Comparison of crystal structures of barley α-amylase 1 and 2: implications for isozyme differences in stability and activity

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The germinating barley seed contains two major α-amylase isozyme families AMY1 and AMY2 involved in starch degradation to provide energy used by the plant embryo for growth. The three-dimensional structure of AMY2 has been solved previously, both in the native state and in complex with acarbose, a pseudo-tetrasaccharide acting as a powerful inhibitor for several glycosidases. Furthermore, the endogenous bifunctional barley α-amylase/subtilisin inhibitor (BASI) present in the mature seeds inhibits both proteases from the subtilisin family and AMY2, but not AMY1. The crystal structure of the AMY2-BASI complex has earlier been solved at 1.9 Å resolution. In addition to this difference, the isozymes AMY1 and AMY2 show several distinctly different properties, despite their high sequence identity. AMY1 and AMY2 thus differ in isoelectric point, in affinity for calcium ions, and in stability at acidic pH and elevated temperature. With regard to the enzymatic properties, large variation is found in the activity towards starch granules and in the affinity for soluble substrates. The crystal structure of AMY1 has recently been established at 1.5 Å resolution, which allows a direct comparison of the two isozymes on the basis of the 3D structures. Detailed analysis shows that the structural organization of the isozymes is virtually identical, and that local changes are very small. AMY1 and AMY2 thus constitute a remarkable case of enzymes displaying very similar structures, but important differences in physico-chemical and enzymatic properties. Here, the recently solved crystal structure of AMY1 is compared to that of AMY2, with focus on isozyme differences at areas of interest, i.e. the active site, the starch-granule-binding surface site, the area where BASI is recognized by AMY2, and the three calcium binding sites.

Key words: α-amylase, barley, isozymes, X-ray crystallography, structure comparison.
Introduction

\(\alpha\)-Amylases (1,4-\(\alpha\)-D-glucan glucanohydrolase; EC 3.2.1.1) are monomeric enzymes widely occurring in animals, higher plants, and micro-organisms. Their enzymatic function is to catalyse hydrolysis of internal \(\alpha\)-\(D\)-(1,4)-glucosidic linkages in starch (amylose and amylopectin), and related oligo- and polysaccharides. The resulting maltotriose, maltose and glucose are released with retained \(\alpha\)-amyluric configuration.

In barley seeds, the physiological role of \(\alpha\)-amylases is to provide energy to the growing plantlet. The \textit{de novo} synthesis of the \(\alpha\)-amylases, and more generally of an array of hydrodrolases, is controlled by a complex pathway involving phytohormones and launched by external parameters such as the temperature and humidity. Two different \(\alpha\)-amylase isozymes encoded by two multigene families can be distinguished in the germinating seeds. These isozymes, known as \(\alpha\)-amylase 1 (AMY1) and \(\alpha\)-amylase 2 (AMY2), contain 414 (MW = 45342 Da) and 403 residues (MW = 45005 Da), respectively (ROGERS et al., 1983; ROGERS, 1985a). They show 80% sequence identity, but possess low sequence similarity with \(\alpha\)-amylases from micro-organisms and animals (ROGERS, 1985b).

AMY1 and AMY2 differ in isoelectric points, and are also known as the low pI and high pI isozymes, respectively (JACOBSEN & HIGGINS, 1982). They show significantly different 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. From an enzymatic point of view, large variation is found in activity and affinity towards starch granules (MacGREGOR & Ballance, 1980; MacGREGOR & Morgan, 1986) and soluble substrates (SOGAARD & SVENSSON, 1990; AJANDOUZ et al., 1992). Finally, only AMY2 has the capacity of binding the endogeous bifunctional inhibitor BASI (barley \(\alpha\)-amylase/subtilisin inhibitor) (MUNDY et al., 1983; SVENSDSEN et al., 1986; LEAH & MUNDY, 1989).

The high affinity of AMY2 for BASI is indicated by a \(K_d\) of \(2.2 \times 10^{-10}\) M as determined at pH 8 and 37°C (AHE et al., 1993; SIDDENIUS et al., 1995), whereas no interaction has been identified between BASI and AMY1. Both structural and biochemical data explain these differences (VALLEE et al., 1998; Rodenburg et al., 2000).

Recently, the three dimensional structure of AMY1 has been determined to 1.5 Å resolution (this manuscript and details in ROBERT, X., HASER, R., GOTTSCHALK, T. E., DRIGUEZ, H., SVENSSON, B. & AGHAJARI, N., to be submitted). The structure of AMY2 has previously been solved to 2.8 Å resolution (KADZIOŁA et al., 1994). Here, the native AMY1 structure is briefly described and compared with that of AMY2.

