Amylosucrase, a Glucan-synthesizing Enzyme from the α-Amylase Family*

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Amylosucrase (E.C. 2.4.1.4) is a member of Family 13 of the glycoside hydrolases (the α-amylases), although its
biological function is the synthesis of amylase-like polymers from sucrose. The structure of amylosucrase from
Neisseria polysaccharea is divided into five domains: an all helical N-terminal domain that is not similar to any
known fold, a (β/α)8-barrel A-domain, B- and B′-domains displaying α/β-structure, and a C-terminal eight-
stranded β-sheet domain. In contrast to other Family 13 hydrolases that have the active site in the bottom of a
large cleft, the active site of amylosucrase is at the bottom of a pocket at the molecular surface. A substrate
binding site resembling the amylase 2 subsite is not found in amylosucrase. The site is blocked by a salt
bridge between residues in the second and eight loops of the (β/α)8-barrel. The result is an exo-acting enzyme.
Loop 7 in the amylosucrase barrel is prolonged compared with the loop structure found in other hydrolases,
and this insertion (forming domain B′) is suggested to be important for the polymer synthase activity of the
enzyme. The topology of the B′-domain creates an active site entrance with several ravines in the molecular
surface that could be used specifically by the substrates/products (sucrose, glucon polymer, and fructose) that
have to get in and out of the active site pocket.

Amylosucrase (AS) is a hexosyltransferase (E.C. 2.4.1.4) produced by non-pathogenic bacteria from the Neisseria genus
and was identified in N. perflava as early as 1946 (1). MacKenzie et al. (2) identified intracellular AS in six other Neisseriae
species, and later an extracellular N. polysaccharea AS was discovered (3). N. polysaccharea was isolated from the throats
of healthy children, and it was suggested that the function of the secreted glucansucrase AS was to produce insoluble polymers.
Until recently AS has only been found in bacteria from the Neisseria genus, but the Deinococcus radiodurans genome
(4) and the Caulobacter crescentus genome (5) actually encodes proteins with a similar length that are 43 and 34%,
respectively, identical to AS from N. polysaccharea.

In the presence of an activator polymer (e.g. glycogen), AS catalyzes the synthesis of an amylase-like polysaccharide composed
of only α-(1→4)-glucosidic linkages using sucrose as the only energy source (6). This glycogen pathway is not found in
e.g. Escherichia coli, which like most bacteria require activated α-D-glucosyl-nucleoside-diphosphate substrates for polysaccharide
synthesis (7). The utilization of a readily available substrate makes AS a potentially very useful glucosylation tool for
the production of novel amylopolysaccharides.

The recent cloning of N. polysaccharea AS in E. coli (8, 9) has made mutational (10) and detailed kinetic studies of highly
purified enzyme possible (11, 12). It has also provided AS in sufficient amounts for successful crystallization experiments
(13). The recombinant AS is derived from a glutathione S-transferase fusion protein and consists of a single polypeptide
chain with 636 amino acid residues including 6 cysteines and 15 methionines.

Based on amino acid sequence comparisons, AS has been suggested to belong to the α-amylase superfamily, the α-retaining
glycoside hydrolase (GH) Family 13 (14). Putative active site residues in the predicted (β/α)8-barrel have also been
pointed out (9). Most members of this family hydrolyze α-(1→4) and α-(1→6)-glucosidic linkages of starch.

The α-amylase reaction mechanism is a general acid catalysis, similar to all of the glucose hydrolases (16), and the same
mechanistic scheme can also accommodate glucan synthesis from sucrose as shown in Scheme 1. The reaction is initiated by
simultaneous protonation of the glycosidic bond by a proton donor and a nucleophilic attack on the anomeric carbon of
the glucose moiety. This leads to the covalently linked substrate-enzyme intermediate. The intermediate can react with either
water or with another saccharide molecule, as shown in the scheme. This implicates that the ratio between hydrolysis and
transglycosylation is determined only by the relative concentrations of water and sugar moieties in the active site.

