding sites which are involved in binding onto starch granules, it has thus been possible by aid of fusion with a starch binding domain (SBD) to greatly increase both the rate by which the barley α-amylase releases soluble products from barley starch granules and the efficiency of the degradation.[12]

In order to facilitate investigation of the role of isozyme specific structural differences in the 80% sequence identical isozymes 1 and 2 (AMY1 and AMY2), it would be an advantage to have access to suitable expression systems for both of the corresponding genes. However, for more than a decade this type of investigation has been limited because AMY2, which is the major isozyme in the germinating seed, is only obtained in small amounts in recombinant form by heterologous expression in the yeasts *Saccharomyces cerevisiae* and *Pichia pastoris*.[13,14] Very recently, however, insights into the relation between a specific part of the AMY2 sequence and the poor yield of recombinant protein, obtained through isozyme chimeras,[15–18] allowed design of a semi-rational combinatorial protein engineering experiment. Combinatorial exchange of AMY2 residues in the sequence stretch implicated to be responsible for the low expression, by the corresponding AMY1 residue and screening for expression of active enzyme secreted by *S. cerevisiae* colonies in plate assays resulted in AMY2 variants with improved expression compared to AMY2 wild-type.[19] For subsequent site-directed mutagenesis in AMY2 and production of AMY2 mutants, the host *P. pastoris* will be used.

Most α-amylases depend on calcium ions for activity and stability and share a structural calcium ion situated in the three-dimensional structure in the near vicinity of the catalytic site. Many α-amylases also contain additional calcium, sodium or chloride ions. Binding of the conserved Ca^{2+} ion is suggested to be irreversible in the sense that if removed *e.g.* by reaction with a chelator, the integrity of the protein structure is weakened to an extent where it cannot be reconstituted by addition of the ion in question. In the barley α-amylases three structural calcium ions are present.[20,21] They superimpose perfectly in AMY1 and AMY2 and also share all protein ligands. Calcium, however, has distinctly different effects on stability and enzymatic activity of AMY1 and AMY2 and the isozymes, therefore, represent a unique source to understand structural features that modulate the impact of calcium on the behaviour of α-amylases. AMY1 and AMY2 show a number of other functional differences one of these being the sensitivity of AMY2 to the endogenous proteinaceous inhibitor barley α-amylase/subtilisin inhibitor (BASI), present in the mature seed and hence during germination.[22]

The AMY2/BASI complex depends on a fourth calcium ion and represents just one of five known types of α-amylase/α-amylase inhibitor complexes involved in regulation of α-amylase activity.[23–25] Noticeably α-amylases of mammalian, insect, plant and microbial origin are inhibited by proteins from a variety of structural families and both biochemical analysis as well as crystallography of different α-amylase/inhibitor complexes reveal their diverse mode of action.[26] An additional type of regulation which might be important is mediated by special regulatory proteins that in turn react with the α-amylase inhibitors. BASI, for example, interacts with barley thioredoxin h that catalyses the *in vitro* reduction of primarily one of the two disulphide bonds present in BASI.[27] Such protein networks will be addressed in the future in relation to understanding the biological systems represented by germinating seeds.

Secondary surface binding sites in AMY1 and AMY2.

Very early, a site on the surface of AMY2 that contained Trp276-Trp277 was identified to bind β-cyclodextrin by using differential labelling of tryptophanyl side chains accessible to chemical modification and identification of modified and protected residues by peptide fingerprinting and sequence analysis.[28] This surface site was subsequently observed in the crystal structure of the AMY 2/acarbose complex to accommodate two rings defined from bound acarbose, a pseudotetrasaccharide inhibitor.[20] A few years ago a new surface site for GH13 was discovered, in this case in AMY1.[21] The central residue was Tyr380 around which the bound oligosaccharide adopted a half-circle conformation. Although Tyr380 and many other residues in its near vicinity are conserved in AMY2, sugar binding to the corresponding area was never observed in AMY2 crystal structures. The site was named "a pair of sugar tongs," since Tyr380 moves more than 3 Å upon oligosaccharide binding at the site (Fig. 1).[21]

