Insights into the "pair of sugar tongs" surface binding site in barley α-amylase isozymes and crystallization of appropriate sugar tongs mutants

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Abstract: Recently, the three-dimensional structure of AMY1 in complex with a thio-maltotetraose (thio-DP4) has contributed to the understanding of the isozyme differences between AMY1 and AMY2, particularly the higher activity of AMY1 on starch granules. Indeed, this structure reveals the presence of an additional surface binding site called a "pair of sugar tongs" due to the sugar capturing by Tyr380 which is situated in domain C of AMY1. For the first time, a biological role for the domain C was suggested as well as a hypothetical explanation of enzymatic differences between the two barley α-amylase isozymes. However, no sugar was bound at the "sugar tongs" site in the AMY2/acarbose complex. Comparative studies of this domain on the basis of sequence, secondary structure and spatial organization allow to propose factors needed for such a site. One of the most obvious is the replacement of $Ser378_{AMY1}$ by $Pro376_{AMY2}$, which could be responsible for the lack of the surface binding site by inducing a more rigid structure of the loop preceding Tyr380. In order to test this hypothesis, two mutants of this particular residue have been engineered: $AMY1_{S378P}$ mimicking the AMY2 and $AMY1_{S378T}$. However, surface plasmon resonance sugar binding experiments have proven unambiguously that this residue cannot totally explain the lack of the "pair of sugar tongs" and other tracks must be studied as, for example, the differences in orientation of Asp381 and the critical role of His395, both good candidates for mutational engineering. Also, the contribution of domain A could be suggested in addition to minor differences implying interacting residues at the "sugar tongs" binding site. Finally, the successful crystallization of five mutants in the "sugar tongs" binding site is reported.

Key words: α-amylase mutants, barley, isozymes, crystallization, surface binding sites, "a pair of sugar tongs".

Abbreviations: AMY1, barley α-amylase 1 - the low pI isozyme; AMY2, barley α-amylase 2 - the high pI isozyme; BASI, barley α-amylase/subtilisin inhibitor; Cl-pNPG_7, 2-chloro-4-nitrophenyl β-D-maltoheptaoside; PDB, Protein Data Bank; rmsd, root mean square deviation; thio-DP4, methyl-4',4",4"'-trithiomaltotetraoside.

Introduction

α-Amylases are present in barley seeds, where their physiological role is to provide energy to the plantlet for growth. Two different α-amylase isozymes, being encoded by two distinct multigene families, are distinguished in the germinating seeds. These isozymes, which are known as α-amylase 1 (AMY1; the low pI isozyme) and α-amylase 2 (AMY2; the high pI isozyme) (Jacobsen & Higgins, 1982), are made up of 414 and 403 amino-acids, respectively (Rogers & Milliman, 1983; Rogers, 1985), and their sequence similarity with α-amylases from micro-organisms and animals is low (Rogers, 1985).

Despite a high sequence identity of 80% between AMY1 and AMY2, a number of significant differences between these isozymes have been observed as exemplified in their affinity for calcium ions (Bertoft et al., 1984; Bush et al., 1989; Rodenburg et al., 1994), stability at acidic pH (Rodenburg et al., 1994), and stability at elevated temperature. Particularly, only AMY2 binds the endogenous bifunctional inhibitor BASI (barley α-amylase/subtilisin inhibitor) (Mundy et al., 1983; Svendsen et al., 1986; Leah & Mundy, 1989) with a

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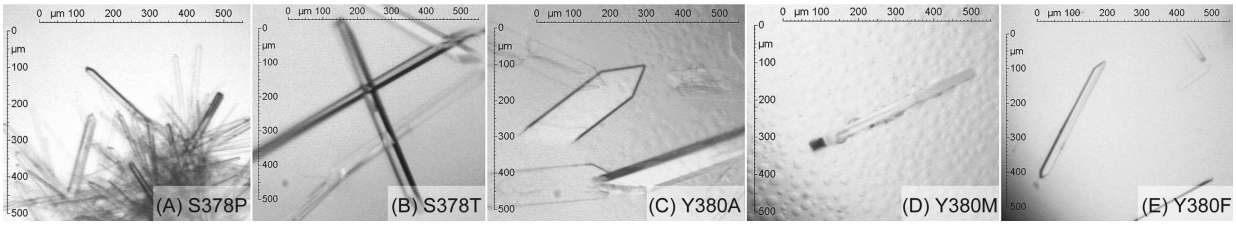

Fig. 1. Crystals of the sugar tongs mutants (A) S378P, (B) S378T, (C) Y380A, (D) Y380M and (E) Y380F, all being rod shaped except mutant $AMY1_{Y380A}$.

K_i of 2.2×10^{-10} M as determined at pH 8 and 37°C (Abe et al., 1993; Sidenius et al., 1995). Structural as well as biochemical data can explain these differences (Vallée et al., 1998; Rodenburg et al., 2000).

The focus of this report is on the activity and affinity differences of these isozymes towards starch granules (MacGregor & Balance, 1980; MacGregor & Morgan, 1986) and soluble substrates (Søgaard & Svensson, 1990; Ajandouz et al., 1992) on the basis of crystallographic studies.

Crystal structures of native AMY1 and AMY2 exist to a resolution of 1.5 Å (Robert et al., 2002b; 2003) and 2.8 Å (Kadziola et al., 1994), respectively, and a vast number of structures of complexes with substrates, substrate analogues and/or inhibitors exist for AMY1 (Kadziola et al., 1998; Robert et al., 2003). The structure of AMY1 in complex with the substrate analogue methyl 4',4",4'''-trithiomaltotetraoside, henceforth referred to as thio-DP4, revealed a new surface sugar binding site called "a pair of sugar tongs" due to its architecture (Robert et al., 2003). Here the "pair of sugar tongs" binding site in the AMY1 structure is described and compared to the corresponding site in AMY2, which despite the presence of an essential tyrosine has not shown the capacity of binding sugar molecules at this surface site. A series of mutants in the "pair of sugar tongs" region have been engineered (Bozonnet et al., 2005; S. Bozonnet et al., unpublished results), and the crystallization of some of these is reported here.

Material and methods

Crystallization of AMY1 sugar tongs mutants

A C-terminal truncated form of AMY1 in which the nine C-terminal residues were lacking, and which resulted in crystals having a morphology and size suitable for X-ray crystallography studies (Robert et al., 2002a) was used. All AMY1 mutants were cloned, expressed and purified (S. Bozonnet et al., unpublished results). Crystals of the mutants studied herein were obtained using the hanging drop vapour diffusion method. The enzyme stock solutions were mixed in a 1:1 ratio with well solutions. Crystals of $AMY1_{S378P}$ were grown in 20% (*w/v*) PEG 8000, 3% MPD, 0.1 M Tris pH 8.0 from a stock solution of 5.1 mg mL^{-1} until they reached the dimensions of $0.3 \times 0.02 \times 0.01$ mm^3,whereas crystals of $AMY1_{S378T}$ were grown to dimensions of $0.7 \times 0.08 \times 0.08$ mm^3 from a solution containing the protein at a concentration of 4.2 mg mL^{-1} and 20% (*w/v*) PEG 8000, 0.1 M Tris pH 8.0. As far as the mutant $AMY1_{Y380A}$ is concerned the crystals were obtained by mixing a solution containing 4.6 mg mL^{-1} protein with an equal volume of reservoir solution consisting of 17% (*w/v*) PEG 8000, 3% isopropanol, 0.1 M MES pH 6.0 and suspended over the reservoir to dimensions of $0.5 \times 0.02 \times 0.01$ mm^3. Finally, crystals of $AMY1_{Y380M}$ and $AMY1_{Y380F}$ were both grown in 20% (*w/v*) PEG 8000, 0.1 M MES pH 6.0 from stock solutions of 4.0 mg mL^{-1} and 3.5 mg mL^{-1} mutant enzyme, respectively. These crystals grew to a dimension of $0.7 \times 0.1 \times 0.01$ mm^3. Crystals suitable for diffraction experiments (Fig. 1) were obtained after approximately one month under all the conditions defined above.