Consistent with Scheme 1, AS catalyzes both sucrose hydrolysis and oligosaccharide and polymer synthesis in the absence
of an activator polymer (11). With 10 mM sucrose as the sole substrate, AS produces glucose (30%), maltose (29%), maltotriose
(18%), turanose (11%), and insoluble polymer (12%).

Apart from AS, the GH Family 13 comprises other enzymes with non-hydrolytic functions. The crystal structure of a cyclo-
the understanding of product profile and substrate specific-

substrate analogue complexes is well suited to provide a basis
for transglycosylation.

oryzae (18) (TAKA-amylase, the first 

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-amylose from Aspergillus oryzae (18) (TAKA-amylase, the first -amylose from Aspergillus oryzae (18) (TAKA-amylase, the first -amylose from Aspergillus oryzae (18) (TAKA-amylase, the first -amylose from Aspergillus oryzae (18) (TAKA-amylase, the first a-amylase structure determined) and thus implicated very similar reaction mecha-
nisms at least for the formation of the covalent intermediate.
The presence of a covalent intermediate has been verified ex-
nperimentally in CGTases (19).

These findings all suggest that the active site of AS is highly
similar to those of the -amylases and CGTases. Structure
determinations of complexes with substrate analogues have
yielded detailed information on the -amylase structure/function
relationships. A stringent nomenclature for enzyme-sub-
strate interactions has been developed, and substrate binding
is usually described in terms of numbered sugar binding sub-
sites (20). The catalytic residues are then located between the
sugar binding subsites -1 and +1, when numbering the poly-
saccharide from the reducing end. In this work, we present
the crystal structure of AS at a resolution of 1.4 Å, which
represents the first crystal structure of a glucansucrase and is
the first structure of a glucan-elongating enzyme from the GH
13 family. The structural alignment of AS and -amylase-
substrate analogue complexes is well suited to provide a basis
for the understanding of product profile and substrate specific-
ity observed for AS.

EXPERIMENTAL PROCEDURES

Crystallization—Expression and purification of recombinant AS was
performed as described previously (9, 12). The production of Se-Met AS
and the crystallization conditions (equal amounts of 4 mg/ml protein
solution (150 mM NaCl, 50 mM Tris-HCl, pH 7.0, 1 mM EDTA, and 1 mM
α-dithiothreitol) and reservoir solution (30% polyethylene glycol Mr 6000 and 0.1 M HEPS, pH 7.0) have been published (13).

Data Collection, Structure Determination, and Refinement—All data
were collected at the ESRF, Grenoble and were processed and scaled
using DENZO and SCALEPACK (21). Multiple wavelength anomalous
dispersion (MAD) data were collected at beamline BM 14. Data were
collected using energies corresponding to the inflection point and peak
of the experimentally determined selenium K edge and a remote high
energy wavelength (Table I). All 15 selenium sites were identified when
the MAD data were analyzed by the SOLVE program (22). Phases were
extended to 1.7 Å using DM (23), and the structure was built with the
automated tracing procedure AR/wARP (24). This tracing located 625
of the total 628 amino acid residues found in the structure. Later a 1.4
Å native data set was obtained at beamline ID 14 EH 1 (Å = 0.934 Å),
and the structure was refined at this level of resolution. Further re-
building was done in program O (25), and refinement was performed
with the CNS program package (26). A total of 628 amino acid residues,
one Tris molecule, one HEPS molecule, a sodium ion, and 751 water
molecules were included in the final model. Refinement statistics are
listed in Table I. Several patches of elongated electron density that
could arise from a polyethylene glycol molecule were not fitted. Thirty-
one of the side chains were fitted with two conformations, and for
sixteen surface side chains some of the outermost atoms displayed high
B-factors. A polymerase chain reaction error was detected by the
sequence of the recombinant DNA identified the residue as an Asp. The
stereochemistry of the final model was analyzed by PROCHECK (27):
91% of the residues were found to lie in the most favorable regions of the
Ramachandran plot and 8.6% in the additional allowed regions. Only
two residues (Glu544 and Phe550) were found in a generously allowed
region. A schematic representation of the enzyme is shown in Fig. 1.
The overall B-factor for the protein is 16.4 Å², whereas it is 15.9 Å²
and 16.5 Å² for the main chain and the side chain atoms, respectively. The
B-factors of the Ca atoms are plotted in Fig. 2. The active site is shown
in Fig. 3 as an example of the quality of the 2Fo-Fc electron density.