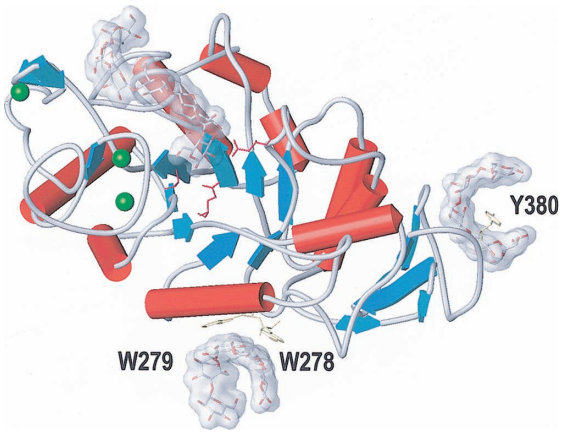

Fig. 1. Surface binding sites of AMY1.

The crystal structure of the inactive catalytic nucleophile utant D180A AMY1 with maltoheptaose bound in the active site cleft and to surface binding sites where only glucose rings with good electron density are shown (Ref. 4). The three residues at the catalytic site are shown in pink, and Tyr380 at the "sugar tongs" surface binding site as well as the two residues Trp278 and Trp279 in the starch granule binding site are shown in yellow. The three green solid spheres represent the Ca^{2+} ions.

In order to study the importance of the "sugar tongs" site, a mutational analysis was designed in which Tyr380 and adjacent residues were replaced. These AMY1 mutants were subsequently analysed for enzymatic properties towards the usual substrates; 2-chloro-4-nitrophenyl β-D-maltoheptaoside; amylose DP17 and DP440; and insoluble Blue Starch. The affinity for β-cyclodextrin was measured using surface plasmon resonance analysis and for barley starch granules using an assay in which the dissociation constant was calculated from the ratio of bound/unbound enzyme after incubation with the starch granules at low temperature. Recently, the "sugar tongs" site was also demonstrated by protein crystallography to bind β-cyclodextrin (Tranier, Aghajari, Haser, Mori and Svensson unpublished results). Among the analysed "sugar tongs" mutants Y380A and Y380M AMY1 both showed 7 fold lower affinity for β-cyclodextrin and Y380A was found to reduce affinity for barley starch granules by more than one order of magnitude. For both of these mutants activity was modestly, albeit significantly affected. In contrast, when the AMY2 residue proline was replacing $Ser378_{AMY1}$ at the only remarkable sequence variation between AMY1 and AMY2 in the vicinity of Tyr380, the AMY2 mimic S378P AMY1 did not loose affinity towards β-cyclodextrin or starch granules. Recent mutation in AMY1 of the first identified surface site Trp278Trp279, which also binds carbohydrate in the crystal structures of AMY1, was found to decrease the affinity for starch granules. The binding is currently analysed for a series of mutants at this site as well as for multiple-site mutants combining both this and the "sugar tongs" site.

Action on polysaccharide substrates.

High molecular mass amylose of DP 440 was previously used to investigate the mode of action of AMY1 and AMY2 towards un-branched polysaccharides. It was found that similarly to some other α-amylases, AMY1 and AMY2 degrade amylose DP440 by a so-called multiple attack mechanism.[6] In this mode of action the initial endo-action and hydrolytic cleavage of the substrate chain is followed by release of one of the polymeric products, whereas the other product remains bound to the enzyme.[29] A relocation of this product in the active site, results in the formation of a new productive complex, from which this time predominantly shorter maltodextrins and -oligosaccharides are released, the major product being maltoheptaose. In an attempt to gain insight into the role of specific enzyme residues in the substrate-binding cleft in the multiple attack mechanism, a series of mutants at subsites along the entire binding site—presumably extending over 10 binding subsites, *i.e.*, 6 or 7 substrate glycone binding subsites and 2–4 aglycone binding subsites—were studied.[6] Surprisingly, although the measured degree of multiple attack (DMA) of the various AMY1 mutants varied from 1 to 3.3 compared to wild-type AMY1 having a DMA of 1.9, the oligosaccharide product profiles from these mutants retained maltoheptaose as the predominant product, while small albeit clear changes occurred in the distribution of some of the less prominent products of DP 1–5. It was proposed therefore that the multiple attack mechanism was associated with structural features found outside the substrate binding cleft. Possible candidates for such sites in AMY1 are the two surface sites. Additional support for the role in multiple attack of sites on the enzyme surfaces was obtained from the fusion protein AMY 1-SBD in which the C-terminus of AMY1 was coupled to the starch-binding domain (SBD) from *Aspergillus niger* glucoamylase *via* a linker sequence found naturally in the latter enzyme.[29] The AMY1-SBD thus has two extra surface binding sites contributed by the SBD moiety[30] and showed increased DMA of 3.0, whereas the oligosaccharide product profiles were very similar to those obtained with wild-type AMY1, although the relative contents of products of DP 8–10 slightly increased. In a preliminary analysis, the DMA of the Y380A mutant was reduced compared to the wild-type value, which supports the hypothesis that binding of polysaccharide substrates at secondary surface sites could be important in the mechanism of multiple attack on these substrates.

