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# Site-directed mutagenesis of key amino acids in the active site of amylosucrase from *Neisseria polysaccharea*

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Amylosucrase from *Neisseria polysaccharea* (AS) is a glucosyltransferase from family 13 of the glycoside hydrolases. In this family, AS shows an unusual specificity for sucrose, which is the best substrate for the enzyme. AS synthesises, from this high-energy substrate, an amylose-like polymer. In addition, it catalyses the transfer of glucose units from sucrose onto acceptor molecules like glucose, maltooligosaccharides or glycogen. Finally, it catalyses the disproportionation of maltooligosaccharides. A structural analysis of a mutated AS complexed with sucrose led to a detailed description of the active site and of the interactions between sucrose and AS at subsites −1 and +1. Site-directed mutagenesis experiments confirmed the essential role of residues always conserved in the $\alpha$-amylase family. The nucleophile Asp286 and the general acid-base catalyst Glu328 are identified unequivocally. The conserved residues Asp393, His187, His 392, Arg284 and the stacking residues Tyr147 and Phe250 are critical for the enzymatic activity. These results support, for AS, an $\alpha$-retaining mechanism via a double-displacement, similar to that described for $\alpha$-amylases. In addition, the salt bridge formed by Asp144 and Arg509 is essential for the architecture of the active site and consequently for the sucrose specificity. Finally, Asp394 and Arg446 which interact with the fructosyl ring are not essential for activity towards sucrose but could be crucial for the binding of acceptor molecules.

Key words: amylosucrase, sucrose, active site, $\alpha$-amylase, site-directed mutagenesis, substrate specificity

## Introduction

Amylosucrase is a hexosyltransferase (EC 2.4.1.4) that synthesises, from sucrose, an insoluble $\alpha$-glucan containing mainly $\alpha$-1,4 glucosidic linkages (Hehre et al., 1949). Amylosucrase has been iden-

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tified in non-pathogenic bacteria of the *Neisseria* genus, first in 1946 in *N. perflava* (HEHRE & HAMILTON, 1946; HEHRE et al., 1949) and later in other strains of the same genus such as *N. polysaccharea* and *N. subflava* (RIOU et al., 1983). Recently, the sequence analysis of the genomes of *Deinococcus radiodurans* (WHITE et al., 1999) and *Caulobacter crescentus* (NIERMAN et al., 2001) enabled the localisation of genes coding for proteins having 43% and 48%, respectively, of amino acid residues identical to those of amylosucrase from *N. polysaccharea*.

Among all the amylosucrases, amylosucrase from *Neisseria polysaccharea* (AS) has been the most studied. The *as* gene has been cloned in *Escherichia coli* and the amylosucrase has been produced and highly purified for enzymatic characterization (POTOCKI DE MONTALK et al., 1999) and for the determination of the three-dimensional structure (SKOV et al., 2000, 2001). Although AS produces an $\alpha$-glucan from sucrose – without any participation of nucleotide-activated sugars, like the glucansucrases from lactic acid bacteria which belong to family 70 of glycoside hydrolases (DAVIES & HENRISSAT, 1995; COUTINHO & HENRISSAT, 1999) – it is not classified in the same family. Indeed, AS belongs to family 13 of the glycoside hydrolases ($\alpha$-amylase family) (POTOCKI DE MONTALK et al., 1999). AS is thus the only glucansucrase that does not contain a circularly-permuted $(\beta/\alpha)_8$-barrel (MACGREGOR et al., 1996). In addition, AS is the first glucansucrase of known three-dimensional structure.

AS is a highly remarkable member of family 13 of the glycoside hydrolases. Unlike most enzymes from family 13, AS shows an unusual specificity for sucrose which is its best substrate, and an ability to catalyse, from this high-energy substrate, the synthesis of an amylose-like polymer and the release of fructose (POTOCKI DE MONTALK et al., 1999). Polymer synthesis, the main reaction, is also accompanied by sucrose hydrolysis and by the formation of oligosaccharides. Maltooligosaccharides (mainly maltose and maltotriose) and sucrose isomers (turanose and trehalulose) are formed by transfer of glucosyl units onto glucose and fructose, respectively (POTOCKI DE MONTALK et al., 2000a). In addition, when glucose, maltooligosaccharides or glycogen are added to the reaction mixture, AS transfers the glucosyl unit of the sucrose donor onto these acceptor molecules (POTOCKI DE MONTALK, 1999). The glycosylation of glycogen occurs at the non-reducing end of the polymer branches and results in a 100-fold activation of sucrose consumption (POTOCKI DE MONTALK et al., 2000b). Finally, AS catalyses the disproportionation of maltooligosaccharides via an exo mode of action (ALBENNE et al., 2002). It cleaves the $\alpha$-1,4 linkage of a maltooligosaccharide donor from its non-reducing end and transfers a glucosyl unit to the non-reducing end of a maltooligosaccharide acceptor. This property testifies that AS is also specific for the cleavage of $\alpha$-1,4 linkages.