Data collection

X-ray diffraction data of all AMY1 sugar tongs variants were collected using a MAR Research CCD detector on the European Synchrotron Radiation Facility (ESRF) BEAMLINE BM30A (Grenoble, France). The crystals were maintained at 100K during the collections.

Data reduction

For all the mutants, diffraction data were integrated and scaled using programs from the XDS package (Kabsch, 1993).

Sequence and structure alignment

The sequence alignment showing the superimposition of secondary structures between domains C of AMY1 and AMY2 was calculated with the program CLUSTALW (Thompson et al., 1994) and presented using the program ESPript (Gouet et al., 1999; 2003). Figures showing overall and detailed structures were generated with the programs TURBO-FRODO (Roussel & Cambillau, 1992), and VIEWERLITE^{TM} (Accelrys, Inc., San Diego, USA).

Accession numbers

The coordinates of the crystal structure of the AMY1/thio-DP4 complex employed within this study corresponds to the entry code 1P6W deposited in the Protein Data Bank at Rutgers (PDB; http://www.rcsb.org). The coordinates of the complex AMY2/acarbose used in this comparative study are present in the PDB under the accession number 1BG9.

Results

Overall structure, calcium ions and active site

The three-dimensional structure of AMY1 (Robert et al., 2002b, 2003) and AMY2 (Kadziola et al., 1994) is made up of domains A, B and C as found in other α-amylases.

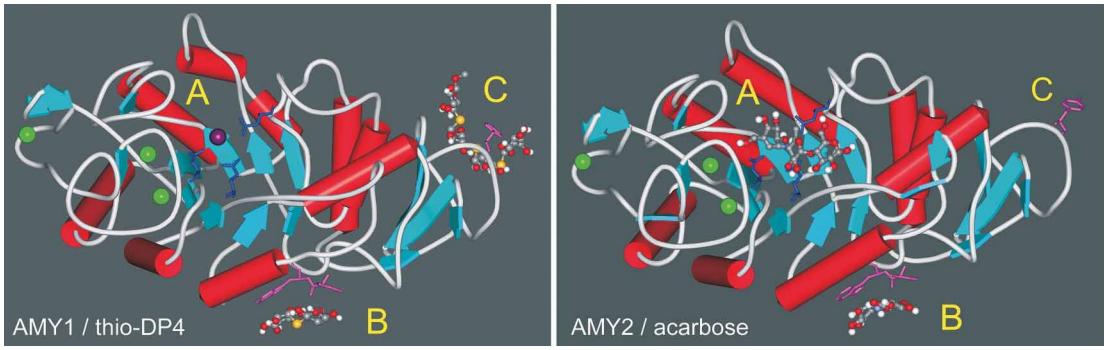

Fig. 2. Representations of the crystal structures of AMY1/thio-DP4 and AMY2/acarbose highlighting the active site (A), the starch granule surface binding site (B) and the domain C "sugar tongs" binding site (C). The active site of AMY1 in complex with the inhibitor thio-DP4 highlights the presence of a fully hydrated calcium ion (Ca503), whereas a sugar molecule is lacking. This fourth calcium ion is coloured in purple to be distinguished from the three others and is conserved in the structure of native AMY1. The three other calcium ions (presented by green spheres) are located at identical places in the two structures. The catalytic residues situated in the active site are coloured in blue while residues defining the surface binding sites are coloured in pink.

When superimposing the native structures of AMY1 on AMY2 a very high similarity is found with a root mean square deviation (rmsd) of 0.53 Å based on main chain atoms (Robert et al., 2002b), the essential differences between AMY1 and AMY2 being within some of the barrel loops. Furthermore, both AMY1 and AMY2 (Kadziola et al., 1994) bind three calcium ions, two of which have ligands only from domain B (Ca501 and Ca502). The third and so-called conserved calcium ion, which is present in all other α-amylase three-dimensional structures (Ca500), has one ligand from domain A and the remaining ones from domain B (Matsuura et al., 1984; Boel et al., 1990; Qian et al., 1993; Brayer et al., 1995; Ramasubbu et al., 1996; Strobl et al., 1997; Aghajari et al., 1998b; Machius et al., 1998; Dauter et al., 1999; Linden et al., 2003). These calcium ions appear to be essential for proper folding, conformational stability and therefore for activity (Bertoft et al., 1984; Jones & Jacobsen 1991).

In the complex AMY1/thio-DP4, a fourth calcium ion was detected in the active site of the enzyme instead of as expected a sugar molecule (Figs 2 and 3) (Robert et al., 2003). This calcium ion is maintained in the active site by interactions with the three catalytic residues *via* water molecules and involving no direct contacts with protein residues (Fig. 3). Surprisingly, this fourth calcium ion is exactly at the same position as the fully hydrated Ca^{2+} ion present at the protein interface in the AMY2/BASI complex (Vallée et al., 1998) but with a different spatial organization of the surrounding water molecules. The fact that this calcium ion was not found in the native structure of AMY1 despite a yet higher calcium concentration in the crystallization media as compared to crystals containing thio-DP4, is far from being understood since the catalytic residues as well as the immediate environments in both isozymes superimpose perfectly. The structure of AMY1/thio-DP4 suggests that the presence of the calcium ion is not induced by the inhibitor

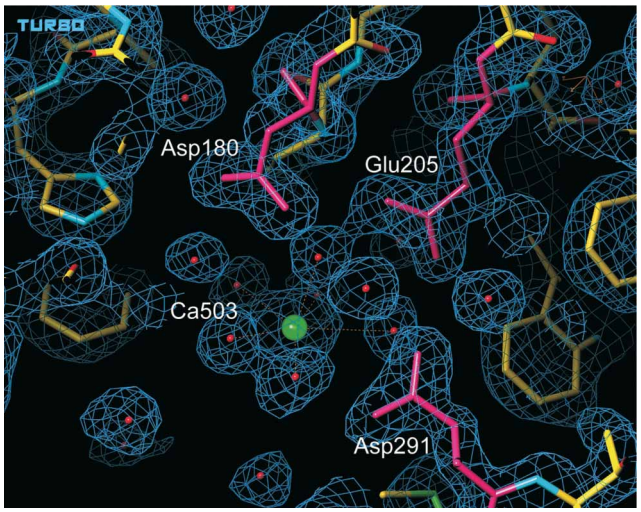

Fig. 3. Close-up on the active site in AMY1/thio-DP4 revealing the water mediated interactions between Ca503 (green sphere) and the catalytic residues coloured in pink. The electron density $2F_o$-F_c map shows the 6 water molecules (red spheres) which define a pyramid with a pentagonal base. Three of these are hydrogen bonded to the catalytic residues. The coordination bonds are represented by dashed red lines.

