Oligosaccharide Binding to Barley α-Amylase 1*

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Enzymatic subsite mapping earlier predicted 10 binding subsites in the active site substrate binding cleft of barley α-amylase isozymes. The three-dimensional structures of the oligosaccharide complexes with barley α-amylase isozyme 1 (AMY1) described here give us a fresh perspective on the key components involved in the catalytic mechanism of this enzyme.

Structural studies of α-amylases in complex with substrate analogues have received much attention in the past decade (1–13). The pseudotetrasaccharide inhibitor acarbose was used for the vast majority of these studies. In contrast, binding of natural substrates was only described in the Bacillus subtilis α-amylase (14, 15), the inactive catalytic nucleophile B. stearothermophilus in complex with a maltose unit that was derived from a maltotriose (17). α-Amylases belong to the glycoside hydrolase family 13 (afmb.cnrs-mrs.fr/CAZY), which together with GH70 and GH77 constitute GH clan H (18). These enzymes share the catalytic site geometry with three invariant acid residues as follows: a catalytic nucleophile, a proton donor, and a third catalytic acidic side chain, which polarizes the glucoside unit at subsite −1 (19). Despite a sequence identity of 80% and a nearly identical overall folding (20), AMY1 and AMY2 exhibit important differences as concerns stability (21, 22), enzymatic properties (23), and sensitivity to the proteinaceous inhibitor barley α-amylase subtilisin inhibitor, belonging to the Kunitz soybean trypsin inhibitor family (24). Most interestingly, three calcium ions are bound to both isozymes sharing identical ligands (20), although calcium affects the activity of AMY1 (10) and AMY2 in distinct manners (21). Numerous AMY1 subsite mutants have been characterized with respect to substrate affinity and catalytic capacity, but structural insight for barley α-amylases was limited until now to subsites −1 through +2 being experimentally defined with the AMY2-acarbose complex (4). Enzymatic subsite mapping (25) and computer-aided modeling (26, 27) suggested that both AMY1 and AMY2 possess 10 subsites spanning from the nonreducing end at subsite −6 to +4 on the aglycon accommodating part. A more recent study in which crystals of AMY1 soaked in a solution containing thio-maltotetraose was expected to expand our knowledge on subsite-binding modes in the catalytic cleft of plant α-amylases. This substrate analogue, however, led to the discovery of a new surface binding site at domain C (Fig. 1), the so-called “pair of sugar tongs,” but did not bind to the active site (28). This site and an earlier discovered surface binding site on the catalytic domain made up of two consecutive tryptophan residues, Trp-278 and Trp-279 (AMY1 numbering), perform distinct interactions with the sugar rings of different oligosaccharide ligands (4, 28). Mutational analyses have confirmed that these sites indeed can bind onto starch granules and that they also bind β-cyclodextrin (29, 62). Here we report the crystal structures of native AMY1 as well as an inactive mutant of the catalytic nucleophile Asp-180 in complex with acarbose (Fig. 2, A and B) and maltoheptaose (Fig. 1), respectively. This is the first report on sugar binding to the active site of barley α-amylase 1 and on binding of the substrate maltotetraose to the active site region.

EXPERIMENTAL PROCEDURES

Preparation of Recombinant AMY1Δ9—A truncated form, AMY1Δ9 (nonapeptide deletion from the C terminus), of recombinant AMY1 was prepared as described previously (30) to overcome difficulties encountered in growing three-dimensional crystals of full-length AMY1 (30). AMY1Δ9 is henceforth referred to as AMY1.

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9 The atomic coordinates and structure factors (codes 1NPk, 1RP6, and 1RP9) have been deposited in the Protein Data Bank, Research Collaboratory for Structural Bioinformatics, Rutgers University, New Brunswick, NJ (http://www.rcsb.org/).

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3 The abbreviations used are: AMY1, barley α-amylase isozyme 1; AMY2, barley α-amylase isozyme 2; MES, 4-morpholineethanesulfonic acid; CGTase, cyclomaltodextrin glucanotransferases; G7, maltoheptaose; G8, malto-octaose.
Preparation of the Inactive Mutant AMY1{D180A}—Escherichia coli DH5α (Invitrogen) was used for standard cloning. Pichia pastoris GS115 and pPICZA (Invitrogen), carrying the Zeocin R selection marker, were used for expression. AMY1 cDNA was from an in-house collection (31). pPICZA-AMY1 harbored the AMY1-encoding insert flanked by EcoRI and KpnI sites (32). Standard culture media were used for E. coli (33) and P. pastoris (31).

Construction, Site-directed Mutagenesis, Transformation, and Screening—The D180A mutation was introduced into pPICZA-AMY1 (32) by the megaprimer procedure (34) as done for other mutations (32, 35). AMY1 cDNA was amplified using primer A (32) and the mutagenesis primer C (5’-CCCCTAGCGAAAGCAAGGCGCCACG-3’, positions 695–719, antisense orientation; mutant anticodon double-underlined) to generate the megaprimer used in the second PCR with primer B (32). All PCRs were performed using high fidelity Pfu DNA polymerase (Stratagene), and the complete sequence of pPICZA-AMY1{D180A} was confirmed (Applied Biosystems 377 DNA Sequencer and Taq DyeDeoxy Terminator Cycle Sequencing kit, PerkinElmer Life Sciences). The plasmid was linearized at the BstXI site for P. pastoris transformation (31).