To further investigate the interaction between high molecular weight substrates and AMY1 and AMY2, the time course of release of products was determined for amylopectin and amylose. It was found that in a certain concentration range of amylopectin, the degradation could be represented by bi-exponential kinetics, whereas that of amylose followed a simple mono-exponential relationship. Deconvoluting the individual rate constants towards the two polysaccharides therefore gave an overlay of reactions with a low and a high rate of hydrolysis for amylopectin, while for amylose one fast reaction was identified. Remarkably, addition of β-cyclodextrin decreased only the fast component in the hydrolysis of amylopectin and influenced neither the rate of amylose nor the slow component in the hydrolysis of amylopectin. It is speculated that the earlier identified surface site with affinity for β-cyclodextrin is involved in the fast reaction component in the degradation of amylopectin. Perhaps this is an effect of attaching the enzyme to the polysaccharide and facilitating action on a special category of substrate bonds, for example those found in the outer A chains in the structure

of the amylopectin molecule. The elaborate kinetics analysis will be extended to include surface site mutants.

Development of efficient heterologous expression and production of recombinant AMY2.

One serious impediment in structure/function relationship investigation of barley α-amylases has been low yields of recombinant AMY2 obtained in the yeast expression systems that work well for AMY1. This has been a particular concern since in germinating barley seeds the content of AMY2 exceeded that of AMY1 by more than an order of magnitude.[31] It thus has been practically impossible to investigate this major isozyme by mutational analysis in combination with protein structure determination. In a very early attempt to counteract this problem, isozyme chimeras were generated by *in vivo* homologous recombination in *S. cerevisiae*.[15–18] The yields of recombinant AMY1/AMY2 chimeras were found to increase with the segments size of the N-terminal AMY1 component in the obtained chimeras that contained varying lengths of the domain A barrel and the domain B from AMY1 and had the remaining C-terminal segments from AMY2. These chimeras allowed mutational analysis of the roles of specific residues in domain B AMY2 for example in the strict target isozyme specificity of BASI for AMY2 connected with Arg128.[18]

This relationship between the length of the AMY1 parts and the yields of recombinant protein recently inspired the development of an AMY2 variant through a combinatorial approach in which 10 AMY1 positions were allowed to replace the corresponding positions in the N-terminal part of AMY2 (Fig. 2).[19] The goal was to obtain a variant of AMY2 which behaved essentially as the wild-type isozyme with respect to stability and enzyme activity, but in which the sequence patch postulated to suppress its heterologous production in yeast was modified. For this purpose a degenerate oligonucleotide gene shuffling (DOGS) method was used, which is characterised by resulting in a low frequency of parent genes and a high recombination. Six AMY2 variants were obtained which represented 6 of the 10 positions targeted for substitution by the corresponding AMY1 residue. These variants were then produced and characterised and only one, A42P AMY2, fulfilled the criteria of maintained AMY2 enzymatic properties. The corresponding mutant gene was transferred to and expressed in *P. pastoris*[14] to increase the amounts of recombinant protein and indeed rather than wild-type AMY2 being produced at about 1 mg/L induction culture, 60 mg/L A42P AMY2 was obtained.[19] It has been the experience that certain AMY1 mutants and isozyme chimeras were produced in significantly lower amounts than the wild-type, but this newly achieved AMY2 A42P expression level avoids such reduction in yields caused by mutations and this variant is therefore suitable for protein engineering experiments addressing AMY2 properties.[18] The expression system will shortly be employed for mutational analysis in A42P AMY2 of the putative "sugar tongs," as well as in various regions assumed to play a role in substrate specificity and thought to be implicated in calcium ion effects.