BASI (Robert et al., 2003). Nevertheless some questions deserve to be raised. For example, does this ion play the role of an inhibitor of AMY1 and if so can this explain the lack of thio-DP4 binding to the active site by a competitive inhibition mechanism? This ion also could be an activity modulator for the protein opposed to thio-DP4, conclusions which are supported by the deficient inhibition of the AMY1-catalyzed hydrolytic reaction (M.T. Jensen & B. Svensson, unpublished results). Calcium binding could be pH dependent and calcium ions may modulate AMY1 activity, explaining the higher affinity of AMY1 for these ions compared to AMY2 (Bertoft et al., 1984; Bush et al., 1989; Rodenburg et al., 1994).

The active site of AMY2, identified in the crystal structures of the AMY2/acarbose complex (the lat-

ter being a pseudotetrasaccharide transition-state analogue) (Kadziola et al., 1998), appears to be a large V-shaped depression on one side of the enzyme, formed by parts of domain A and domain B and relatively well conserved between the two isozymes. This topology of the active site is also highlighted in complexes between AMY1/acarbose and an inactive mutant, $AMY1_{D180A}$, complexed with maltoheptaose which is a real substrate (Robert et al., 2005).

The starch granule-binding surface site

In the two isozymes a pair of tryptophans, Trp276 and 277 (Trp278 and 279 in AMY1), define the so-called starch granule binding site (Gibson & Svensson, 1987) on the surface of the enzyme (Kadziola et al., 1994). This site, which is independent of the active site, has been confirmed by UV difference spectroscopy (Gibson & Svensson, 1987; Søgaard et al., 1993) and constitutes the first surface binding site discovered. Enzymatically, its low binding affinity for acarbose and β-cyclodextrin, which compete with starch granules (Weselake & Hill, 1983; Søgaard et al., 1993), suggests a role in binding of the enzyme to starch *in vivo*. This site has been proven also by X-ray crystallography with the AMY2/acarbose complex, solved to 2.8 Å resolution (Kadziola et al., 1998) in which a disaccharide unit was stacking onto the two tryptophan side chains.

Trp278 and Trp279 in AMY1 superimpose remarkably well with the AMY2 counterparts as defined in the electron density. This secondary binding site is also found in the various AMY1 complexes (Robert et al., 2005). In fact, each aromatic side-chain defines a plane and the angle between the two planes was determined to be about 135°. This angle is conserved in all three-dimensional structures of the two isozymes solved to date and was proposed to be responsible for the substrate selection by acting like a geometric filter (Robert et al., 2003). In other words, substrates are selected if geometrically compatible with this surface site.

This surface site situated in domain A was the first to be described and studied, whereas the second surface binding site, the so-called "pair of sugar tongs" was discovered more recently (see the next section). Thus, all properties revealed by mutational engineering, when knowledge on sugar tongs was lacking, were assigned to the consecutive Trp278 and Trp279 (Søgaard et al., 1993), ignoring the impact of the "sugar tongs" binding site.

The "pair of sugar tongs"

The crystal structure of AMY1 at high resolution in complex with the substrate analogue, thio-DP4, resulted in the discovery of a new sugar binding site at the surface of AMY1: "a pair of sugar tongs" (Robert et al., 2003). The electron density showed unambiguously an entire molecule of thio-DP4 in the vicinity of Tyr380 (AMY1 numbering) in domain C (Fig. 2). It is worth mentioning that a raw starch binding domain localised at the C-terminal region has been identified previously (Tibbot et al., 2000; Wong et al., 2000). This site seemed to retain sufficient function in the absence of the Trp278/Trp279 region. However, sequencing of this fragment revealed that it extends from Ala283 to Lys354 and, consequently, the binding properties studied cannot stem from "the sugar tongs" site.

In the AMY1/thio-DP4 complex, the interaction of the sugar molecule with the enzyme is ensured by 9 direct hydrogen bonds to residues Lys375, Tyr380, Asp381, Val382, His395, and Asp398 in addition to 8 hydrophobic contacts with Tyr380, Thr392, Tyr399, and Trp402 (Fig. 4). Particularly, Tyr380 plays a key role in the binding of thio-DP4 to domain C due to its participation in 8 contacts out of a total of 17 formed by the phenol ring and the carbonyl oxygen. This residue makes hydrophobic contacts to the two sulphur atoms of the trisaccharide unit at the reducing end reinforced by numerous hydrogen bonds to Lys375, Asp381, Val382, and His395. All these interactions contribute to the stabilization of the trisaccharide as opposed to the glucose unit at the nonreducing end for which the ring is only making one hydrogen bond to Asp398. This might explain the high average B factor (57.9 $Å^2$) of the glucose unit at the nonreducing end as compared to those of the three others (32.0 $Å^2$, 14.4 $Å^2$ and 24.6 $Å^2$). The importance of the above-mentioned tyrosine in the binding of the substrate analogue is underlined when superimposing the structure of the complex AMY1/thio-DP4 on native AMY1. The Tyr380 side chain actually undergoes a shift of 1.2 Å and 3.1 Å from the C_α and the phenol oxygen, respectively, to entrap the substrate, giving rise to the name "a pair of sugar tongs" (Fig. 5 in pink). It also should be emphasized that Tyr380 is conserved only in plant α-amylases (Robert et al., 2003).

Electron density in the corresponding "sugar tongs" binding site area in the complex AMY2/acarbose (Kadziola et al., 1998) did not show any binding of the oligosaccharide, despite a high degree of similarity of these sites (see the next section). It is therefore tempting to imagine that this site in AMY1 could be a crystallization artefact. Nevertheless, crystal structures of various AMY1 complexes as well as an inactive mutant of the catalytic nucleophile $AMY1_{D180A}$ with acarbose highlight the presence of this site. These structures have confirmed the ability of the "sugar tongs" surface site to recognize and bind natural substrates, and show similar values for the tyrosine shift and a high conservation of sugar protein interactions (Robert et al., 2005).

Why is the pair of sugar tongs not found in AMY2?

All the above-mentioned interacting amino acid residues have their counterparts in AMY2, with the exception of $Thr392_{AMY1}$ being replaced by a valine (Fig. 5 in

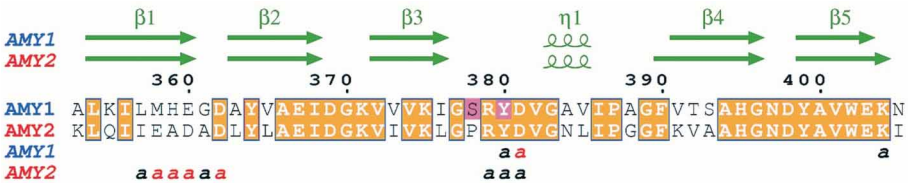

Fig. 4. Sequence alignment of barley AMY1 and AMY2 extending from residues Ala353$_{\mathrm{AMY1}}$ to Asn405$_{\mathrm{AMY1}}$ (domain C). Identical residues are highlighted in yellow. The secondary structure annotation is indicated over the alignment coloured in green. Mutated residues of the sugar tongs described herein are coloured by pink scares. Finally, for both enzymes, residues involved in crystallographic contacts are indicated with an "***a***" and are coloured as follows: red (distances less than 3.2 Å) and black (distances between 3.2 and 4.0 Å).