Production and Purification of AMY1{D180A}—The selected transformant was grown (0.5 liters of BMGY, 2 days in a 5-liter flask) to A_{600} = 20, and the medium was replaced by BMMY (1 liter) for induction for 32 h under vigorous shaking. The supernatant was kept, and the cells were resuspended in BMMY (1 liter) for a second induction culture (39 h). AMY1{D180A} was purified from the combined supernatants by affinity chromatography on β-cyclodextrin-Sepharose (31, 32) and anion exchange chromatography.
Barley α-Amylase Oligosaccharide Complexes

**Table One**

| Protein data bank entry code | AMY1-acarbose | AMY1D180A-acarbose | AMY1D180A-maltoheptaose |
|-------------------------------|---------------|---------------------|-------------------------|
| Data collection temperature (K) | 100           | 100                 | 100                     |
| Unit cell dimensions, a, b, c (Å) | 93.4, 72.9, 61.4 | 93.7, 73.5, 61.1 | 93.0, 72.5, 62.2 |
| Space group | P2₁2₁2₁ | P2₁2₁2₁ | P2₁2₁2₁ |
| Resolution range (Å) | 17.4 to 2.0 | 39.5 to 2.0 | 39.1 to 2.0 |
| Completeness of data (%) | 94.2 (94.7) | 88.0 (88.9) | 99.9 (100.0) |
| Multiplicity of data (overall) | 3.5 (3.1) | 4.4 (4.4) | 7.5 (3.3) |
| Outermost shell (Å) | 2.05 to 2.00 | 2.05 to 2.00 | 2.05 to 2.00 |
| Total no. reflections | 96,013 | 114,564 | 211,756 |
| No. unique reflections | 27,344 | 25,774 | 29,146 |
| Rsym | 18.3 (36.6) | 8.3 (31.5) | 9.5 (31.1) |
| Rfree | 21.3 | 23.1 | 22.1 |

**Enzyme Activity Assays**—Activity toward soluble potato starch (Sigma), 9 mg x ml⁻¹ in 20 mM sodium acetate buffer, 5 mM CaCl₂, pH 6.8, 0.02% (v/v) sodium azide at 4 °C. Isoelectric focusing (pI 4–6.5) and SDS-PAGE (PhastGels and Phast-System, Amersham Biosciences) of AMY1D180A were silver-stained according to the manufacturer’s recommendation (36). Enzyme concentrations were calculated from amino acid contents of protein (25 μg) hydrolysat (32). Purified AMY1D180A was obtained (6.5 mg x liter⁻¹) and gave one band in SDS-PAGE (45 kDa) and native PAGE (data not shown).

**Crystallographic Data Collection and Refinement**—The complex was crystallized (Cen-triPrep YM10, Millipore, Bedford, MA) to ~2 mg x ml⁻¹ and stored in 10 mM MES, 5 mM CaCl₂, pH 6.8, 0.02% (v/v) sodium azide at 4 °C. Isoelectric focusing (pI 4–6.5) and SDS-PAGE (PhastGels and Phast-System, Amersham Biosciences) of AMY1D180A were silver-stained according to the manufacturer’s recommendation (36). Enzyme concentrations were calculated from amino acid contents of protein (25 μg) hydrolysat (32). Purified AMY1D180A was obtained (6.5 mg x liter⁻¹) and gave one band in SDS-PAGE (45 kDa) and native PAGE (data not shown).

**Crystallization, Soaking, and Cryo-protection**—The complex between AMY1 and the inhibitor acarbose was obtained by co-crystallization at conditions derived from those of the native enzyme (30). 0.5 μl of a 100 mM acarbose stock solution was mixed with 2 μl of protein stock and 3 μl of well solution, thus resulting in a final concentration of 9.1 mM acarbose in the drop, and crystals grew to a final size of 0.35 x 0.1 x 0.05 mm³ within 3 weeks.

The inactive AMY1D180A mutant was crystallized by the hanging drop vapor diffusion method at 19 °C, using protein stock (2.9 mg x ml⁻¹) in 10 mM MES, pH 6.7, 5 mM CaCl₂, and 0.02% NaN₃. Drops were prepared by mixing 2 μl of protein stock with 0.5 μl of 3% (v/v) isopropyl alcohol and 2.5 μl of well solution containing 20% (v/v) polyethylene glycol 8000. Thin crystals typically grew to a final size of 0.8 x 0.1 x 0.05 mm³ after ~6 months. Soaking was done by adding a solution of maltotetraose (Roche Applied Science) directly to the drop to a final concentration of 10 mM and leaving it for 24 h at 19 °C.

The AMY1D180A-acarbose complex was obtained by adding acarbose directly to the drop at a final concentration of 10 mM and leaving it for 24 h at 19 °C. All crystals were cryo-protected prior to data collection by rapid soaking in three successive steps in mother liquor containing increasing concentrations of ethylene glycol (5, 10, and 15% (v/v)) and 10 mM acarbose or maltotetraose, respectively.

**Diffraction Data Collection**—AMY1-acarbose diffraction data were collected on a MAR Research 345 Image Plate System associated to a Nonius FR591 rotating anode (CuKα radiation), operating at 44 kV and 100 mA and coupled to Osmic confocal mirrors. Data processing and reduction was carried out using DENZO (38) and SCALA from the CCP4 package (39).

AMY1D180A-maltoheptaose data were collected at the ID14-1 beamline at the European Synchrotron Radiation Facility, Grenoble, France, on a MarCCD detector, and data on the AMY1D180A-acarbose were collected at the FIP BM30A beamline (European Synchrotron Radiation Facility) on a MarCCD detector. Diffracted intensities were integrated with the program MOSFLM (40) as implemented in the CCP4 software package (39) and scaled with SCALA (39). All crystals belong to the orthorhombic space group P2₁2₁2₁, and one molecule is present in the asymmetric unit. Data collection statistics are presented in TABLE ONE.

**Structure Determination and Refinement**—Because of crystal isomorphism with AMY1 (28), the latter was used as starting model (Protein Data Bank entry 1HT6) in a difference Fourier, where all water molecules, calcium ions, and ligand molecules had been removed. For all data sets, an initial rigid body refinement, including data to 4 Å resolution, was performed, and in the remaining refinements a simulated annealing protocol was used extending the data to 2.0 Å resolution. All refinements were done with the program CNS (41). In order to avoid over-refinement, free and conventional R-factors were monitored (42). Alternating with these refinement steps, visual examination of electron density maps and manual building was carried out using the graphic software TURBO-FRODO (43). Based on the inspection of 2Fo – Fe and Fo – Fe maps (contoured at 1 and 3σ, respectively), calcium ions were inserted, and water molecules were manually added respecting hydrogen bonds with standard distances and angles formed to appropriate atoms. Ligands (sugar moieties from acarbose or maltotetraose) were manually inserted in the electron density maps. Because of
the high quality and resolution of the diffraction data, the orientation and nature of each sugar ring was unequivocally determined. Models of acarbose and glucose units were found in the HIC-Up data base (44). The geometry and coordinate errors of the three final structures were examined with the programs PROCHECK (45) and WHATCHECK (46). Refinement statistics are listed in Table One.