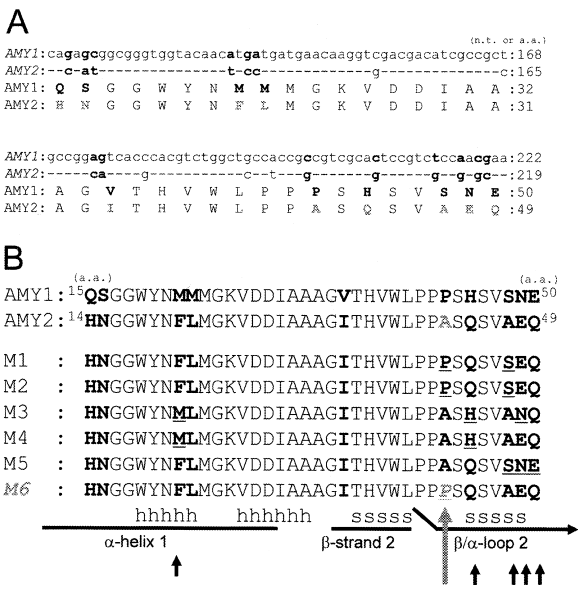

Fig. 2. Engineering of AMY2 by DOGS.

Partial cDNA and amino acid alignments of AMY1 and AMY2 (A) and selected mutants (B) (Ref. 19). The variable amino acid residues between AMY1 and AMY2 are shown in bold style and arrows point to the sequence diversity achieved by DOGS. Residues of AMY1 origin are underlined. "h" and "s" indicate residues part of α-helix and β-strand secondary structure. AMY2 enzymatic properties and stability characteristics were achieved only for the single mutant M6 (A42P) shown in grey italics, which in shake flask cultivations resulted in 15 fold increase in production levels.

Role of calcium ions in AMY1 and AMY2 stability and activity.

Crystal structures of barley α-amylases contain three Ca^{2+} (Ca500, Ca501, Ca502) bound in domain B with all

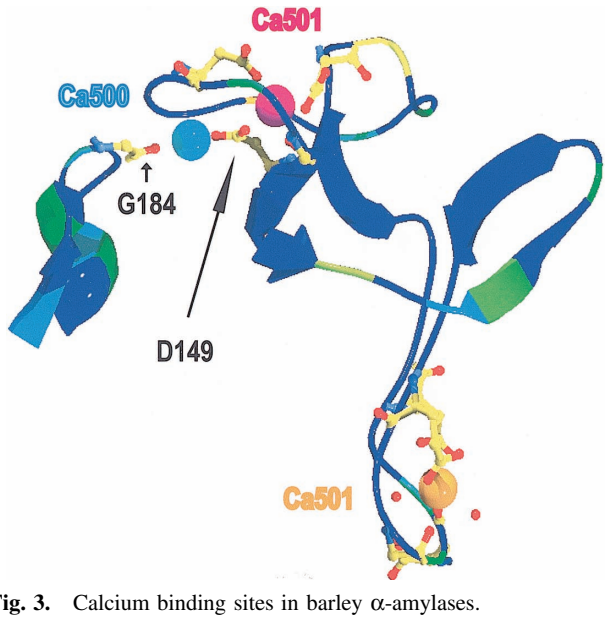

Fig. 3. Calcium binding sites in barley α-amylases.

The figure features a ribbon representation of domain B in AMY1 and AMY2 coloured according to the primary structure alignment diversity with the consensus in blue, through higher temperature colours with increased side chain diversity. As evident from the figure the vast majority of residues are conserved and there are only minor differences shown in green and turquoise between the two isozymes. The red small spheres are solvent ligands of Ca501, and some protein ligands of the three Ca^{2+} are shown including the common ligand Asp149 and the only domain A ligand Gly184 using AMY1 numbering for both ligands.