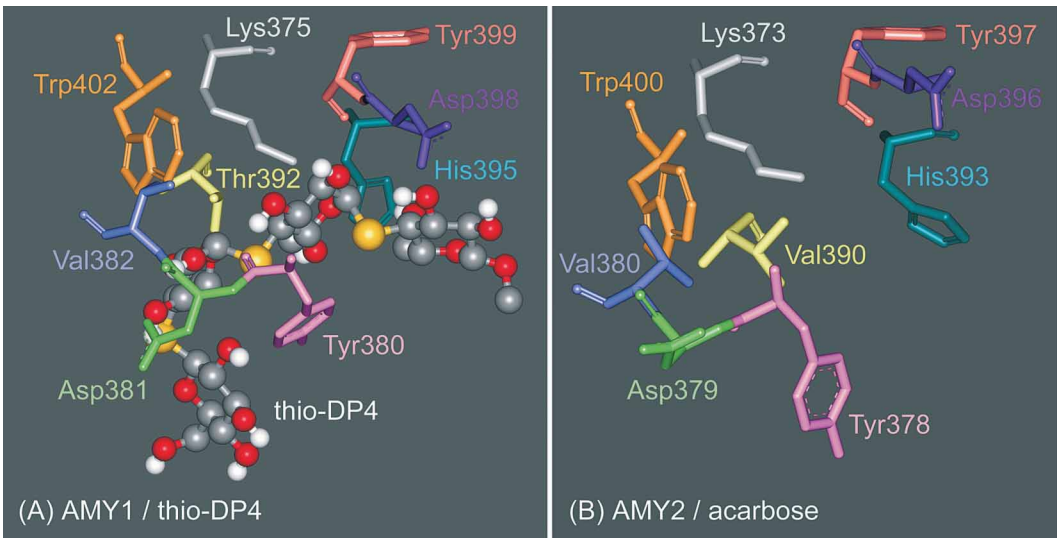

Fig. 5. Close up on the "sugar tongs" surface binding site in the AMY1/thio-DP4 complex (A) and the corresponding site in AMY2/acarbose (B). The picture shows clearly the shift of the key residue Tyr380$_{\mathrm{AMY1}}$ coloured in pink to entrap the sugar molecule in AMY1/thio-DP4, while it remains in its native position in AMY2/acarbose. The distinct orientations of Asp381$_{\mathrm{AMY1}}$/Asp379$_{\mathrm{AMY2}}$ as well as those of His395$_{\mathrm{AMY1}}$/His393$_{\mathrm{AMY2}}$ are also emphasized. Finally, it should be noted that Thr392$_{\mathrm{AMY1}}$ is replaced by a valine, Val$_{390}$ in AMY2.

yellow), but surprisingly as mentioned earlier no maltooligosaccharide binding to domain C seemed to take place in the three-dimensional structure of the complex between AMY2 and the pseudo-tetrasaccharide acarbose (Kadziola et al., 1998).

Moreover, in the region around Tyr380, both the secondary structure elements and the sequences are well superimposable with a few exceptions of amino acids being replaced by physicochemical equivalents (Fig. 4):

Gly361$_{\mathrm{AMY1}}$→Ala359$_{\mathrm{AMY2}}$
Ala363$_{\mathrm{AMY1}}$→Leu361$_{\mathrm{AMY2}}$
Val365$_{\mathrm{AMY1}}$→Leu363$_{\mathrm{AMY2}}$
Val373$_{\mathrm{AMY1}}$→Ile371$_{\mathrm{AMY2}}$
Ile376$_{\mathrm{AMY1}}$→Leu374$_{\mathrm{AMY2}}$
Val385$_{\mathrm{AMY1}}$→Leu383$_{\mathrm{AMY2}}$.

Nevertheless, these differences do not seem to be determinant for why only AMY1 binds sugar substrate to the "pair of sugar tongs". Solely, one residue from this region, Ser378 which belongs to the short loop preceding Tyr380 is replaced by an imino acid, Pro376$_{\mathrm{AMY2}}$, and this difference was thought to be a possible explanation for the lack of such a binding site in AMY2 (Robert et al., 2003). This loop is flexible in AMY1, whereas it seems to be more restrained in AMY2 possibly due to the rigid structure of the proline. The second residue upstream of Tyr380 (Ser378$_{\mathrm{AMY1}}$/Pro376$_{\mathrm{AMY2}}$) was thus proposed to be a major determinant of an isozyme-specific difference in the flexibility of this loop, which in turn may control maltooligosaccharide binding to domain C (see the next section).

Comparative studies between AMY1 and AMY2 of residues involved in the interaction network with the substrate analogue thio-DP4 highlight new possible explanations for the formation of the "sugar tongs" binding site. Primarily, at first sight, it appears relatively clear that Lys375, Tyr399, Asp398 and Trp402 (AMY1 numbering) are spatially conserved in the two structures. They define a cavity where the substrate is lying being entrapped by Tyr380 (Fig. 5). Secondly, Tyr399 seems to be closer to the substrate chain in AMY1/thio-DP4 with its aromatic ring oriented in a parallel way with respect to the sugar rings. Distances are nevertheless too important to be referred to as

stacking interactions but the hydrophobic contact provided by this residue may not exist in AMY2/acarbose, thus destabilizing the sugar molecule. Thirdly, Thr392 is making a hydrophobic contact to a carbon atom in thio-DP4. In AMY2, this residue is replaced by a valine, thus a residue with altered hydrophobicity and moreover the functional group is turning away from the sugar molecule. This residue belongs to a tripeptide being completely different in AMY2 (Fig. 4). Finally, when comparing domain C surfaces of AMY1 and AMY2 coloured as a function of charges, it appears distinctly that a large negatively charged area is present in AMY1, close to the key residue Tyr380 and lacking in AMY2. This interesting difference seems to stem from the different orientations of $Asp381_{AMY1}$/$Asp379_{AMY2}$ being oriented in the same direction or away from $Tyr380_{AMY1}$/$Tyr378_{AMY2}$, respectively (Fig. 5 in light green). Moreover, when comparing native AMY1 and AMY1/thio-DP4, it appears that the carboxyl group of the side chain undergoes a slight shift in order to form a hydrogen bond to the sugar molecule in AMY1/thio-DP4. Indeed, the backbone of $Asp379_{AMY2}$ is more than 1 Å closer to the β-sheet formed by the five β-strands than the backbone of $Asp381_{AMY1}$ in the structures of native AMY1 and AMY1/thio-DP4.

Starch binding domains in α-amylases from other species

In the α-amylase family, besides a common structure composed of the central $(\beta/\alpha)_8$ barrel (domain A), domains B and C, some enzymes contain one or two additional domains, D and E. Particularly, domain E has a raw starch binding function which facilitates degradation of starch granules, which is often referred to as the SBD, the starch-binding domain (Svensson et al., 1989; Janecek & Sevcik, 1999; Sauer et al., 2000). Such a structural module is found in a variety of starch-degrading enzymes which belong to family 13, including some amylopullulanases (EC 3.2.1.41), maltogenic α-amylases (EC 3.2.1.133), maltotetraose-forming exo-amylases (EC 3.2.1.60), maltopentaose-forming amylases (EC 3.2.1.60), almost all CGTases (EC 2.4.1.19) and a few α-amylases (EC 3.2.1.1) (Juge et al., 2002; Janecek et al., 2003). This module is nowadays well characterized, studied and described (Ohdan et al., 2000; Sumitani et al., 2000; Janecek et al., 2003) whereas the finding of the domain C surface binding site is far more recent and therefore appears obscured.