Structural superimposition of the structures was performed using the "rigid" option in TURBO-FRODO (43). Figures shown in this paper were rendered using ISIS-Draw (freeware from Elsevier MDL, San Leandro, CA; www.mdli.com), TURBO-FRODO (43), and VIEWER-LITE™ 4.2 (freeware from Accelrys Inc., San Diego; www.accelrys.com).

RESULTS

AMY1 in Complex with Acarbose and Comparison with AMY2-Acarbose—The three-dimensional structure of the complex between AMY1 and acarbose was solved to 2.0 Å resolution. As for AMY2 (4), three substrate binding subsites are occupied by a molecule of component 2 (a pseudotrisaccharide derived from acarbose after cleavage of a glucose unit from the reducing end) meaning that unit A to C occupies subsites −1 to +2. The starch granule-binding surface site containing Trp-278 and Trp-279 also binds a component 2 molecule, whereas the "sugar tongs" site at domain C (28) displays the entire acarbose molecule. Direct hydrogen bonds between enzyme and inhibitor molecules are listed for the three sites in Table Two.

The electron density map clearly suggests that acarbose was cleaved by AMY1 resulting in a component 2 molecule binding to the active site. At the starch granule-binding surface site, the fourth sugar ring is not defined, reflecting either that this sugar partly points into the solvent and therefore is flexible or a difference in affinity for this and the sugar tongs-binding site, respectively. This conclusion is supported by the structures, the surface site with Trp-278 to Trp-279 being widely opened and exposed to solvent. Only a relatively low number of interactions are present for stabilizing long flexible substrates; therefore, a short and less flexible substrate may have a higher affinity. In contrast, the sugar tongs site is defined by a cleft in which oligosaccharide is captured by Tyr-380 and has an increased number of interactions compared with the former surface site, thus a priori providing superior stabilization for longer ligands. Remarkably, when comparing the active sites for AMY1-acarbose and AMY2-acarbose, structural conservation between the enzymes is high as only two amino acid side chains differ a priori providing superior stabilization for longer ligands.

### Table Two
Comparison of direct hydrogen bond contacts in AMY1-acarbose and AMY2-acarbose

| AMY1 atom | Acarbose atom | Distance (Å) | AMY2 atom | Acarbose atom | Distance (Å) |
|-----------|---------------|--------------|-----------|---------------|--------------|
| A. Active site |
| Tyr-52-O | aca-O6A | 3.6 | Tyr-51-O | aca-O6A | 3.2 |
| His-93-Ne2 | aca-O6A | 3.0 | His-92-Ne2 | aca-O6A | 3.2 |
| Arg-178-Nη2 | aca-O2A | 3.0 | Arg-177-Nη1 | aca-O2A | 3.0 |
| Asp-180-O62 | aca-O6A | 2.8 | Asp-179-O62 | aca-O6A | 2.9 |
| Arg-183-Nη1 | aca-O2B | 2.8 | Lys-182-Nζ | aca-O3C | 3.0 |
| Glu-205-Or1 | aca-O3B | 2.7 | Glu-204-Or1 | aca-O4B | 2.8 |
| Glu-205-Or2 | aca-O4B | 2.8 | Glu-204-Or2 | aca-O3B | 2.6 |
| Trp-207-O | aca-O3C | 2.7 | Trp-206-O | aca-O3C | 2.8 |
| Asn-209-N | aca-O2C | 2.8 | Ser208-N | aca-O2C | 3.5 |
| Asn-209-61 | aca-O2C | 3.6 | Ser208-Or | aca-O2C | 3.2 |
| His-290-Ne2 | aca-O2A | 2.8 | His-288-Ne2 | aca-O2A | 2.9 |
| His-290-Ne2 | aca-O3A | 2.9 | His-288-Ne2 | aca-O3A | 2.8 |
| Asp-291-61 | aca-O3A | 2.6 | Asp-289-61 | aca-O3A | 2.8 |
| Asp-291-62 | aca-N4B | 3.1 | Asp-289-62 | aca-N4B | 3.2 |
| Asp-291-62 | aca-O2A | 2.5 | Asp-289-62 | aca-O2A | 2.7 |
| B. Starch granule surface binding site |
| Gln-227-Or1 | aca-O3C | 3.0 | Gln-226-Or1 | aca-O3B | 2.8 |
| Gln-227-Ne2 | aca-O2C | 2.7 | Gln-226-Ne2 | aca-O2B | 2.9 |
| Asp-234-61 | aca-O3B | 2.7 | Asp-233-61 | aca-O2A | 2.9 |
| Asp-234-62 | aca-O2B | 2.5 | Asp-233-62 | aca-O3A | 3.1 |
| Trp-276-O | aca-O6A | 3.0 |
| C. Sugar tongs surface binding site |
| Lys-375-Nζ | aca-O2B | 2.9 |
| Lys-375-Nζ | aca-O3B | 3.2 |
| Tyr-380-O | aca-O2A | 2.8 |
| Tyr-380-O | aca-O3B | 2.5 |
| Asp-381-61 | aca-O3A | 3.0 |
| Val-382-N | aca-O2A | 2.8 |
| Thr-392-Or1 | aca-O6A | 3.0 |
| Asp-398-N | aca-O3C | 2.9 |
| His-395-N81 | aca-O6C | 2.9 |