RESULTS AND DISCUSSION

Description of the Structure—The single polypeptide chain
(628 amino acid residues) is folded into a tertiary structure
with five domains named N, A, B, B', and C (Fig. 1). Residues
1–90 comprise the all -helical N-domain. It contains six am-
phiphilic helices that we have chosen to name nh1 to nh6. The
helices consist of the residues Pro7-Leu137, Thr26-Thr127, Ser26-
Ser51, Pro51-Gly68, Leu68-Arg95, and Ser95-Asn102 as defined by
the Kabsch-Sander algorithm (28) in the program PRO-
CHECK (27). No known structures or domains were found to be
similar to the AS N-domain in a database search with the DALI
(29) server. Two helices from the N-domain (nh4 and nh5) are
packed against two helices (h3 and h4) from the central (h/α)8-
barrel (domain A) forming a four-helix bundle. The interface
between the helices in the bundle is almost entirely hydropho-
bic, and no solvent molecules are located in the interface.

Domain A (residues 98–184, 261–395, and 461–550) is made up of eight alternating -sheets (ε1-ε8) and a-helices (h1-h8)
giving the catalytic core (the well characterized (h/α)8-barrel)
common to the GH Family 13 (Figs. 1 and 4). A characteristic of the (h/α)8-barrel enzymes is that the loop region connecting
strands to helices (labeled loop1 to loop8) are much longer on
average than those connecting helices to strands. In particular
AS has two loops (loop3 and loop4) flanked by two a-helices (residues 193–201 and
A B-domain is found in many a-amylases. In TAKA-amylase the main structural feature is a short three-stranded antiparallel $\beta$-sheet. There are also a-amylases that do not have a B domain. For example, barley a-amylase (30) has a short hairpin at this position.

Domain B (residues 395–460) starts with two $\alpha$-helices (residues 400–407 and 410–422) (Figs. 1 and 4). They are followed by a short $\beta$-sheet (residues 433–436 and 446–449) where the strands are separated by a hairpin-like stretch of amino acid residues. A short $\alpha$-helix (residues 451–456) terminates the domain. The domain starts immediately after two catalytically important residues (His$^{392}$ and Asp$^{393}$, see below) found in all related enzymes.

Domain C is an eight-stranded $\beta$-sandwich found C-terminal to the ($\beta/\alpha$)$_5$-barrel (residues 555–628). A C domain is found in other a-amylases, for example TAKA-amylase and barley a-amylase. Several of these domains are found in the CGTases, but so far the functional role of the C domain is unknown.

Although the complete AS sequence contains six cysteine residues no disulfide bridges are found in the structure. Some of the cysteines are exposed on the surface, but no tendency to multimerization has been reported.

The $C_a$ displacement parameters (B-factors) are plotted in Fig. 2. The enzyme displays low overall thermal vibration with a mean B-factor for all atoms of 16.4 Å$^2$. The plot shows that especially the region 250–400 (from the start of $h_3$ to the beginning of the B$_9$ domain) has low displacement parameters and that the regions of the molecule with the highest displacement parameters are localized far from the substrate binding pocket.

The Ca displacement parameters (B-factors) are plotted in Fig. 2. The enzyme displays low overall thermal vibration with a mean B-factor for all atoms of 16.4 Å$^2$. The plot shows that especially the region 250–400 (from the start of $h_3$ to the beginning of the B$_9$ domain) has low displacement parameters and that the regions of the molecule with the highest displacement parameters are localized far from the substrate binding pocket.