To further analyze the implication of domain C in malto-oligosaccharide binding, several α-amylase three-dimensional structures from different species were compared to that of AMY1 and AMY2 (Robert et al., 2003). At the primary structure level, noticeably, $Tyr380_{AMY1}$ is only present in the two isozymes from barley. Furthermore, among the set of compared structures, AMY1 and AMY2 have the smallest domain C with only five β-strands whereas microorganisms, insects and mammals, for which this domain is comparable, have respectively seven/eight and ten strands. Consequently, even if domains C in α-amylase structures solved to date are mostly composed of antiparallel β-strands, it is logical to think that the smaller structural organization may be one of the major determinants conferring the ability of domain C in AMY1 to bind sugar molecules. The same study has also prominently displayed a different spatial organization, despite an apparent secondary structure conservation of aligned β-strands. This unequivocal difference could be the key to the understanding of why the "sugar tongs" site is not found in other known α-amylase three-dimensional structures. Particularly, the additional β-strands in non-plant α-amylases and their spatial location prevent the presence of a putative domain C "sugar tongs" by sterically hindering the access to this site. One part of the "pair of sugar tongs" is the motif $\beta4$/turn/$\beta5$, where $\beta4$ is specific to AMY1/AMY2 and equivalent to two distinct β-strands in non-plant α-amylases. This motif constitutes an antiparallel β sheet. The other part of the site corresponds to the loop containing Tyr380 between $\beta3$ and $\beta4$ which is perpendicular to the antiparallel β-sheet. Together these features define a small cavity where sugars can bind and wind around Tyr380. Moreover, residues 386-390 connecting $\eta1$ and $\beta4$ form a barrier that forces the malto-oligosaccharide to adopt the curvature seen in Figure 5.

Recently, however, crystal structures of wild-type TVAI in complex with acarbose and an inactive mutant $TVAI_{D356N\text{-}E396Q}$ with malto-tridecaose (G13) have been solved at 2.6 and 1.8 Å, respectively (Abe et al., 2004). Besides the active site, four additional sugar binding sites were observed on the surface of the enzyme: one in the N-terminal region in domain N preceding the catalytic domain, the interface between domains N and A, the centre of domain A and domain C. However, this latter one is not well defined in either of the complexes due to poor electron density. As in AMY1, it seems that an aromatic residue (Trp485) possibly forms the domain C surface site (Abe et al., 2004) but this residue is located at the beginning of the $\alpha7_{bA}$ helix and consequently belongs not to domain C but to domain A. Furthermore, the sugar molecule is not entrapped in the same way as in AMY1/thio-DP4 since the side chain orientation of Trp485 is perpendicular to the sugar moieties in the complex with acarbose, and close to being parallel in the complex with G13, but without stacking interactions. Moreover, the disaccharide from the acarbose complex is perpendicular to the β-strands as opposed to the thio-DP4 sugar in AMY1/thio-DP4.

Enzymatic properties and crystallization of sugar tongs mutants

Based on the above mentioned speculations a series of mutants were constructed to further investigate the structure/function/activity relationships of "the pair of sugar tongs". First, enzymatic studies

Table 1. Crystal data and data collection statistics for the sugar tongs mutants.

	Mutants				
	S378P	Y380A	Y380M	S378T	Y380F
Resolution (Å)	1.9	1.9	1.9	2.0	2.8
Space group	$P2_12_12_1$	$P2_12_12$	$P2_12_12$	$P2_12_12$	$P2_12_12$
Unit-cell parameters					
a (Å)	72.6	91.5	91.4	89.7	89.0
b (Å)	84.7	72.6	72.5	72.5	73.0
c (Å)	127.0	62.1	61.7	61.6	62.3
V (Å^3)	780643.6	412524.1	409129.8	400600.2	404763.1
Solvent content (%)	44	47	47	45	46
Molecules in AU [a]	2	1	1	1	1
No. of reflections	385637	146929	124845	195577	67295
Unique reflections	58762	31965	28264	27280	9620
Completeness (%)	94.2	98.0	85.5	97.6	91.8
$R^{b}_{\mathrm{merge(F)}}$ (outermost shell) (%)	11.6 (27.9)	9.8 (20.3)	7.2 (16.4)	6.5 (27.7)	9.1 (29.3)
$I/\sigma(I)$	12.8	8.4	14.2	20.3	13.9

[a] Number of molecules in the asymmetric unit.

[b] $R_{\mathrm{merge(F)}} = \dfrac{\sum \left| A_{I_{h,P}} - A_{I_{h,Q}} \right|}{0.5 \sum A_{I_{h,P}} + A_{I_{h,Q}}}$ with $I_{h,P} = \dfrac{1}{n_{h,P}} \sum_{i \varepsilon P}^{n_{h,P}} I_{h,i}$, $I_{h,Q} = \dfrac{1}{n_{h,Q}} \sum_{i \varepsilon Q}^{n_{h,Q}} I_{h,i}$ and the pseudo-amplitudes A_I defined as following in order to overcome the problem of negative intensities: $A_I = \begin{cases} \sqrt{I} & \text{if } I \geq 0 \\ -\sqrt{I} & \text{if } I < 0 \end{cases}$ according to Diederichs & Karplus (1997).

of mutants of Tyr380$_{\mathrm{AMY1}}$ (AMY1$_{\mathrm{Y380A}}$, AMY1$_{\mathrm{Y380M}}$ and AMY1$_{\mathrm{Y380F}}$) by surface plasmon resonance analysis confirmed that this residue is critical for β-cyclodextrin binding (Bozonnet et al., 2005; S. Bozonnet et al., unpublished results). Indeed, the Kd of the starch mimic β-cyclodextrin determined by surface plasmon resonance increased from 0.20 mM of AMY1 wild type to 1.4 mM of the mutant Y380A, reflecting a smaller affinity for β-cyclodextrin probably due to the loss or weakening of the "sugar tongs".

Several enzymatic studies in the past showed clearly that AMY1 and AMY2 differ remarkably in terms of affinity and activity towards substrates. Compared to AMY2, AMY1 has higher activity on starch granules and generally higher affinity on substrates. Meanwhile, AMY2 is more active on amylose substrates (MacGregor & Balance, 1980; Bertoft et al., 1984; MacGregor & Morgan, 1986; Søgaard & Svensson, 1990; Ajandouz et al., 1992; MacGregor et al., 1994). The AMY2 mimicking mutant, AMY1$_{\mathrm{S378P}}$ had a Kd for β-cyclodextrin of 0.25 mM being similar to that of wild type AMY1 and a slightly lower affinity towards 2-chloro-4-nitrophenyl β-D-maltoheptaoside (Cl-pNPG$_7$) compared to AMY1 but significantly higher than wild type AMY2 (Bozonnet et al., 2005; S. Bozonnet et al., unpublished results). Furthermore the mutant S378T was constructed with the aim of introducing a semi-conservative mutation at this particular position. Primarily the enzymatic results show that the semi-conservation does not induce significant effects on the kinetic parameters (S. Bozonnet et al., unpublished results). Hence, a particular residue at this position seems not to be mandatory and this result is in agreement with that of the S378P mutation.