Material and methods

Crystallization of full-length and truncated AMY1

AMY1 (full-length) was first crystallized as previously described (ROBERT et al., 2002) and only two-dimensional, ultra-thin plate-form crystals were obtained. These were mechanically unstable due to their morphology and thus very difficult to handle. Since the failure in obtaining three-dimensional crystals of AMY1 is thought to be due to a flexible C-terminus as compared to AMY2, a C-terminal truncated form of AMY1, henceforward referred to as AMY1\(\Delta 9\) (ROBERT et al., 2002) was used afterwards. Crystals suitable for X-ray diffraction studies were obtained after approximately one month in PEG 8000 with 2-methyl-2,4-pentanediol (MPD) as additive and 3 weeks using 2-propanol as additive, as previously described (ROBERT et al., 2002). Crystals leading to the high resolution structure of AMY1\(\Delta 9\) were obtained under the latter conditions.

Data collection, structure solution, refinement

Crystals of full-length AMY1 as well as AMY1\(\Delta 9\) belong to the orthorhombic space group P2\(_1\)2\(_1\)2\(_1\). As earlier described (ROBERT et al., 2002) the molecular replacement method using the AMoRe software (Navaza, 2001), as implemented in the CCP4 suite (CCP4 COLLABORATIVE COMPUTATIONAL PROJECT, 1994), was used to solve the phase problem of the full length, as well as the AMY1\(\Delta 9\), form. The 3D structure of AMY2 (KADZIOŁA et al., 1994), without the bound calcium ions and water molecules, was used as a search model. Refinement of the models, alternating with manual building, were done using the simulated annealing routine as implemented in the software CNS version 1.0 (BRUNGER et al., 1998). The final R factor for the high resolution AMY1\(\Delta 9\) structure was 13.6% with an R\(_\text{free}\) of 16.3%.

Sequence and structure alignment

The sequence alignment showing the superimposition of secondary structures and domains between AMY1 and AMY2 was rendered using the program ESPript (GOUET et al., 1999). Structural superimposition of AMY1 and AMY2 C\(_\alpha\) backbones was performed with the “rigid body fit” option in TURBO-FRODO (ROUSSEL & CAMBILLAU, 1989). Figures showing overall and detailed structures were generated with the programs TURBO-FRODO (ROUSSEL & CAMBILLAU, 1989), GRASP (NICHOLLS et al., 1991), and VIEWERLITE (freeware from Accelrys Inc., San Diego, USA – http://www.accelrys.com).
Accession number
The coordinates and structure factors of the AMY1Δ9 crystal structure solved to 1.5 Å resolution have been deposited at the RCSB (Research Collaboratory for Structural Bioinformatics) Protein Data Bank (PDB – http://www.rcsb.org) under the entry code 1HT6. The coordinates of the native AMY2 structure used in this comparative study are present in the PDB under the accession number 1AMY.

Results

Overall structure
The three-dimensional structure of AMY1Δ9 consists of 404 amino-acid residues, and all side-chains are clearly identified in the electron density map. It turned out that quite a large number of amino acid residues (16) throughout the entire polypeptide display double conformations (Robert, X., Haser, R., Gottschalk, T.E., Driguez, H., Svensson, B., & Aghajani, N., to be submitted). The structure confirms the sequence correction to AMY1 (as deposited in SWISS-PROT, with entry name AMY1_HORVU and primary accession number P00693): Ala308 in AMY1 (2.4 Å deviation between the summit of these loops can be explained by an important sequence difference at the beginning of this loop (sequence IDPG in AMY1, RGTD in AMY2), with a proline (Pro268) being present only in AMY1. Due to its rigid nature, this residue can promote a slightly different local backbone folding in AMY1 compared to AMY2. Secondly, a significant deviation in the backbone was observed in the region located between Val206 and Pro218 (AMY1 numbering), which forms a large loop between β2 and α5, and protrudes from the barrel. The maximum deviation between Cα's of AMY1 and AMY2 for this loop is 3.4 Å and is located at the level of the Gly214AMY1 residue (Gly213AMY2). This shift can be explained by the fact that the neighbouring region of this residue in AMY2 is involved in crystal contacts (residue 209 to 214 in AMY2 numbering) as well as in AMY1 (residue 212 to 216) – see sequence alignment Figure 2. Nevertheless, due to different crystal packings, symmetry-related molecules involved in inter-molecular contacts differ in AMY1 and AMY2, in terms of number and strength of interactions. In AMY2, inter-molecular interactions are Tyr211-N/Asp113-O#2 (2.9 Å) and Gly213-N/Asp360-O#4 (3.1 Å) (Kadziola et al., 1994) where #2 and #4 are symmetry related molecules in AMY2. In AMY1, inter-molecular interactions are: Thr212-Oγ1/Arg156-Np1#4 (3.3 Å), Gly214-O/Asp118-O#4 (3.3 Å), Asp215-N/Asp118-O#4 (3.3 Å) and Gly216-N/Arg156Ne#4 (4 Å) where #4 is a symmetry related molecule in AMY1.
Fig. 1. Superimposition of AMY1 on AMY2. AMY1 and AMY2 in backbone presentations are shown in red and blue, respectively. The three calcium ions (presented by green spheres) are perfectly superimposable in the two structures.