**Relation to Family 13 Enzymes**—A comparison of the full-length enzyme with known protein structures using the DALI server (29) showed that the glycoside hydrolyase Family 13 exo-acting enzyme oligo-1,6-glucosidase (31) had the highest similarity to AS. A total of 458 Ca atoms could be superimposed with an rms of 2.7 Å. The superimposable residues are almost all found in the A and C domains. The structural similarity to
Family 13 α-amylases is also high. In particular, the TAKA-amylase-acarbose complex (32) had 368 superimposable Cα atoms with a rms of 2.8 Å. A structural-based sequence alignment between TAKA-amylase, AS, and oligo-1,6-glucosidase starting after the unique N-domain is shown in Fig. 4. The boxed sequence patches represent regions of genuine structural similarity. Because of the high structural similarity the alignment can be used to propose AS-substrate interactions from enzyme-substrate investigations performed on related enzymes.

Active Site Architecture—The general acid residue Glu328 and the nucleophile Asp286 have been identified using conventional sequence alignment (9) and mutational studies (10). These results are supported by the structural alignment found in Fig. 4, which shows that the Cα positions of the two residues coincides with catalytic residues from both TAKA-amylase and oligo-1,6-glucosidase. Asp286 and Glu328 are found at the tips of β-sheets 4 and 5 in the (β/α)8-barrel of AS (Fig. 1), as required for Family 13 members. The distance between Asp286 Ca and Glu328 Co is 5.4 Å in accordance with AS being an α-retaining enzyme (14). A Tris molecule is bound at the active site (Fig. 3) with a short hydrogen bond (2.6 Å) between Oδ2 of Asp286 and Oδ1 of the Tris molecule. Tris has previously been found to be a very good probe for the active site of α-amylases (33).

The TAKA-amylase-acarbose complex (32) has identified a number of enzyme-substrate active site interactions. Around the −1 subsite the following interactions are reported (conserved residues at an equivalent position in AS given in parentheses): The nucleophile Asp206 Oδ2 (Asp286) is forming a hydrogen bond to the O6 of the I-ring of the modified acarbose (Fig. 5). His122 (His187) also forms a hydrogen bond to O6I. Arg204 (Arg284) forms a salt-bridge to the Oδ1 of the nucleophile and is very important for the correct positioning of the nucleophile. Arg204 (Arg284) has an additional weak hydrogen bond to O′1. Glu230 (Glu328) is the general acid/base. It forms a hydrogen bond to the acarbose N. Asp297 (Asp393) Oδ1 and Oδ2 forms hydrogen bonds to O2′ and O3′ respectively. Tyr82 (Tyr147) provides an important stacking platform for the substrate ring at the −1 position. Finally His92 (His296) forms a short hydrogen bond to the hydroxyl O of Tyr22 (Tyr147) an interaction suggested to be pivotal for the positioning of the stacking platform (32). All of these residues can be found at identical Cα positions in TAKA-amylase, AS, and oligo-1,6-glucosidase (Figs. 4 and 5). As seen in Fig. 5, the side chains of these residues are found in identical spatial positions as well. Thus α-amylases and AS have very similar active site architecture with respect to the immediate surroundings of the scissile bond (subsite −1). This is in agreement with the general mechanism outlined in Scheme 1. The mechanism for the formation of the covalent intermediate is similar. But how does AS ensure specificity for sucrose as the first substrate, and how does AS
suggests that Phe250 is sandwiching the I-ring of the modified pocket includes the TAKA-amylase-acarbose structure it can be seen that the when examining the AS structure superimposed with the elongated acarbose molecule is for clarity labeled at the +3 and −3 subsite. Distances (from the superimposition) between the nucleophile (Asp286 O61) and the anomeric carbon at subsite −1 in acarbose and between the general acid (Glu328 Oε2) and acarbose N are shown. Furthermore, the distance (in Å) in the salt bridge found in AS between Asp144 Oε2 and Arg509 Nε2 is shown.

Specificity for Sucrose as the First Substrate—TAKA-amylase is an endo-acting enzyme. It has a total of six specific binding sites for linked α-(1→4)-glucosyl moieties. The TAKA-amylase residues reported to be involved in enzyme-substrate contacts in subsites +1 and +2 are not structurally conserved in AS. TAKA-amylase His217 in subsite +1 donating a hydrogen bond from Ne2 to O5J is a Phe in AS, whereas TAKA-amylase Lys309 with hydrogen bonds to the modified acarbose hydroxyls OK2 and OK3 is an Ala in AS. This suggests that the +1 subsite in AS is modified to accommodate specificity for the furanosyl ring of sucrose.