* The inversions in hydrogen bonding partners between AMY1 and AMY2 have no functional significance.
hydrogen bonding interactions exist between AMY1D180A and the site, and the sugar tongs. The high quality of the electron density maps in sites as follows: the active site, the starch granule-binding surface mutant AMY1D180A showed a specific activity of 6.7

ues found in the AMY2-acarbose complex may be related to the lower

sional structure of inactive AMY1D180A-maltoheptaose exhibits the

H9251

genic bonding between His-93 in subsite

sequence of the mutation of the catalytic nucleophile is the loss of hydro-

resulting in (Φ,Ψ) = (118°,−113°) – Glc2006(−7) to Glc2005(−6); (123°,−111°) – Glc2005(−6) to Glc2004(−5); (−62°,−73°) – Glc2004(−5) to Glc2003(−4); (122°,−122°) – Glc2003(−4) to Glc2002(−3); (69°,−148°) – Glc2002(−3) to Glc2001(−2); (83°,−155°) – Glc2001(−2) to Glc2000(−1). When compared with the analogue values in AMY1D180A-acarbose, Φ and Ψ are rather similar between subsites −3 to −1 as expected. However, when approaching the nonreducing end of the glycone part of the active site, only Φ,Ψ values between subsites −2 to −1 are similar to the analogue ones as found in other complexes (47). Hereafter they differ, which is particularly remarkable when comparing to (Φ,Ψ) for the interglycosidic bond between subsites −3 to −2 in the B. subtilis α-amylase-maltopentaose structure (16) being (123°,−107°). This seems to be mainly due to the torsion angles between subsite −5 to −4 glucoses, being subjected to a very drastic shift resulting in (Φ,Ψ) = (−62°,−73°) deviating to a very high degree from those observed in a regular helical structure of amylose. In this structure the repeating unit consists of a maltotriose where the confor-

mation of the glycosidic linkage is (91.8°,−153.2°), (85.7°,−145.3°),

and (91.8°,−151.3°) (51). The interactions between maltosheptaose and active site residues are listed in TABLE THREE, part A. Eight of the 16 direct hydrogen bonds between maltosheptaose and AMY1D180A involve the reducing end ring (Glc2006), whereas Glc2004 has no direct interac-

ions with the enzyme, and an aromatic stacking exists between Trp-10 and Glc2005 (subsite −2). In comparison to this relatively low number of contacts, AMY1-acarbose (see TABLE TWO, part A) has 15 direct hydrogen bonds between the enzyme and the three observed rings of acarbose, as well as a certain number of interactions mediated by water molecules. Fig. 4 shows a schematic representation, summarizing these interactions. Furthermore, a number of internal hydrogen bonds are observed for maltosheptaose in the complex (see TABLE THREE, part D). A summary of residues defining subsites −1 to −7 is given in TABLE FOUR. The fact that no direct interactions exist between sugar moieties in subsite −3 (Glc2004) and the enzyme is in excellent agreement with the binding kinetics of malto-oligosaccha-

rides, which allows determination of subsite affinities as listed in TABLE FOUR. Two independent groups have arrived at somewhat different results (25, 50), although some features are shared. These studies show that the affinity at subsite −3 is low compared with the other subsites (25) and even negative (50). An unexplained density around Cys-95 in the native structure of AMY1 (28) is seen in the electron density here as well but does not seem to affect the interaction with Glc2002.

The presence of subsite −7 in the AMY1D180A-maltoheptaose complex was not unequivocally defined by subsite mapping (25, 50). In this subsite, His-45 and Val-47 make direct hydrogen bonds to the nonreducing end of maltoheptaose (Glc2000), see Fig. 4. Furthermore, two indirect hydrogen bonds to Arg-56 and Lys-64 mediated by Wat-1592 and Wat-1088, respectively, are observed. The ensemble of glucose units 2000, 2001, 2002, and 2003 adopt a half-circle conformation with the Val-47-Cy1 atom as the approximate center. Clearly defined elec-
tron density for the glucose unit, Glc2000, strengthens the existence of subsite —7. The mean value of $B$-factors for the atoms of this ring is 42.9 Å$^2$, which is in accordance with a relatively flexible ring.

Most interestingly, Glc2001 is located between two hydrophobic residues Val-47 and Tyr-105, but no hydrophobic contacts exist to either of these. The space between these two residues may be considered as the substrate “entrance” to the active site cleft. The plane of ring Glc2001 is not parallel to that of the aromatic group of Tyr-105 and an imperfect aromatic stacking is present between these two entities. Probably, this stacking would be optimized if the additional glucose unit (Glc2000) at
### TABLE THREE

Direct hydrogen bond contacts in the complexes AMY1D180A-maltoheptaose and AMY1D180A-acarbose

Atom labeling convention for glucosyl residues and acarbose is presented in Fig. 2.