Fig. 2. Sequence alignment of barley AMY1 and AMY2, calculated with the program CLUSTALW (THOMPSON et al., 1994) and presented using the program ESPript (GOUET et al., 1999). Identical residues are highlighted in orange. The secondary structure annotation is indicated over the alignment, coloured in blue, green and red for domains A, B and C respectively. Catalytic residues are highlighted in blue, whereas AMY2 residues involved in the interaction with BASI are highlighted in red. The asterisks indicate residues interacting with calcium ions and are coloured as a function of length of interaction (red, distances less than 3.2 Å and black, distances between 3.2 and 4.0 Å). Finally, for both enzymes, residues involved in crystallographic contacts are indicated with an “a”, the colour code being the same as for the calcium ligand distances.
Table 1. Calcium-ion-binding sites in AMY1 and AMY2.

<table>
<thead>
<tr>
<th>Calcium ions</th>
<th>Ligands in AMY1</th>
<th>Distances in AMY1 (Å)</th>
<th>Ligands in AMY2</th>
<th>Distances in AMY2 (Å)</th>
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<td>Wat788</td>
<td>2.30</td>
<td>a</td>
<td>a</td>
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</table>

*An electron density which could correspond to a water molecule with low occupancy was reported (KADZIOLA et al., 1994).*

Thirdly, a deviation occurs in the vicinity of Gly243 (β1), one of the two residues inserted in AMY1 compared to AMY2 (Fig. 2). Interestingly, this insertion does not cause major structural changes in β1, and from Val245 the backbones of both isozymes are perfectly superimposable. Also, the secondary structures are perfectly conserved around Gly243.

**Calcium binding sites**

Both AMY1 and AMY2 (KADZIOLA et al., 1994) structures contain three Ca$^{2+}$, two of which have ligands only from domain B (Ca501 and Ca502). The third so-called conserved calcium ion is found in all other α-amylase 3D structures (Ca500), and 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). Calcium ions appear to be essential for proper folding, conformational stability and therefore for activity (BERTOFT et al., 1984; JONES & JACOBSEN, 1991).

Superimposition of the structure of AMY1 on AMY2 demonstrates both the perfect spatial identity of the three calcium ions and of all the amino-acid and water ligands. These three calcium-ion-binding sites of AMY1 are presented in Figure 3. The only structural difference seen between the isozymes, when comparing the calcium binding sites, is the presence of an extra water molecule (Wat788) as a ligand in AMY1 for Ca502, but it should be mentioned that a water molecule with low occupancy was seen in the Fo-Fc electron density map of AMY2, but not included in the refinement (KADZIOLA et al., 1994). In AMY1, Ca500 has 7 interactions with its ligands, one of these being a water molecule (Wat630). This hepta-coordination forms a distorted pentagonal bipyramid and is thus similar but not identical to the homologous site in other α-amylases, which all are octa-coordinated. Ca501 has 8 ligands including 2 water molecules (Wat604 and Wat656) and finally, Ca502 is hepta-coordinated including 1 water molecule (Wat788) as ligand, determining a pentagonal bipyramid geometry. Interactions between calcium ions and their ligands, both in AMY1 and AMY2, are summarized in Table 1. Two of the calcium ions (Ca500 and Ca502) are...
Fig. 3. Calcium binding sites in AMY1. Ca$^{2+}$ ligand interactions are indicated as dot-and-dash lines. (a) is an overall view of the Ca500 and Ca502 binding sites for which Asp 149 is a ligand in both cases. (b) is a view of the Ca501 binding site.