The TAKA-amylase-2 subsite has been completely disrupted in AS. Asp144 from loop2 forms a salt bridge with Arg509 and thus occupies the subsite. An equivalent salt bridge is observed in the exo-acting oligo-1,6-glucosidase. An Ala and an Asp are found in these positions in TAKA-amylase. The salt bridges give the active site a pocket topology in AS and oligo-1,6-glucosidase, in contrast to the cleft observed in TAKA-amylase. The result of the pocket topology is an exo-acting enzyme. The α-amylase cleft is closed by residues from domain B, domain B’, and loop2. The bottom of the pocket is quite thin-walled with a solvent accessible dent in the protein surface right behind the Asp144–Arg509 salt bridge. Without this blockage the active site topology would be a tunnel. In conclusion, the assumed furanosyl specificity at the +1 site and the salt bridge creates the sucrose specificity in AS.

Oligosaccharides as Second Substrates—The pocket topology in AS greatly reduces the solvent accessibility to the active site. When examining the AS structure superimposed with the TAKA-amylase-acarbose structure it can be seen that the pocket includes the −1 and +1 subsites. The superposition also suggests that Phe250 is sandwiching the I-ring of the modified acarbose at the −1 subsite with Tyr147 in AS. TAKA-amylase has a Gly at this position, oligo-1,6-glucosidase also have a Phe. This could implicate a more stable covalent intermediate in AS and oligo-1,6-glucosidase compared with α-amylases. This in turns could reflect the reduced accessibility of the active site. The intermediate simply has to exist long enough for the fructose to leave the active site and the second substrate (oligosaccharide or water) to enter.

The pyranosyl ring bound at the TAKA-amylase +2 subsite can just be seen in the surface plot (Fig. 6). Compared with the exo-acting hydrolase oligo-1,6-glucosidase the pocket of AS is very narrow leaving little room for water to enter when an oligosaccharide such as elongated acarbose is in the pocket. In the superimposition of acarbose into the AS structure the modified acarbose molecule fills the pocket almost completely. The surface area of the enzyme around the pocket-entrance is however quite open (Fig. 6). In fact several ravines in the surface leads to the pocket. Hence, it could be speculated that the growing glucan polymer is embedded in one ravine, whereas sucrose/fructose approaches/leaves the active site through another ravine. An architecture like this with a number of remote glucose binding subsites (securing a high “effective” concentration of the oligosaccharide chain) could be responsible for the transferase rather than hydrolase activity of AS. The unique AS domain-B’ is involved in the formation of these ravines (Fig. 6, dark gray surface). Because of the close proximity to the active site and the high content of aromatic residues (7 Phe, 3 Tyr, and 1 Trp out of 54 residues) it is tempting to propose that the B’-domain is essential for the binding of the growing glucan polymer. However, this hypothesis has to be tested by experiments involving complex formation with different oligosaccharides.

Calcium Independence—No calcium ions were found in the peptide.
structure. This also makes AS more similar to oligo-1,6-glucosidase than to TAKA-amylase. However, most of the Ca\(^{2+}\) site arrangement found in many amylases is conserved. In TAKA-amylase a calcium ion is hepta-coordinated by oxygen atoms from Asp\(^{175}\) (O\(\delta 1\) and O\(\delta 2\)), Asn\(^{121}\) (O\(\delta 1\)), Glu\(^{162}\) (backbone O), His\(^{210}\) (backbone O), and three water molecules. For both AS and oligo-1,6-glucosidase the calcium ion found in \(\alpha\)-amylases is replaced with a presumable protonated lysine N\(\zeta\) (Lys\(^{203}\) in AS and Lys\(^{206}\) in oligo-1,6-glucosidase). The two side chains involved in calcium binding (Asn\(^{121}\) and Asp\(^{175}\)) in TAKA-amylase (32). The last of the three hydrogen bonds found for Lys\(^{203}\) N\(\zeta\) comes from a water molecule. The lack of calcium has also been observed in neopullulanase (34) and maltogenic \(\alpha\)-amylase (35).

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