The recently-solved 3D-structure of AS revealed that it is organized in five domains named N, A, B, B' and C (Fig. 1) (SKOV et al., 2001). Domains A, B and C are characteristic of family 13 whereas the two additional domains, N and B', are unique. Domain A (residues 98–184, 261–395 and 461–550) is the characteristic $(\beta/\alpha)_8$-barrel catalytic domain. In the $\alpha$-amylase family, the conserved catalytic residues and the substrate binding residues are found at the C-termini of the $\beta$-strands in the $(\beta/\alpha)_8$-fold and in loops that extend from these strands (SVENSSON, 1994). Diversity in specificity could arise by variation in substrate binding at the $\beta \rightarrow \alpha$ loops. In particular, the protruding loop 3 inserted between $\beta$-strand 3 and $\alpha$-helix 3 which forms the separate domain B (residues 185–260 in AS) could be involved in functional diversity and stability. The C-terminal domain (residues 555–628) is composed of an eight-stranded $\beta$-sandwich. The N-terminal domain (residues 1–90) contains six amphiphilic helices and shows no structural similarities with any known domain. Its function has not been elucidated. The B' domain (residues 394–460) is formed by an extended loop between the seventh $\beta$-strand and $\alpha$-helix of the barrel. It contains two helices followed by a short $\beta$-strand and is terminated by a short $\alpha$-helix. It starts right after highly-conserved residues in the family and is thought to play an important role in the enzyme specificity.

Crystal structures of AS complexed with D-glucose and sucrose enabled the precise localisation of the active site and the mapping of subsites $-1$ and $+1$ of AS (using the subsite numbering used for $\alpha$-amylases) to be achieved (MIRZA et al., 2001). The active site is located at the bottom of a narrow pocket that contains the proposed catalytic residues, in particular the catalytic triad described in $\alpha$-amylases (SVENSSON, 1994). This architecture is mainly due to the presence of the unique domain B' which partially covers the active site entrance. In the AS:sucrose complex, the sucrose molecule is very strongly bound to the bottom of the pocket, with the glucose ring occupying subsite $-1$, while the fructose ring binds subsite $+1$. Among the three glucose residues found

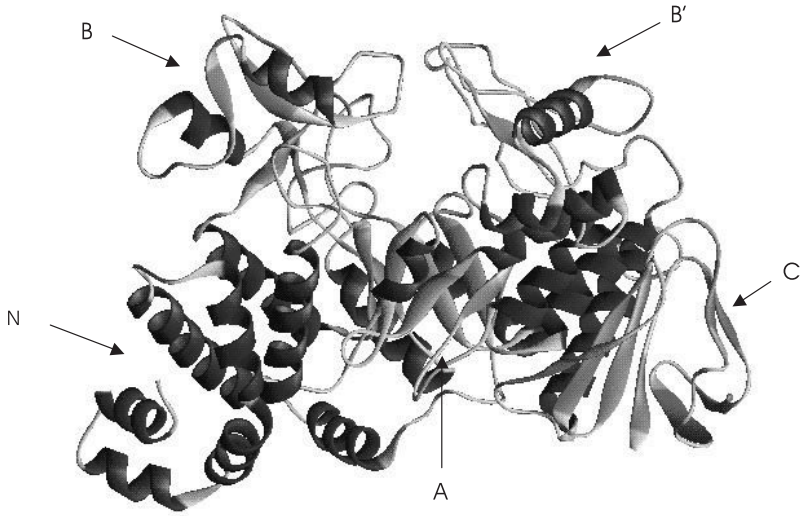


Fig. 1. Overall three-dimensional structure of amylosucrase of *Neisseria polysaccharea*.