Crystallization of all "sugar tongs" mutants was successfully achieved (Fig. 1) and data collection has been performed as shown in Table 1. Interestingly, all mutants display similar crystal parameters with the exception of the S378P mutant. Its space group, unit-cell dimensions and the number of molecules in the asymmetric unit differ from the other mutants and AMY1 (Robert et al., 2002a). The three-dimensional structures which have been solved using the phases from the native AMY1Δ9 (1HT6) and which are currently under refinement will be described elsewhere.

Discussion

As it was already shown in the AMY1/thio-DP4 structure, electron density underlined the presence in domain C of malto-oligosaccharide binding being maintained by the key residue Tyr380 and a high number of non-covalent interactions. This new binding site was yet not seen in the AMY2/acarbose structure and has been definitively confirmed by structural studies of AMY1 complexes with a true substrate and a transition state analogue (Robert et al., 2005). Actually, mutants in the sugar tongs site are under functional and structural studies in order to understand which residues are being specific and what kind of environment are essential for the formation of this surface binding site. These appropriate "sugar tongs" mutants were constructed on the basis of observations from comparative studies of AMY1/thio-DP4 and AMY2/acarbose crystal structures and from the isozyme sequences. The results of these observations can be divided in two categories (ter-

tiary and primary structure) and described as follows.

First, the presence of Tyr380 in a flexible region seems essential, but not sufficient for creating a sugar binding site in domain C in AMY1. Analysis of the structural environment brings up new features, particularly His395 not mentioned until now. For this residue which seems to be a major determinant for the existence of this binding site, its imidazole ring undergoes a nearly 45° shift in order to form a hydrogen bond to thio-DP4. Two significant movements take place upon malto-oligosaccharide binding as seen when superimposing domains C of native and complexed AMY1 structures: the first one within the vicinity of Tyr380 and the second close to His395. It should also be emphasized that His395 is not conserved in α-amylases of known three-dimensional structure from other species. A good candidate for obtaining a mutant lacking the sugar tongs binding site would be this histidine residue. Moreover, the importance of the substitution $Thr392_{AMY1}/Ser390_{AMY2}$ has to be considered since this residue participates in a direct hydrogen bond with acarbose (Robert et al., 2005) in addition to the hydrophobic contact already seen in AMY1/thio-DP4 (Robert et al., 2003). An overall comparison of the site shows subtle differences in terms of tertiary structure already described earlier. Interacting residues are either spatially conserved and belong to strand $\beta 3$ of domain C, or are not conserved and situated in the loop containing Tyr380.

At the sequence level, it should be highlighted that even if most of the residues substituted between the two isozymes are physicochemically equivalent, some are drastically different such as the tripeptide 391-393, which probably deserves to be subjected to more intensive studies and mutational engineering. Moreover, the 9 additional terminal residues present in the natural full-length AMY1 are highly flexible (Robert et al., 2002a) and it would be interesting to know the impact of this property on the organization and interactions network in domain C, a work which in part has been started (Tibbot et al., 2002).

Finally, the actual enzymatic results as concerns S378P do not explain the lack of malto-oligosaccharide binding to the "sugar tongs" binding site in AMY2 (Bozonnet et al., 2005; S. Bozonnet et al., unpublished results). The point variations in primary and tertiary structures described earlier may not be sufficient for abolishing the binding capacity, and most probably combinations of all these divergences rather than point mutations should be sought. To study the incidence of the nine C-terminal residues in AMY1 on the "sugar tongs" binding site formation, progressive deletion mutations from the C-terminal should be made.

Until now, we essentially focused our analysis on required elements relative to domain C, however, the influence of domain A on the site should also be considered. It has been shown previously in a study on the capacity of AMY1 in binding raw starch that the fragment 353-414 was necessary but not sufficient by itself (Tibbot et al., 2002). Consequently, three hypotheses were set-up: (i) the 72 residue-long fragment Ala281-Ser352 could define the starch binding domain; (ii) residues belonging to domain A may participate in binding; or (iii) the portion $\beta_7\alpha_7$-$\beta_8\alpha_8$ (Ala283-Asn346) of domain A may be required for an appropriate folding, and in turn be responsible for the binding activity. Meanwhile, the two first hypotheses are definitely refuted by the crystal structure of AMY1/thio-DP4, whereas the last supposition could be plausible. One of a series of mosaic chimeras produced by restriction enzyme "site-based isozyme segment" recombination in yeast, called AMY1-12221 ($AMY1_{1-91}/AMY2_{91-322}/AMY1_{325-404}$) displays kinetic parameters towards Cl-pNPG_7 and DP440 being nearly identical to those of AMY2 (Bozonnet et al., 2003; T.J. Kim et al., in preparation). These results, being surprising at first sight, may hence be correlated to the hypothesis mentioned above. Indeed, a part of domain A supposed to be necessary for the folding of domain C in order to make appropriate interactions stems from AMY2 in this chimera.

Domain C structure, roles and functions have been largely studied in term of folding integrity and enzymatic process, subjected to multiple hypotheses but still remain nearly totally obscure. This domain is missing in the glycoside hydrolase family GH77 – as shown in the structures of amylomaltase from *Thermus aquaticus* (Przylas et al., 2000) and *Thermus thermophilus* HB8 (PDB codes 1FP8 and 1FP9; J.C.M. Uitdehaag et al., unpublished results) – and lacking in its common form in the family GH70, for which the glycosyltransferase structures have a circularly permuted catalytic $(\beta/\alpha)_8$-barrel (MacGregor et al., 1996; Janeček et al., 2003). This terminal domain is the most variable in term of sequence (Brayer et al., 1995) and secondary structure (5 to 12 β-strands). Multiple roles have been assigned to domain C, like its implication in thermostability of extremophilic α-amylases (Aghajari et al., 1998a; Machius et al., 1998), substrate recruitment and stabilization of the catalytic domain (MacGregor et al., 2001), and recently the crystal structure of AMY1/thio-DP4 highlighted its biological role in barley α-amylase isozyme 1. The discovery of this site has been the first guideline for rational understanding of the higher affinity of AMY1 on starch granules as described approximately two decades ago (MacGregor & Morgan, 1986). Its binding activity has been logically linked to the enzymatic functions. A possible mechanism could be a cooperative system between this site and the domain A (surface site) to select and orient the substrate chain to the active site for the enzymatic process. It might also help to localize the enzyme to the surface of insoluble substrates and thereby enhance processivity as well as disentanglement and exposure of the glucan polymers for hydrolysis (Robert et al., 2003).

Finally, three-dimensional structural data on new plant α-amylases and new complexes with substrate analogues or inhibitors in conjunction with site-directed mutagenesis and biochemical data should help us further to understand the distinct and remarkable differences between the two barley α-amylase isozymes.

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References

Abe, A., Tonozuka, T., Sakano, Y. & Kamitori, S. 2004. Complex structures of *Thermoactinomyces vulgaris* R-47 α-amylase 1 with malto-oligosaccharides demonstrate the role of domain N acting as a starch-binding domain. J. Mol. Biol. **335:** 811–822.