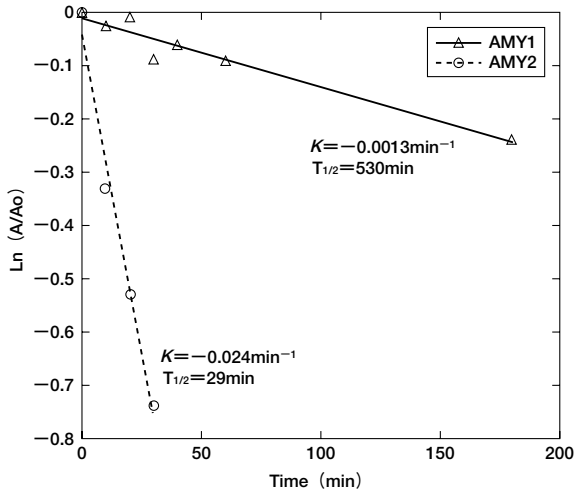

Fig. 4. Kinetic stabilities of AMY1 and AMY2.

Thermal inactivation of AMY1 and AMY2 at 37°C in 10 mM Hepes buffer pH 7.5 without any added Ca^{2+} and without treatment with chelating agents. The data fitting conforms to a first order kinetics (straight solid and dashed lines) for determination of the inactivation rate constants and half times.

of their 22 ligands except one, which is from domain A. It is the conserved Ca500 site in α-amylases that has the single ligand contributed by domain A (the carbonyl oxygen of $Gly184_{AMY1}$ or $Gly183_{AMY2}$) (Fig. 3).[20,21] Ca500 is at a distance of 7 Å from another Ca^{2+} (Ca502) with ligands only in domain B, including the common ligand with Ca500 ($Asp149_{AMY1}$, $Asp148_{AMY2}$). Ca501 has all ligands coming from a short loop in domain B ($Phe108_{AMY1}$-$Asp118_{AMY1}$) and has also two water molecules as ligands. Activity and conformational stability of AMY1 and AMY2 depend differently on Ca^{2+} even though all Ca^{2+}-ligands are shared. Previously, the effect of Ca^{2+} on the stability was analysed by denaturation in urea in the presence of $CaCl_2$ or EDTA and AMY2 was found to have the higher stability of the two isozymes.[32] To further analyse the role played by calcium the thermal stability at a [Ca^{2+}] range was monitored using differential scanning calorimetry, indicating that AMY1 has higher stability than AMY2 in the presence of stoichiometric Ca^{2+}, where only high affinity sites with K_D values $<10^{-8}$ M are essentially saturated. The difference in kinetic stability between AMY1 and AMY2 was far more pronounced, and the rate of inactivation of AMY2 was much faster than that of AMY1 in agreement with higher affinity of AMY1 for Ca^{2+} (Fig. 4). The activity towards insoluble Blue Starch in the presence of Ca^{2+}, Mg^{2+} and Na^{+} showed that AMY1 and AMY2 react differently in the presence of these ions. It became clear from parallel experiments that rather than the putative high affinity calcium binding sites seen in the crystal structures, nonspecific effects of ionic strength and pH may alter the charge distribution on the enzyme surface and elicit activity differences on insoluble Blue Starch.

Reactions involving α-amylases, α-amylase inhibitors and thioredoxin h.

Binding of calcium ions is also of special importance for the interaction between barley α-amylase/subtilisin inhibitor (BASI) and the target isozyme AMY2, since a fully hydrated Ca^{2+} (Ca503) is located at the AMY2/BASI interface and mediates the contact between inhibitor side chains and catalytic groups of the enzyme.[23–25] Remarkably, a Ca^{2+} is bound at the same position at the catalytic site in AMY1/methyl 4′, 4″, 4‴-thiomaltotetraoside complex at the "sugar tongs",[21] albeit with a slightly different coordination geometry. The complex formation of AMY2 and BASI was described using surface plasmon resonance analysis which also facilitated studies of the effect of calcium on the stability of the AMY2/BASI complex.[24,25] While increasing [Ca^{2+}] from the low μM range to 20 mM evidently stabilised the complex by an order of magnitude, the dependence of the stability on pH suggested that a group in BASI of pK_a around 6 participates in the binding to AMY2. The three-dimensional structure of the AMY2/BASI pointed to Glu168 as an obvious candidate, since this residue through hydrogen bonds with the molecules in the water shell around Ca503 was indirectly in contact with the enzyme. Indeed mutation of this residue to glutamine showed both substantially reduced effect of increasing [Ca^{2+}] on the stability of the complex and secondly the E168Q BASI mutant had superior affinity for AMY2 at <5 mM Ca^{2+} compared to BASI wild-type.[25] This latter property suggests that Glu168 weakens the binding to AMY2 due to the weakened network of direct and solvent-mediated hydrogen bonds centred around the fourth calcium ion bound at the AMY2/BASI interface. Further explanation of this weakening seems also to be the electrostatic repulsion from the catalytic carboxyl groups, which Ca503 on the protein interface screens and hence this calcium contributes to stabilisation of the complex.