in the AS:glucose complex, one occupies the same position as above with the glucosyl ring at subsite −1. The glucose ring in the −1 position is maintained by stacking on Tyr147 and Phe250 and by a strong hydrogen-bonding network. Eight direct hydrogen bonds are formed and only one water molecule associates with the pyranosyl ring at O4. The direct hydrogen bonds involve the conserved residues Asp286, His187, Arg284, Asp393 and His392 and the two unconserved residues Asp144 and Arg509. The distance between Asp286 and the C1 of the pyranosyl ring (3.1 Å) is short enough to make a nucleophilic attack possible. The hydrogen bonds involving conserved residues are also found in other $\alpha$-amylases such as TAKA-amylase ($\alpha$-amylase from *Aspergillus oryzae*) which possesses the well-conserved residues in identical positions. Asp144 and Arg509 reinforce this network by interacting with O4 of the glucose ring. They form a salt bridge and block the bottom of the pocket. Subsite +1 involves fewer residues than subsite −1; this results in a weaker hydrogen network between AS and the fructosyl ring. Interactions between the fructosyl moiety and AS involve four direct hydrogen bonds and four water-mediated hydrogen bonds. Direct hydrogen bonds involve residues Asp393, Asp394 and Arg446. These bonds affect the sucrose $\varphi$ and $\psi$ angles. The distortions induced enable the alignment of the lone pair of sucrose O1 with the acidic hydrogen of Glu328. The presence of four water-mediated hydrogen bonds is in agreement with the fact that fructose must leave the active site easily during the catalysis.

The involvement of conserved, but also unconserved, residues of AS in the interaction with sucrose shows that the network of interactions is partly similar to that observed in other $\alpha$-amylases but also presents some particularities. Asp144, Arg509, Asp 394 and Arg446 which are not conserved in the family are thought to primarily determine the specificity for sucrose. Both conserved and unconserved residues were submitted to site-directed mutagenesis experiments. Here, we present the results of these mutations. The activities in the presence of sucrose were measured, to identify the residues essential for catalysis and those which control the specificity. The effects of the mutations are analysed in relation to the three-dimensional structure.

## Material and methods

*Bacteria and plasmid*

*E. coli* JM109 was used as a host for site-directed mutagenesis and for purification of wild-type and mutated AS. The strain was maintained and grown on Luria-Bertani (LB) agar plates containing ampicillin (100 $\mu$g/mL). The pGST-AS (Potocki de Montalk et al., 1999) encoding GST fused to AS, was used for site-directed mutagenesis and for expression of the fusion gene. Plasmid DNA was prepared using a Qiaprep Spin miniprep kit (Qiagen).

*Site-directed mutagenesis*

Site-directed mutagenesis of the AS gene was carried out with the QuikChange$^{TM}$ site-directed mutagenesis kit (Stratagene). The procedure utilises the pGST-AS double-stranded DNA vector and two synthetic oligonucleotide primers containing the desired mutation. The oligonucleotide primers (synthesised by Isoprim, Toulouse, France), each complementary to opposite strands of the vector, were extended during temperature cycling by *Pfu* Turbo DNA polymerase

(Stratagene). On incorporation of the oligonucleotide primers, a mutated plasmid containing staggered nicks is generated. Following temperature cycling (95 °C, 30 s; 55 °C, 1 min; 68 °C, 15 min for 15 cycles), the product is treated with *DpnI*. The *DpnI* endonuclease (target sequence: 5'-G$^{m}$ATC-3') is specific for methylated and hemimethylated parental DNA templates and is used to select for mutation-containing synthesised DNA. The nicked vector DNA incorporating the desired mutation is then transformed into *E. coli* JM109 competent cells. Mutagenic oligonucleotides were designed to create a restriction site which was used to screen the correct mutation. The mutations were confirmed by DNA sequencing (Genome Express, Grenoble, France).

*Enzyme extraction methods*
*E. coli* carrying the recombinant plasmid encoding wild-type and mutated AS gene was grown on LB containing ampicillin (100 $\mu$g/mL) and isopropyl-$\beta$-thiogalactopyranoside (IPTG) (1 mM) for 10 h. The cells were harvested by centrifugation (8000 × g, 10 min, 4 °C), resuspended and concentrated to an $OD_{600nm}$ of 80 in PBS buffer (140 mM NaCl, 2.7 mM KCl, 10 mM $Na_2HPO_4$, 1.8 mM $KH_2PO_4$, pH 7.3). The intracellular enzyme was extracted by sonication and 1% (v/v) Triton X-100 was added to the extract and mixed for 30 min at 4 °C. After centrifugation (10000 $\times g$, 10 min, 4 °C), the supernatant obtained was used as the source for enzyme purification. The overexpression was verified by sodium-dodecyl-sulfate-polyacrylamide gel electrophoresis (SDS-PAGE).

*Purification of wild-type and mutated AS*
Purification of wild-type and mutated AS was performed by affinity chromatography of the GST/AS fusion protein on glutathione-Sepharose 4B (Amersham Pharmacia Biotech) as previously described (Potocki de Montalk et al., 1999). The enzyme was obtained in PreScission buffer (50 mM Tris-HCl pH 7.0, 150 mM NaCl, 1 mM EDTA, 1 mM dithiothreitol). The protein content was determined by the micro-Bradford method, using bovine serum albumin as standard.