Fig. 4. Close-up of the active site of AMY2 in complex with the acarbose inhibitor (KADZIOŁA et al., 1998) showing catalytic residues (labeled in red) along with the remaining substrate-binding residues. Direct hydrogen-bond interactions are shown by dot-and-dash lines. The three sugar rings of acarbose seen in the structure (coloured in yellow) are located in subsites $-1$, $+1$ and $+2$.

very close to each other, and share a ligand (Asp 149). The third calcium ion (Ca500) is located about 20 Å from the two others. All amino acid residues interacting with these calcium ions belong to domain B, except Gly184, which is located in loop $\beta_4\alpha_4$ of domain A.
Table 2. Active site residues in AMY2 and the corresponding residues in AMY1.

<table>
<thead>
<tr>
<th>Residues of AMY2 involved in the active site</th>
<th>Corresponding residues on AMY1</th>
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<tr>
<td>Tyr51 O</td>
<td>Tyr52 O</td>
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<tr>
<td>His92 Ne1</td>
<td>His93 Ne1</td>
</tr>
<tr>
<td>Arg177 Nγ1</td>
<td>Arg178 Nγ1</td>
</tr>
<tr>
<td>Asp179 O2</td>
<td>Asp180 O2</td>
</tr>
<tr>
<td>Lys182 Nc</td>
<td>Arg183</td>
</tr>
<tr>
<td>Glu204 Oe2</td>
<td>Glu205 Oe2</td>
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<tr>
<td>Glu204 Oe1</td>
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<td>Asp291 Oε1</td>
</tr>
<tr>
<td>Asp289 Oε2</td>
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</tr>
</tbody>
</table>

a The trio of catalytic residues is presented in bold. Non-conserved residues between AMY1 and AMY2 are underlined.

Active site

The active site of AMY2, identified in the crystal structure of a complex between the enzyme and acarbose (a pseudotetrasaccharide resembling a transition-state analogue) (KADZIOLA et al., 1998), appears to be a large V-shaped depression (KADZIOLA et al., 1998a) (see Fig. 4). When examining the structures, it seems that the AMY1 Arg183, despite its difference in orientation at this position, does not represent a drastic difference since the residues are of approximately the same length and possess nitrogen atoms capable of forming a hydrogen bond with an oxygen in acarbose or a sugar substrate. A similar conclusion can be drawn for the couple Ser208/Asn209 (AMY2/AMY1 numbering). In the AMY2/ acarbose complex, one of the hydrogen bonds between Ser208 and aca1-O2C at subsite +2 (KADZIOLA et al., 1998) involves the nitrogen of the peptide bond (see Fig. 4). AMY1 has this atom at the same location, due to the almost perfect overlay of the backbone in this region. A second hydrogen bond is formed by Ser208 Oγ2. Due to the comparable lengths of Ser and Asn and to the presence of an oxygen atom in both side chains, it seems possible to establish the equivalent hydrogen bond in AMY1-substrate and/or substrate analogue complexes.

In the AMY2/ acarbose complex (KADZIOLA et al., 1998), a chain of five water molecules was observed running from the active site cleft, towards the interior of the protein. The first water molecule in this chain, Wat607, bridges the carboxylate groups of Glu204 and Asp289, two of the three catalytic residues. This water has been proposed earlier as candidate for participation in the enzymatic process as the first catalytic water molecule in hydrolysis of substrates (KADZIOLA et al., 1994; AGHAJARI et al., 1998b). The presence of these five water molecules, defining a water pocket, is not correlated with binding of a substrate, a substrate analogue like acarbose or of the bifunctional α-amylase/subtilisin inhibitor (BASI). Indeed, these water molecules were found in the native AMY2 structure (KADZIOLA et al., 1994) and in the structure at 1.9 Å resolution of the AMY2/BASI complex (VALLEE et al., 1998), whereas a like chain of buried water molecules linked to a chloride effector ion was observed in the psychrophilic α-amylase, AHA (AGHAJARI et al., 1998a). The 3D structure of AMY1 has a water pocket similar to the one found in AMY2.