| AMY1D180A atom | Maltoheptaose atom | Distance | AMY1D180A atom | Acarbose atom | Distance |
|----------------|-------------------|----------|----------------|---------------|----------|
|                |                   | Å        |                |               | Å        |
| A. Active site |                   |          |                |               |          |
| His-45-N/e2    | Glc2000-O3        | 3.0      | Ala-146-O     | Aca-O3A       | 3.0      |
| Val-47-O      | Glc2000-O4        | 3.3      | Ala-146-O     | Aca-O4A       | 3.3      |
| Cys-95-Sy      | Glc2002-O2        | 3.1      | Arg-178-N/e1  | Aca-O1D       | 3.0      |
| Ala-96-O      | Glc2001-O2        | 2.6      | Arg-178-N/e2  | Aca-O2D       | 3.0      |
| Ala-146-O     | Glc2002-O2        | 2.9      | Phe-181-N     | Aca-O1D       | 3.3      |
| Ala-146-O     | Glc2003-O4        | 2.8      | Glu-205-Oe1   | Aca-O1D       | 2.4      |
| Arg-178-N/e1  | Glc2006-O1        | 3.0      | His-290-N/e2  | Aca-O2D       | 2.8      |
| Arg-178-N/e2  | Glc2006-O2        | 3.0      | His-290-N/e2  | Aca-O3D       | 3.0      |
| Phe-181-N     | Glc2006-O1        | 3.4      | Asp-291-Oe1   | Aca-O3D       | 2.7      |
| Glu-205-Oe1   | Glc2006-O1        | 2.6      | Asp-291-Oe1   | Aca-O3D       | 2.7      |
| His-290-N/e2  | Glc2006-O2        | 3.1      | His-290-N/e2  | Aca-O3D       | 3.0      |
| His-290-N/e2  | Glc2006-O3        | 3.1      | Asp-291-Oe1   | Aca-O3D       | 2.7      |
| Asp-291-Oe1   | Glc2006-O3        | 2.8      | Asp-291-Oe1   | Aca-O3D       | 2.7      |
| Asp-291-Oe2   | Glc2006-O2        | 2.6      | Asp-291-Oe2   | Aca-O2D       | 2.5      |
| Glu-296-Oe1   | Glc2005-O2        | 2.9      | Gln-296-Oe1   | Aca-O2C       | 2.8      |
| B. Starch granule surface-binding site | | | | | |
| Gln-227-N/e2  | Glc4003-O2        | 2.5      | Gln-227-N/e2  | Aca-O3C       | 3.0      |
| Gln-227-O/e1  | Glc4003-O3        | 2.8      | Gln-227-O/e1  | Aca-O2C       | 2.6      |
| Asp-234-O/e1  | Glc4002-O2        | 2.7      | Asp-234-O/e1  | Aca-O3B       | 2.7      |
| Asp-234-O/e2  | Glc4002-O2        | 2.7      | Asp-234-O/e2  | Aca-O2B       | 2.6      |
| Lys-375-N/e2  | Glc3002-O2        | 3.0      | Lys-375-N/e2  | Aca-O2B       | 2.9      |
| Lys-375-N/e2  | Glc3002-O3        | 3.3      | Lys-375-N/e2  | Aca-O3B       | 3.1      |
| Tyr-380-O     | Glc3001-O2        | 2.8      | Tyr-380-O     | Aca-O2A       | 2.8      |
| Tyr-380-O     | Glc3001-O3        | 2.8      | Tyr-380-O     | Aca-O3B       | 2.6      |
| Asp-381-O/e1  | Glc3001-O3        | 2.7      | Asp-381-O/e1  | Aca-O3A       | 2.6      |
| Val-382-N     | Glc3001-O2        | 3.1      | Val-382-N     | Aca-O2A       | 2.9      |
| Thr-392-O/e1  | Glc3001-O6        | 2.9      | His-392-O/e1  | Aca-O6C       | 2.7      |
| His-395-N/e1  | Glc3003-O6        | 2.7      | His-395-N/e1  | Aca-O6C       | 2.7      |
| Gly-396-O     | Glc3003-O6        | 3.3      | Gly-396-O     | Aca-O2A       | 2.9      |
| Asp-398-N     | Glc3003-O3        | 2.8      | Asp-398-N     | Aca-O3C       | 2.9      |
| Asp-398-O/e1  | Glc3003-O3        | 3.2      | Asp-398-O/e1  | Aca-O3C       | 3.3      |
| D. Intramolecular hydrogen bonds | | | | | |
| Maltoheptaose (active site) | | | Acarbose (active site) | | |
| Glc2000-O2    | Glc2001-O3        | 2.7      | aca-O2A        | aca-O3B       | 2.9      |
| Glc2001-O2    | Glc2002-O3        | 2.7      | aca-O2A        | aca-O3B       | 2.9      |
| Glc2001-O5    | Glc2002-O6        | 3.3      | aca-O2B        | aca-O3C       | 3.1      |
| Glc2003-O2    | Glc2004-O3        | 2.5      | aca-O2A        | aca-O3B       | 3.5      |
| Glc2004-O6    | Glc2005-O6        | 3.0      | aca-O2B        | aca-O3C       | 3.1      |
| Maltoheptaose fragment (starch granule-binding surface site) | | | Acarbose (starch granule-binding surface site) | | |
| Glc4000-O2    | Glc4001-O3        | 3.1      | aca-O2A        | aca-O3B       | 3.5      |
| Glc4001-O2    | Glc4002-O3        | 3.0      | aca-O2A        | aca-O3B       | 3.5      |
| Glc4002-O2    | Glc4003-O3        | 2.9      | aca-O2B        | aca-O3C       | 3.1      |
| Maltoheptaose fragment (sugar tongs binding site) | | | Acarbose (sugar tongs binding site) | | |
| Glc3002-O2    | Glc3003-O3        | 3.2      | aca-O2A        | aca-O3B       | 3.7      |
| Glc3003-O2    | Glc3004-O6        | 3.4      | aca-O2B        | aca-O3C       | 3.1      |
| Glc3003-O2    | Glc3004-O6        | 3.4      | aca-O2C        | aca-O6D       | 3.5      |
the nonreducing end of Glc2001 in maltoheptaose was lacking. It seems that Glc2000 forces Glc2001 to move away from Tyr-105 and thereby weakens the aromatic stacking interaction.

Comparative studies of active site residues in AMY1D180A-maltoheptaose and native AMY1 show that the backbones are superimposable and that no drastic reorientation of side chains occurred. All residues involved in interactions with the substrate and their neighbors are highly superimposable with the exception of Arg-183. In native AMY1, this residue displays a double conformation, for which the side chain approaches toward Glc2006. Although Arg-183 does not interact directly with Glc2006, it contributes to the formation of a network of water molecules in the catalytic cleft of the AMY1D180A-maltoheptaose complex. Arg-183 is orientated in an opposite direction in the complex AMY1D180A-maltoheptaose. This reorientation results in a slight modification of the water molecule network surrounding ring D in acarbose, without any major influence on the indirect water-mediated protein-inhibitor interactions. As it was shown in the AMY1D180A-maltoheptaose complex, acarbose ring B, which corresponds to glucose unit Glc2004, occupies subsite −3 and has no interaction at all with the protein. Once again, these two sugar rings are perfectly superimposed.

Finally, ring A from acarbose is located in subsite −4 and its conformation slightly differs from Glc2003, its counterpart in AMY1D180A-maltoheptaose, thus leading to an additional interaction with Ala-146-O. This difference is due to the inter-cyclic nitrogen atom in the acarvi-rose unit. Moreover, the presence of extra glucose units in maltoheptaose occupying subsites −5 to −7 seems to force the substrate to adopt a half-circle conformation centered on Val-47 (see above), which is not the case for acarbose. This is consistent with residues Val-47 and Tyr-105 defining the entrance of the catalytic pathway, thus serving as “guides” for the substrate and conferring the spatial organization for its approach toward the active site.