In the germinating seeds the disulphide reductase thioredoxin h[33] is capable of reducing disulphide bonds in a series of target proteins, including BASI as well as different α-amylase inhibitors of the CM-protein family shown to act on mammalian and insect α-amylases.[27] In the case of BASI, it has been demonstrated that one of its two disulphide bonds, actually situated in the three-dimensional structure in the vicinity of residues interacting with AMY2 in the complex is particularly prone to reduction by thioredoxin h. The second disulphide bond in BASI is near the protease-binding site and seems to be only slightly affected. It is not yet known if this reduction of the disulphide bond actually is accompanied by loss of the inhibitory activity of BASI, although it has been proposed that these reactions contribute to the system of reactions that controls and regulates the action of α-amylases and other amylolytic enzymes in mobilisation of seed storage starch during germination.

Conclusion and prospects.

The insight into the action of α-amylases at the molecular level and the understanding of how these enzymes tackle the degradation of the very different substrates they act on in nature continues to progress rapidly. A highly challenging area is that of the degradation of polysaccharides which has fundamental importance in the natural mobilisation in the seeds or leaves of starch for plantlet growth and energy. This opens up a whole system of enzymes interacting with different specificity on α-1,4 and

α-1,6 linkages in amylose and amylopectin as well as on degradation products there of and which are also under the influence of inhibitors and regulatory proteins. Industrial processes and other commercial applications as well may benefit from the improved insight into these conversions of starch and related sugars. Ultimately the specificity and multivalent substrate binding of the natural enzymes acting on natural substrates can guide rational design of engineered enzymes to achieve novel substrate specificities, stability, sensitivity to regulation and recognition of inhibitors as well as modulation by calcium ions of these various properties.

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〔質　問〕　鹿児島大　安部

1) Can Ca^{2+} be replaced with Mg^{2+} ion? (in the fluorescence titration study)
2) Have you replaced two glycines near Y380?

〔答〕

1) No we don't think so, since the effect is not the same for these two ions.
2) No we haven't and this is a very good question. We are also making other mutants in the sugar tongs binding site, these mutant proteins however are still under characterisation and they do not include the glycines.

〔質　問〕　阪大　松浦

Your enzyme has two surface carbohydrate binding sites. What happens when either one of them is deactivated?

〔答〕

In barley α-amylase 1 we are currently characterising site-directed mutants at either the sugar tongs binding site or of the two adjacent tryptophanyl residues in the surface binding site on the catalytic domain, as well as double mutants addressing together these two surface sites. It is certain that the aromatic groups mentioned in both of the surface binding sites contribute to binding onto starch granules. We are not sure if also the tryptophans at the surface site on the catalytic domain, however, are critical for binding β-cyclodextrin as measured by surface plasmon resonance analysis of the binding using the enzyme being biotinylated and bound to the streptavidin-chip and the analyte, β-cyclodextrin, being used in a wide concentration range.

〔質　問〕　江崎グリコ・生化研　栗木

Your idea pointing out the importance of the enzyme surface is very interesting. Have you ever had any observations that different substrate recognition under the different ionic strength of the reaction mixture which may change the substrate-binding of the enzyme at the surface?

〔答〕

No we haven't done any systematic experiments on the effect of the ionic strength, but we are confident that there is an effect on activity at *e.g.* different concentrations of calcium ions, and that these effects presumably stem from electrostatics properties of the protein surface rather than the three structural calcium ions as identified by crystallography.