The starch granule-binding surface site

In AMY2, two contiguous tryptophan residues, Trp276 and 277 (Trp278 and 279 in AMY1) constitute the so-called starch-granule-binding site (GISSON & SVENSSON, 1987) on the surface of the enzyme (KADZIOLA et al., 1994). This site (Figs 5a, b), which is independent from the active site, was previously confirmed by UV difference spectroscopy (GISSON & SVENSSON, 1987; SOGAARD et al., 1993b). Its low binding affinity for acarbose and β-cyclodextrin, which competes with starch granules (WESELAKE & HILL, 1983; SOGAARD et al., 1993b), suggests a role in binding of the enzyme to starch in vivo. This site has been proven also by X-ray crystallography in the AMY2/acarbose complex, solved to 2.8 Å resolution (KADZIOLA et al., 1998) in which a disaccharide unit was stacking to the indole rings of the two tryptophans.
Fig. 5. Close-up on the starch-granule-binding surface site in AMY2 (a) and AMY1 (b) showing the two adjacent tryptophan residues. The electron density 2Fo-Fc map contoured at 1σ is shown in both cases, for which the resolutions are 2.8 Å and 1.5 Å, respectively.

Fig. 6. Overall view of AMY1 and AMY2 surfaces coloured as a function of charges where positive and negative charges are presented in blue and red, respectively. The active site and the BASI recognition area of AMY2, and its corresponding area in AMY1, are highlighted in this figure, where domain B occupies the upper part of the molecule, and domain C, the lower part. Representations for AMY1 as well as AMY2 are generated on the same scale with the program GRASP (Nicholls et al., 1991).

Trp278 and Trp279 in AMY1 superimpose remarkably well with the AMY2 counterparts. Each aromatic side-chain defines a plane and the angle between the two planes was determined to be about 135°. This angle is preserved in AMY1 and AMY2 in the native state and in the AMY2 complex with acarbose. The conservation of the spatial position and orientation of the two residues can be explained in that tryptophans are large bulky residues, and that these particular tryptophans are locked by the neighbouring residues: Trp278 in AMY1 is situated next to Arg226 (Arg225 in
AMY2) and Trp279 next to Lys282 (Lys280 in AMY2). These basic residues, which are held in a fixed position by several hydrogen bonds and have little space for moving, lock the position of the tryptophans and thus this local structure is perfectly conserved between AMY1 and AMY2.

The interface of the protein-protein complex AMY2-BASI and its counterpart in AMY1 BASI belongs to the soybean trypsin inhibitor family and inhibits AMY2, but not AMY1. The crystal structure of AMY2–BASI was determined at 1.9 Å resolution (Valée et al., 1998), in which BASI contains a 12-stranded β-barrel structure and inhibits AMY2 by sterically occluding access of substrate to the active site. The total buried surface area in AMY2-BASI is 2355 Å² and comprises three interacting regions situated in the neighborhood of the catalytic site of AMY2. BASI, however, does not interact directly with the three catalytic residues, Asp179Amy2, Glu204Amy2 and Asp289Amy2 (Valée et al., 1998).

Remarkably, a large cavity, in which a solvated calcium ion is trapped, is created at the AMY2-BASI binding interface and stabilized by six water molecules in a trigonal bipyramidal geometry. Five water molecules make hydrogen bonds to the three catalytic residues (Asp179, Glu204 and Asp289) and to two oxygen atoms of Glu168 and Tyr170 from BASI (Tab. 3). Six of eight residues interacting with BASI by direct hydrogen bonds are changed in AMY1 (Tab. 3). Six of eight residues interacting with BASI by direct hydrogen bonds are changed in AMY1 (Tab. 3).

Examination of the surfaces of AMY1 and AMY2 (Fig. 6) colored as a function of charge shows that the BASI recognition area in AMY2 is mainly negatively charged, and that the corresponding region in AMY1 displays a notably wider negatively-charged area. The structural superimposition of the two native isoforms and the examination of residues involved in direct contacts with BASI in AMY2 show that these residues and their counterparts in AMY1 can be classified in two categories (Tab. 3): 1) residues not necessarily conserved between AMY1 and AMY2, but which are capable of making the same interactions in case a complex between AMY1 and BASI could be established. These residues include: Asp143Amy1, which is perfectly superimposed onto Asp142Amy2: the peptide bond nitrogen atom of Ala145-Namy1, superimposable with Gly144-Namy2; Thr212-Namy1 superimposable on Tyr211-Oamy2; Gly216-Oamy1 and Gly215-Oamy2, where the position of the peptide bond oxygen atom is conserved, and finally, Ala297-Oamy1 and His295-Oamy2 with the same kind of conservation; 2) residues that display drastic differences between the two isoforms: Thr129Amy1, not only has a shorter side chain com-