### Table Four

| Amino acid residues defining subsites in the active site cleft of AMY1 based on the structural studies of AMY1D180A/maltoheptaose, AMY1D180A-acarbose, and AMY1-acarbose complexes |
|---------------------------------------------------------------|
| **Residues involved in direct hydrogen bond**                 |
| His-45 Val-47 | Ala-96 | Cys-95 Ala-146 | Ala-146 | Gln-296 | Tyr-52 His-93 Arg-178 Asp-180 Phe-181 Glu-205 His-290 Asp-291 |
| **Residues involved in indirect hydrogen bonds**              |
| Ser-46 Arg-56 Lys-64 | Asp-97 Ala-96 Tyr-105 | Lys-130 Tyr-131 Ala-145 | Glu-50 Glu-50 Glu-296 Ser-48 | Ser-48 Glu-50 Glu-205 Trp-207 Trp-207 ND ND |
| **Residues involved in aromatic stacking**                    |
| Tyr-105 | Tyr-52 | Trp-207 ND ND |
| **Subsite affinity (kJ mol⁻¹)**                               |
| −0.26 | 7.68 | 5.19 | 4.42 | 1.25 | 8.46 | −10.20 | 6.50 | 4.94 | 0.32 | 3.24 |
| Based on MacGregor et al. (50) |
| **Subsite affinity (kJ mol⁻¹)**                               |
| 1.6 | 11.0 | 1.8 | 0.5 | −0.9 | 3.5 | ND | ND | 6.0 | −1.2 |
| Based on Ajandouz et al. (25) |

### Figure 4

Schematic representation of hydrogen bonding network in the catalytic cleft of the AMY1D180A-maltoheptaose complex. Amino acid residues are in rectangles and water molecules in ellipsoids. Catalytic residues are highlighted. Figure was rendered using the ISIS-Draw software.

Amino acid residues are in lighted rectangles. Catalytic residues are highlighted. Figure was rendered using the ISIS-Draw software.
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Starch Granule Surface-binding Site—A secondary binding site at the surface of the enzyme was visualized earlier in crystal structures of AMY2 (4) and AMY1 (28) in complex with the substrate analogues acarbose and thio-maltotetraose, respectively. Two adjacent tryptophans, 278 and 279 (Trp-276 and Trp-277 in AMY2), define this so-called starch granule-binding site and were identified by differential labeling and UV spectroscopy (52). It has been shown that these two tryptophan residues are spatially locked because of their environment. An angle of 135° found between the two planes defined by the aromatic tryptophan side chains appears to be constant in both AMY1 and AMY2, in complexed as well as in native states (28). It was therefore suggested that Trp-278 and Trp-279 (AMY1) function as a kind of “geometric filter” determining whether substrates should bind to the active site.

The complex between the inactive mutant AMY1D180A and maltoheptaose displays electron density corresponding to five of the seven glucose units (Glc4000 to Glc4004, numbered from the nonreducing to the reducing end, Fig. 3B). For this sugar molecule which forms a half-circle, no electron density was observed for the two remaining glucose rings, probably because of their location in the bulk solvent, and thus suggesting a highly disordered state for these two rings. Gln-227 and Asp-234 are performing two major interactions as shown (TABLE THREE, part B). Furthermore, stacking interactions are present between Trp-278/Glc4003 and Trp-279/Glc4002, respectively, and a network of water molecules mediates several indirect contacts between AMY1 and maltoheptaose. Comparative studies show that the only differences between native and complexed AMY1 at this site concern a small reorientation of the side chain of Gln-227 (to interact with Glc4003), which in turn gives rise to a small reorientation of Asn-231.

In the complex between the inactive mutant and acarbose, the starch granule-binding surface site also accommodates an intact acarbose molecule with rings B and C stacking onto Trp-279 and Trp-278, respectively. These interactions are in excellent agreement with those observed for acarbose rings A and D, leading to a less curved conformation of the molecule as compared with maltoheptaose. Consequently, an additional interaction is found between Lys-271 and acarbose (see TABLE THREE, part B).

The Pair of Sugar Tongs—The third substrate-binding site is the AMY1-specific sugar tongs located in domain C (28). At this site, identified in a complex between AMY1 and a thio-maltotetraose substrate analogue, thio-DP4, Tyr-380 shows a shift upon sugar binding, resulting in a movement of the short loop defined by residues surrounding Tyr-380. This substrate binding ability is lacking in the known structures of AMY2, and in part may stem from Pro-376AMY2 which replaces Ser-378AMY1 and thus rigidifies the loop and impedes its movement. Moreover, comparative studies of the tertiary structure of domain C in several α-amylases (28) showed that this domain C-binding site is unique to AMY1-type plant α-amylases. In fact, the presence of two additional β-strands in α-amylases from other species blocks the putative access for the substrate at this site. Moreover, Tyr-380, one of the key residues in the sugar tongs site, is conserved only in plant α-amylases (28).

In the AMY2D180A-maltoheptaose complex, electron density is seen for five of the seven glucose units (labeled Glc3000 to Glc3004, numbering from the nonreducing end to the reducing end), see Fig. 3C. As in AMY1-acarbose, the sugar molecule has the shape of a half-circle with the Tyr-380 side chain pointing into the center. This malto-oligosaccharide is exposed into the solvent, which explains the lack of electron density for two sugar moieties. Direct hydrogen bond interactions are listed in TABLE THREE, part C along with intramolecular hydrogen bonds stabilizing the molecule (TABLE THREE, part D). Moreover, several indirect interactions mediated by water molecules are present (results not shown). The binding of the substrate in this site causes small rearrangements as indicated by shifts of 0.8 and 3 Å for Tyr-380-Cα and Tyr-380-Oh, respectively. Finally, Thr-392 reorients its side chain to interact with Glc3001.