Abe, J., Sidenius, U. & Svensson, B. 1993. Arginine is essential for the α-amylase inhibitory activity of the α-amylase/subtilisin inhibitor (BASI) from barley seeds. Biochem. J. **293:** 151–155.

Aghajari, N., Feller, G., Gerday, C. & Haser, R. 1998a. Crystal structures of the psychrophilic α-amylase from *Alteromonas haloplanctis* in its native form and complexed with an inhibitor [published erratum appears in Protein Sci. 1998, **7:** 1481]. Protein Sci. **7:** 564–572.

Aghajari, N., Feller, G., Gerday, C. & Haser, R. 1998b. Structures of the psychrophilic *Alteromonas haloplanctis* α-amylase give insights into cold adaptation at a molecular level. Structure **6:** 1503–1516.

Ajandouz, E. H., Abe, J., Svensson, B. & Marchis-Mouren, G. 1992. Barley malt-α-amylase. Purification, action pattern, and subsite mapping of isozyme 1 and two members of the isozyme 2 subfamily using *p*-nitrophenylated maltooligosaccharide substrates. Biochim. Biophys. Acta **1159:** 193–202.

Bertoft, E., Andtfolk, C. & Kulp, S.E. 1984. Effect of pH, temperature, and calcium-ions on barley malt α-amylase isoenzymes. J. Inst. Brew. **90:** 298–302.

Boel, E., Brady, L., Brzozowski, A.M., Derewenda, Z., Dodson, G.G., Jensen, V.J., Petersen, S.B., Swift, H., Thim, L. & Woldike, H.F. 1990. Calcium binding in α-amylases: an X-ray diffraction study at 2.1 Å resolution of two enzymes from *Aspergillus*. Biochemistry **29:** 6244–6249.

Bozonnet, S., Bønsager, B.C., Kramhøft, B., Mori, H., Abou Hachem, M., Willemoës, M., Jensen, M.T., Fukuda, K., Nielsen, P.K., Juge, N., Aghajari, N., Tranier, S., Robert, X., Haser, R. & Svensson, B. 2005. Binding of carbohydrates and protein inhibitors to the surface of α-amylases. Biologia, Bratislava **60 (Suppl. 16):** 27–36.

Bozonnet, S., Kim, T.J., BØnsager, B.C., Kramhøft, B., Nielsen, P.K., Bak-Jensen, K.S. & Svensson, B. 2003. Engineering of barley α-amylase. Biocatal. Biotransform. **21:** 209–214.

Brayer, G.D., Luo, Y. & Withers, S.G. 1995. The structure of human pancreatic α-amylase at 1.8 Å resolution and comparisons with related enzymes. Protein Sci. **4:** 1730–1742.

Bush, D.S., Sticher, L., van Huystee, R., Wagner, D. & Jones, R.L. 1989. The calcium requirement for stability and enzymatic activity of two isoforms of barley aleurone α-amylase. J. Biol. Chem. **264:** 19392–19398.

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

Diederichs, K. & Karplus, P.A. 1997. Improved R-factors for diffraction data analysis in macromolecular crystallography. Nat. Struct. Biol. **4:** 269–275.

Gibson, R.M. & Svensson, B. 1987. Identification of tryptophanyl residues involved in binding of carbohydrate ligands to barley α-amylase 2. Carlsberg Res. Commun. **52:** 373–379.

Gouet, P., Courcelle, E., Stuart, D.I. & Metoz, F. 1999. ESPript: analysis of multiple sequence alignments in PostScript. Bioinformatics **15:** 305–308.

Gouet, P., Robert, X. & Courcelle, E. 2003. ESPript/ENDscript: Extracting and rendering sequence and 3D information from atomic structures of proteins. Nucleic Acids Res. **31:** 3320–3323.

Jacobsen, J.V. & Higgins, T.J.V. 1982. Characterization of the α-amylases synthesized by aleurone layers of Himalaya barley in response to gibberellic-acid. Plant Physiol. **70:** 1647–1653.

Janecek, S. & Sevcik, J. 1999. The evolution of starch-binding domain. FEBS Lett. **456:** 119–125.

Janecek, S., Svensson, B. & MacGregor, E. A. 2003. Relation between domain evolution, specificity, and taxonomy of the alpha-amylase family members containing a C-terminal starch-binding domain. Eur. J. Biochem. **270:** 635–645.

Jones, R.L. & Jacobsen, J.V. 1991. Regulation of synthesis and transport of secreted proteins in cereal aleurone. Int. Rev. Cytol. **126:** 49–88.

Juge, N., Le Gal-Goëffet, M.F., Furniss, C.S.M., Gunning, A.P., Kramhøft, B., Morris, V.J., Williamson, G. & Svensson, B. 2002. The starch binding domain of glucoamylase from *Aspergillus niger*: overview of its structure, function, and role in raw-starch hydrolysis. Biologia, Bratislava **57 (Suppl. 11):** 239–245.

Kabsch, W. 1993. Automatic processing of rotation diffraction data from crystals of initially unknown symmetry and cell constants. J. Appl. Crystallogr. **26:** 795–800.

Kadziola, A., Abe, J., Svensson, B. & Haser, R. 1994. Crystal and molecular structure of barley α-amylase. J. Mol. Biol. **239:** 104–121.

Kadziola, A., Søgaard, M., Svensson, B. & Haser, R. 1998. Molecular structure of a barley α-amylase-inhibitor complex: implications for starch binding and catalysis. J. Mol. Biol. **278:** 205–217.

Leah, R. & Mundy, J. 1989. The bifunctional α-amylase/subtilisin inhibitor of barley: nucleotide sequence and patterns of seed specific expression. Plant. Mol. Biol.**:** 673–682.

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

MacGregor, A.W. & Ballance, D.L. 1980. Hydrolysis of large and small starch granules from normal and waxy barley cultivars by α-amylases from barley malt. Cereal Chem. **57:** 397–402.

MacGregor, A.W. & Morgan, J.E. 1986. Hydrolysis of barley starch granules by α-amylases from barley malt. Cereal Foods World **31:** 688–693.

MacGregor, E.A., Janecek, S. & Svensson, B. 2001. Relationship of sequence and structure to specificity in the α-amylase family of enzymes. Biochim. Biophys. Acta **1546:** 1–20.

MacGregor, E.A., Jespersen, H.M. & Svensson, B. 1996. A circularly permuted α-amylase-type α/β-barrel structure in glucan-synthesizing glucosyltransferases. FEBS Lett. **378:** 263–266.

MACGREGOR, E.A., MACGREGOR, A.W., MACRI, L.J. & MORGAN, J.E. 1994. Models for the action of barley α-amylase isozymes on linear substrates [published erratum appears in Carbohydr. Res. 1994, 264**:** C7]. Carbohydr. Res. **257:** 249–268.

MACHIUS, M., DECLERCK, N., HUBER, R. & WIEGAND, G. 1998. Activation of *Bacillus licheniformis* α-amylase through a disorder $\diamondsuit$ order transition of the substrate-binding site mediated by a calcium-sodium-calcium metal triad. Structure **6:** 281–292.

MATSUURA, Y., KUSUNOKI, M., HARADA, W. & KAKUDO, M. 1984. Structure and possible catalytic residues of Taka-amylase A. J. Biochem. **95:** 697–702.