<table>
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<th>AMY2 residues</th>
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<th>Corresponding residues in AMY1</th>
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<td>Gly215 O</td>
<td>Trp162 Nε1</td>
<td>Gly216 O</td>
</tr>
<tr>
<td>Gln223 Oε1</td>
<td>Asn26 Nδ2</td>
<td>Ala224</td>
</tr>
<tr>
<td>His295 O</td>
<td>Arg106 Nε</td>
<td>Ala297 O</td>
</tr>
<tr>
<td>His295 O</td>
<td>Arg106 Nq1</td>
<td>Ala297 O</td>
</tr>
</tbody>
</table>

* Non-conserved residues between AMY1 and AMY2 are presented in bold.
pared to its counterpart in AMY2 (Arg128\textsubscript{AMY2}), but also has different charge properties; Ala224\textsubscript{AMY1} compared to Gln223\textsubscript{AMY2} is a hydrophobic, uncharged residue that is unable to interact with Asn26\textsubscript{BASI}.

Finally Arg183\textsubscript{AMY1}/Lys182\textsubscript{AMY2} can be considered as having comparable charges and side-chain lengths. One can therefore not conclude that this mutation is a severe hindrance to the creation of an interaction with Glu168\textsubscript{OE1BASI} as observed in the AMY2/BASI complex.

A part of the AMY2/BASI complex formation is certainly the solvated calcium ion found at the interface between the two proteins. This calcium only has water molecules as ligands, five of which are interacting with the three catalytic residues of AMY2. The fact that this calcium ion was not found in the native structure of AMY1 is far from being understood, in that the catalytic residues as well as the environments for the calcium ion in both isozymes superimpose perfectly. Furthermore, it is presently difficult to reach a conclusion on the exact role of this calcium in the non-inhibition of AMY1 by BASI.

**Discussion**

The three-dimensional structure of barley AMY1 is the second structure of a plant α-amylase solved to date, the first being that of AMY2 (KADZIOŁA et al., 1994). These two isozymes display conspicuous biochemical, biophysical and enzymatic differences. The crystal structure of AMY1 described here represents the first step in a study aiming at an improved understanding of these dissimilarities. It seems, however, difficult to explain any of these differences based on the two native structures. Indeed, the three-dimensional structures of AMY1 and AMY2 are virtually the same, with the three domains A, B and C of the two structures perfectly superimposable (Fig. 1), with a few variations occurring in specific loops. However, the spatial location of these loops makes it unlikely that the observed structural deviations are responsible for such differences in biochemical properties. For example, the AMY1 and AMY2 contain three calcium ions with exactly the same environment in both isozymes; however they display significantly different calcium affinities.

As concerns the differences in stability at acidic pH and at elevated temperature, they might be related to subtle structural particularities as is generally the case for proteins of different thermostability. Thus a thorough structure comparison including surface electrostatic potential and dynamic studies is required to address such questions.

As described above, the active sites of AMY1 and AMY2 display some differences, but the catalytic machinery seems to be identical. However, on the basis of the native structure of AMY1 and AMY2, it remains difficult to understand the large differences in activity and affinity for starch granules (MACGREGOR & BALLANCE, 1980; MacGREGOR & MORGAN, 1986) and affinity for soluble substrates (SOGAARD & SVENSSON, 1990; AJANDOUZ et al., 1992). The affinity for these substrates is dictated by the availability, the number and the structure of the subsites. The AMY2/αcarbose complex (KADZIOŁA et al., 1998) (Fig. 4) shows occupancy in the three subsites −1, +1 and +2 (nomenclature according to DAVIES et al., 1997). Moreover, it has been found by enzymatic subsite mapping (AJANDOUZ et al., 1992) and predicted by computer-aided modelling (ANDRE et al., 1999), that both AMY2 and AMY1 possess 10 subsites, namely −6 through −1 towards the non-reducing end from the site of cleavage and subsites +1 through +4 towards the reducing end of the substrate.

A detailed structural comparison of all subsites in AMY1 and AMY2 appears to be an essential approach for understanding the differences in enzymatic properties. This, however, requires three-dimensional structures at high resolution of various enzyme/oligo-saccharide complexes. Such studies are under way.

The study of barley α-amylase isozymes AMY1 and AMY2 represents a very interesting case of the collaborative work between structural determination/analysis, biochemistry and molecular biology. This couple is a still unique example of enzymes with very close primary and tertiary structure and with such different biochemical and biophysical properties. The explanation for these differences implies an important work based on structural analyses combined with rational protein engineering experiments in order to understand the activity and the specificity of both isozymes. Finally, AMY1 and AMY2 are two major candidates in bio-engineering with focus on industrial and bio-technological applications using genetically modified and improved barley α-amylases.

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