When comparing to the AMY1D180A-acarbose complex, an entire acarbose molecule superimposes quite well with maltoheptaose on glucose units Glc3001, 3002, 3003, and 3004, respectively, with a large number of conserved interactions for the inactive mutant complexes at this site (see TABLE THREE, part C). Ring A in acarbose is not perfectly superimposed with Glc3001 because of the different chemical structure of the valienamine compared with a glucose ring. Ring D is rotated 180° compared with Glc3004, resulting in its hydroxyl group at C6 pointing in the opposite direction of its homologue in Glc3004. Remarkably, the locations of backbone and side chains, including Tyr-380, are fully conserved between the two complexes. Compared with the native structure of AMY1, the shift observed for Tyr-380-Cα and Tyr-380-Oh atoms in the acarbose complex is 1.2 and 3.4 Å, respectively, which is in excellent agreement with those observed in AMY2D180A-maltoheptaose, as well as with those reported for AMY1-thio-DP4 being 1.2 and 3.1 Å, respectively (28).

DISCUSSION

The combination of structural studies of enzyme-substrate and enzyme-inhibitor complexes as revealed by the AMY1D180A-maltoheptaose, AMY1D180A-acarbose, and AMY1-acarbose complexes leads to a structural definition of sites −7 to +2, as summarized in TABLE FOUR. The present study reveals subsite −7, which was not unambiguously determined neither by substrate mapping (25) nor by molecular modeling (26, 27). Accordingly, only subsites +3 and eventually subsite +4 remain to be visualized experimentally by a complex in AMY1 or AMY2. Our results are predominantly consistent with earlier observations from computer-aided modeling studies showing the interaction of a maltodecaose in the active site cleft of AMY2 (26) and AMY1 (27). These modeling studies proposed a hypothetical fork shape of the non-reducing end of the binding area, which should allow the substrate to reach the catalytic site by two distinct ways, or accommodation of α-1,6-branched substrates. Neither is confirmed by the present structures because no electron density corresponding to sugar rings is observed in the putative alternative binding region. The subsite mapping studies show low affinity at subsite −7, but biochemical data can prove the existence of a functional −7 subsite as small amounts of p-nitrophenyl and substantial amounts of glucose are released from PNPG7 (25) and malto-octaose (50), respectively. AMY1 has similar Km values for G7 and G8 (1.9-fold higher for G7) despite the 18-fold lower kcat/Km for G7 compared with G8 (50), which may be explained by nonproductive binding of G7 to subsites −7 to −1 as illustrated here in the AMY1D180A-maltoheptaose complex. Binding modes of G7 and acarbose covering subsites −7 to −1 and −4 to −1, respectively, in AMY1D180A are unexpected, however, from the subsite mapping. The affinity at subsite +2 is obviously higher than at subsites −3 and −4 (25, 50), suggesting acarbose binding to occur in subsites −2 to +2, or otherwise that the nonreducing end valienamine ring that mimics the distorted glucose should be accommodated in subsite −1 as found for the wild-type AMY1-acarbose complex. The actual binding modes therefore must be due to the loss of the nucleophile. In both complexes the glucose moiety at the reducing end is located in subsite −1, is undistorted, and lacks the hydrogen bond between O6 and His-93, as observed in all complexes of the α-amylase family enzymes including including...
the wild-type AMY1-acarbose complex. Asp-180 therefore seems to be essential both for distortion of the glucose ring in subsite −1 and for binding this distorted glucose ring in the appropriate orientation, resulting in substrate binding in a productive mode spanning subsites −1 and +1. In AMY1_D180A, the loss of Asp-180 hence causes lack of distortion and thereby from binding in the productive binding mode.

AMY1 is more efficient than AMY2 in degrading starch granules and has higher affinity for substrates in general. Structural analysis of individual subsites within the substrate binding crevice of the two isoforms does not provide an explanation for this variation. However, the presence of the sugar tongs-binding site in AMY1, so far not observed in AMY2, makes a fundamental difference. This study demonstrates the ability of the sugar tongs surface site to recognize and bind natural substrates, whereas the previous study only showed binding of a substrate analogue (28). It also confirms binding of acarbose or maltoheptaose to the starch granule surface-binding site. The orientation of maltoheptaose molecules on the two surface substrate-binding sites in AMY1 suggests that both of them a priori are independent of each other. First, they are separated by a considerable distance, and it is difficult to imagine a single polysaccharide chain connecting these sites. Second, the reducing ends of oligosaccharides located in these two sites are facing each other if we try to build a link between these sites by the shortest path. The same conclusion is made for connecting molecules at the active site and the surface sites. This is only possible if the polysaccharide chain coils up around the enzyme, which seems highly improbable. AMY1 thus could interact with three distinct sugar chains, as opposed to AMY2 binding only two sugar chains.

The identification of two surface binding sites in AMY1 leads to the question of their role in vivo. Hypothetically, these two sites may allow interaction of the enzyme with amylase and amylpectin molecules in starch. We have shown that the substrates have a salient tendency to circularize themselves when binding to these sites, and if trying to complete this curved sugar chain to obtain a full cycle, it can be shown that β-cyclodextrin (7 glucose rings) and most probably α-cyclodextrin (6 glucose rings) are obtained at the sugar tongs-binding site. At the starch granule-binding site, β-cyclodextrin seems the more probable, in accordance with earlier studies demonstrating that AMY2 can bind β-cyclodextrin (52, 53). This, however, needs experimental confirmation. The structure of amylase is a helicoidally arranged chain, each turn containing 6 glucosyl residues (54). It therefore seems plausible that the two surface sites in AMY1 can interact with amylase in its natural conformation.

Because of the specificity of these two sites, they may locate/orient the enzyme in order to facilitate access to the active site for polysaccharide chains. In addition, the sugar tongs surface site could also disentangle polysaccharide chains, Tyr-380 acting as “molecular tweezers” by their insertion in the helical and/or lamellar structure of starch substrates (55).