MUNDY, J., SVENDSEN, I. & HEJGAARD, J. 1983. Barley α-amylase/subtilisin inhibitor. Isolation and characterization. Carlsberg Res. Commun. **48:** 81–90.

OHDAN, K., KURIKI, T., TAKATA, H., KANEKO, H. & OKADA, S. 2000. Introduction of raw starch-binding domains into *Bacillus subtilis* α-amylase by fusion with the starch-binding domain of *Bacillus* cyclomaltodextrin glucanotransferase. Appl. Environ. Microbiol. **66:** 3058–3064.

PRZYLAS, I., TOMOO, K., TERADA, Y., TAKAHA, T., FUJII, K., SAENGER, W. & STRATER, N. 2000. Crystal structure of amylomaltase from *Thermus aquaticus*, a glycosyltransferase catalysing the production of large cyclic glucans. J. Mol. Biol. **296:** 873–886.

QIAN, M., HASER, R. & PAYAN, F. 1993. Structure and molecular model refinement of pig pancreatic α-amylase at 2.1 Å resolution. J. Mol. Biol. **231:** 785–799.

RAMASUBBU, N., PALOTH, V., LUO, Y.G., BRAYER, G.D. & LEVINE, M.J. 1996. Structure of human salivary α-amylase at 1.6 Å resolution: implications for its role in the oral cavity. Acta Crystallogr. **D52:** 435–446.

ROBERT, X., GOTTSCHALK, T.E., HASER, R., SVENSSON, B. & AGHAJARI, N. 2002a. Expression, purification and preliminary crystallographic studies of α-amylase isozyme 1 from barley seeds. Acta Crystallogr. **D58:** 683–686.

ROBERT, X., HASER, R., GOTTSCHALK, T.E., RATAJCZAK, F., DRIGUEZ, H., SVENSSON, B. & AGHAJARI, N. 2003. The structure of barley α-amylase isozyme 1 reveals a novel role of domain C in substrate recognition and binding: a pair of sugar tongs. Structure **11:** 973–984.

ROBERT, X., HASER, R., MORI, H., SVENSSON, B. & AGHAJARI, N. 2005. Oligosaccharide binding to barley α-amylase 1. J. Biol. Chem. **280:** 32968–32978.

ROBERT, X., HASER, R., SVENSSON, B. & AGHAJARI, N. 2002b. Comparison of crystal structures of barley α-amylase 1 and 2: implications for isozyme differences in stability and activity. Biologia, Bratislava **57 (Suppl. 11):** 59–70.

RODENBURG, K.W., JUGE, N., GUO, X.J., SØGAARD, M., CHAIX, J.C. & SVENSSON, B. 1994. Domain B protruding at the third β strand of the α/β barrel in barley α-amylase confers distinct isozyme-specific properties. Eur. J. Biochem. **221:** 277–284.

RODENBURG, K.W., VALLÉE, F., JUGE, N., AGHAJARI, N., GUO, X., HASER, R. & SVENSSON, B. 2000. Specific inhibition of barley α-amylase 2 by barley α-amylase/subtilisin inhibitor depends on charge interactions and can be conferred to isozyme 1 by mutation. Eur. J. Biochem. **267:** 1019–1029.

ROGERS, J.C. 1985. Conserved amino acid sequence domains in α-amylases from plants, mammals, and bacteria. Biochem. Biophys. Res. Commun. **128:** 470–476.

ROGERS, J.C. & MILLIMAN, C. 1983. Isolation and sequence analysis of a barley α-amylase cDNA clone. J. Biol. Chem. **258:** 8169–8174.

ROUSSEL, A. & CAMBILLAU, C. 1992. TURBO-FRODO. Biographics, AFMB, Marseille, France.

SAUER, J., SIGURSKJOLD, B.W., CHRISTENSEN, U., FRANDSEN, T.P., MIRGORODSKAYA, E., HARRISON, M., ROEPSTORFF, P. & SVENSSON, B. 2000. Glucoamylase: structure/function relationships, and protein engineering. Biochim. Biophys. Acta. **1543:** 275–293.

SIDENIUS, U., OLSEN, K., SVENSSON, B. & CHRISTENSEN, U. 1995. Stopped-flow kinetic studies of the reaction of barley αtamylase/subtilisin inhibitor and the high pI barley α-amylase. FEBS Lett. **361:** 250–254.

SØGAARD, M., KADZIOLA, A., HASER, R. & SVENSSON, B. 1993. Site-directed mutagenesis of histidine 93, aspartic acid 180, glutamic acid 205, histidine 290, and aspartic acid 291 at the active site and tryptophan 279 at the raw starch binding site in barley α-amylase 1. J. Biol. Chem. **268:** 22480–22484.

SØGAARD, M. & SVENSSON, B. 1990. Expression of cDNAs encoding barley α-amylase 1 and 2 in yeast and characterization of the secreted proteins. Gene **94:** 173–179.

STROBL, S., GOMIS-RUTH, F.X., MASKOS, K., FRANK, G., HUBER, R. & GLOCKSHUBER, R. 1997. The α-amylase from the yellow meal worm: complete primary structure, crystallization and preliminary X-ray analysis. FEBS Lett. **409:** 109–114.

SUMITANI, J., TOTTORI, T., KAWAGUCHI, T. & ARAI, M. 2000. New type of starch-binding domain: the direct repeat motif in the C-terminal region of *Bacillus* sp. no. 195 α-amylase contributes to starch binding and raw starch degrading. Biochem. J. **350:** 477–484.

SVENDSEN, I., HEJGAARD, J. & MUNDY, J. 1986. Complete amino acid sequence of the α-amylase/subtilisin inhibitor from barley. Carlsberg Res. Commun. **51:** 43–50.

SVENSSON, B., JESPERSEN, H., SIERKS, M.R. & MACGREGOR, E.A. 1989. Sequence homology between putative raw-starch binding domains from different starch-degrading enzymes. Biochem. J. **264:** 309–311.

THOMPSON, J.D., HIGGINS, D.G. & GIBSON, T.J. 1994. CLUSTALW: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. Nucleic Acids Res. **22:** 4673–4680.

TIBBOT, B.K., WONG, D.W. & ROBERTSON, G.H. 2000. A functional raw starch-binding domain of barley α-amylase expressed in *Escherichia coli*. J. Protein Chem. **19:** 663–669.

TIBBOT, B.K., WONG, D.W.S. & ROBERTSON, G.H. 2002. Studies on the C-terminal region of barley α-amylase 1 with emphasis on raw starch-binding. Biologia, Bratislava **57 (Suppl. 11):** 229–238.

VALLÉE, F., KADZIOLA, A., BOURNE, Y., JUY, M., RODENBURG, K.W., SVENSSON, B. & HASER, R. 1998. Barley α-amylase bound to its endogenous protein inhibitor BASI: crystal structure of the complex at 1.9 Å resolution. Structure **6:** 649–659.

WESELAKE, R.J. & HILL, R.D. 1983. Inhibition of α-amylase-catalyzed starch granule hydrolysis by cycloheptaamylose. Cereal Chem. **60:** 98–101.

WONG, D.W., BATT, S.B., TIBBOT, B.K. & ROBERTSON, G.H. 2000. Isolation of a raw starch-binding fragment from barley α-amylase. J. Protein Chem. **19:** 373–377.

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