These conclusions are supported by comparative studies with other family 13 glycoside hydrolases. For example, the structures of T. vulgaris R-47 α-amylase 1 (TVAl) complexed with malto-oligosaccharides reveal the presence of a domain “N” putatively acting as a starch-binding domain (56). As compared with the α-amylase domains A–C, this extra N-terminal domain N was shown to bind malto-oligosaccharides at two distinct sites, site N and site NA. The first one could interact with the outer surface of the starch helix, mainly through stacking interactions to aromatic residues, whereas the second one holds the saccharide units from both outside and inside of the helix by stacking interactions and hydrogen bonds. These authors suggest that site N is implicated in recognizing the surfaces of rigid helical structure of starch, whereas site NA may recognize the loose helical structure region or contribute to unravel helical starch. Also, specific hydrolytic activity of TVAI compared with that of α-amylase from Aspergillus oryzae (lacking domain N) is around 18-fold higher on raw starch, supporting the crucial role of domain N (56). The architecture of sites N and NA exhibit no similarity to that of the sugar tongs site in AMY1, because the substrate is not captured by a flexible aromatic residue entering its inner curvature. These sites resemble more closely the starch granule-binding surface site in AMY1 and AMY2, notably by the implication of at least one tryptophan residue performing aromatic stacking interactions with substrate. We suggest that the sugar tongs from AMY1 and site NA from TVAl may share a common role, whereas the starch granule-binding site in AMY1/AMY2 and site N could have a similar function.

Structural comparative studies were furthermore performed to cyclomaltodextrin glucanotransferases (CGTase, EC 2.4.1.19), also belonging to the glycoside hydrolase family 13, and having, in addition to the property of hydrolyzing α-1,4-glycosidic bonds, the ability of circularizing oligosaccharides into α-, β-, or γ-cyclodextrins. The crystal structure of the double mutant E257Q/D229N of CGTase from Bacillus circulans (strain 251) in complex with γ-cyclodextrins showed that Tyr-195 in the active site is essential for the circularization mechanism (57). Remarkably, the phenolic ring of this residue is very close to the center of the γ-cyclodextrin, and the binding of the molecule induces an important shift (2.6 Å) of this key residue. The similarity with the AMY1 sugar tongs, however, ends here, as Tyr-195 from the CGTase only makes hydrogen bonds with γ-cyclodextrin but no hydrophobic interactions. CGTases bind α- or β-cyclodextrins on two distinct surface sites located in domain E (58, 59) of the carbohydrate-binding module family 20 (18). Two adjacent tryptophans (Trp-616 and Trp-662) in the CGTase perform aromatic stacking onto two glucosyl units, constituting one of the binding sites of this domain (site 1). Tyr-663, which makes hydrophobic interactions, and Leu-600, which inserts in the cyclodextrin cylinder (59), define the second site. The structure of binding site 1 shares the two tryptophanyl residues with the starch granule surface-binding sites in AMY1 and AMY2, but the CGTase has a very different environment, and the two tryptophans are less stabilized by neighboring residues. The side chains of these two residues possess a high degree of flexibility, allowing the accommodation of both α-, β-, and γ-cyclodextrins in contrast to AMY1 and AMY2 counterparts, which are in a perfectly locked position (20, 28). Evolutionary aspects may explain these common structural features between this CGTase and AMY1, as CGTases derived from α-amylases, keeping their hydrolytic activity and gaining their circularization property by the addition of new domains (60).

C domains of AMY1 and the glucosyltransferase amylosucrase from Neisseria polysaccharea have been compared. Amylosucrase bind substrates to its domain C (61), but with a distinct binding mode and location of the substrate as compared with AMY1. The β-sandwich domain C of amylosucrase does not confer enough space for accommodating the substrate, and the polysaccharide chain is bound onto the side of the domain, implicating among others a hydrophobic interaction with a phenylalanine residue.

Finally, binding modes in a complex between acarbose and amylo maltase from Thermus aquaticus (47) were compared with those reported herein. Amylomaltase is a member of GH 77, which together with GH 13 is a part of the clan H. It catalyzes either the transglycosylation with transfer from one α-1,4-glucan to another or an intramolecular cyclization resulting in much larger cyclodextrins than produced by CGTases. Two acarbose molecules are bound in this structure (47), one in the active site and a second close to the active center at a distance of 14 Å. In this latter site, key interactions determining the conformation and bind-
ing of the inhibitor are the hydrophobic contacts of Tyr-54 and Tyr-101 with unit C from acarbose. The authors propose that Tyr-54 may help in curving the glucan chain, thus favoring synthesis of cyclic products. When leaving the catalytic center, the chain could "wrap around" Tyr-54 before returning to the active site, which appeared consistent with the formation of the smallest cycloamyloses having less than 22 glucan units. Recognition, binding, and circularization schemes of acarbose around Tyr-54 are very close to those observed at the sugar tongs site in AMY1. Because this region of amylo maltase cannot be considered as a separate domain, but as an extension of the active site, the similarities end here. Moreover, the role of this region seems to be circularization of the glucan chain, rather than recognizing such a conformation as we suggest for the AMY1 sugar tongs.

Major conclusions drawn from this comparative study are that recognition and binding modes of both surface sites in AMY1 are common to family members. These binding sites do not have their own catalytic properties but appear to contribute to enhance the activity of the enzyme. AMY1 seems to be the simplest enzyme in terms of the three-dimensional structure that contains two distinct surface binding sites indirectly implicated in the catalytic activity. As an example, CGTase from B. circulans has also two surface sites, but they are located in the extra domain E and are not present in AMY1. Thus, AMY1 appears to be a highly "optimized" enzyme with an excellent compromise between properties but appear to contribute to enhance the activity of the enzyme. AMY1 seems to be the simplest enzyme in terms of the three-dimensional structure that contains two distinct surface binding sites indirectly implicated in the catalytic activity. As an example, CGTase from B. circulans has also two surface sites, but they are located in the extra domain E and are not present in AMY1. Thus, AMY1 appears to be a highly "optimized" enzyme with an excellent compromise between properties but appear to contribute to enhance the activity of the enzyme. AMY1 seems to be the simplest enzyme in terms of the three-dimensional structure that contains two distinct surface binding sites indirectly implicated in the catalytic activity.

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