Electrophoresis of pure enzyme was carried out with the PHAST system (Amersham Pharmacia Biotech), using PhastGel$^{TM}$ gradient 8–25 (Pharmacia Biotech) ready-made gels under denaturing conditions. Staining with 0.5% (w/v) $AgNO_3$ led to single band profiles.

*AS activity assays*
All the assays were carried out with pure enzyme excepted for those mentioned in the text, which were carried out with crude supernatant.

The standard amylosucrase assay was carried out at 30 °C in PreScission buffer (50 mM Tris-HCl pH 7.0, 150 mM NaCl, 1 mM EDTA, 1 mM dithiothreitol) supplemented with 50 g/L sucrose and 0.1 g/L glycogen (Potocki de Montalk et al., 2000b). For some mutants, the initial rate of sucrose consumption was also determined in the presence of: i) 50 g/L sucrose alone, ii) 50 g/L sucrose and 30 g/L glycogen.

Initial rates are measured in $\mu$mol of reducing sugars (fructose and glucose) released from sucrose per minute and per gram of pure AS or per liter of crude supernatant. In both cases, activities are expressed as a percentage of the wild-type enzyme activity. The concentration of reducing sugars (fructose and glucose) was measured by the dinitro-salicylic acid method, using fructose as a standard. Protein concentration was determined by the micro-Bradford method, using bovine serum albumin as the standard.

*Carbohydrate analysis*
Sucrose, glucose and fructose concentrations from standard assays were measured by ion exchange chromatography at 25 °C using an Aminex HP87H column (Bio-Rad), the eluant being 8.5 mM $H_2SO_4$ at 0.5 mL/min.

## Results and discussion

*Site-directed mutagenesis of conserved residues*
Amylolytic enzymes possess eight highly-conserved regions containing ten invariant amino acid residues: two structurally-important glycines and eight residues at the active site (Svensson, 1994). The corresponding residues in AS were first identified by multiple sequence alignments and their assignment was later confirmed by the structural data. They are Gly128, Pro134, Asp182, His187, Arg284, Asp286, Glu328, His392, Asp393 and Gly480 (Potocki de Montalk et al., 1999).

Hydrogen network in subsite −1
The structural description of the active site revealed that Asp286, Glu328, Asp393, His187, His392 and Tyr147 define subsite −1 by interacting through hydrogen bonds with the glucosyl ring of sucrose. The superimposition of AS and TAKA-amylase active sites revealed that both $C_\alpha$ and side chains of these residues are found at identical positions (Fig. 2) (Brzozowski & Davies, 1997; Skov et al., 2001).

The position of Glu328 and Asp286 at the tips of $\beta$-strands 4 and 5 in the $(\beta/\alpha)_8$-barrel supports an $\alpha$-retaining mechanism via the formation of a $\beta$-glucosyl enzyme intermediate, involving Glu328 as the general catalyst and Asp286 as the nucleophile. Site-directed mutagenesis experiments of these residues have been carried out to confirm these predictions (Sarcabal et al., 2000). Replacement of the putative nucleophile Asp286 by Asn or Glu, as well as the replacement of the putative catalyst Glu328 by Gln, led to a complete loss of AS activity (Tab. 1). This result demonstrates that the presence and the positioning of the functional carboxylate of these two carboxylic acid residues are crucial for activity. Indeed, the

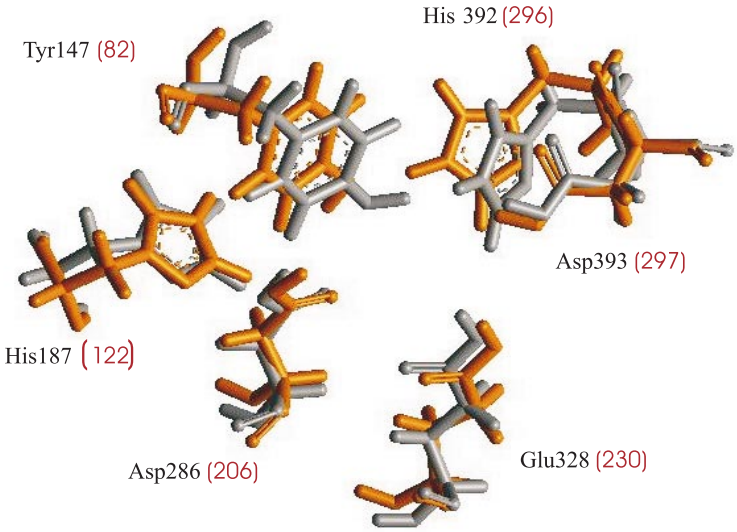


Fig. 2. Superimposition of conserved active site residues of AS (grey) and TAKA-amylase (orange).

replacement of Asp286 by Glu does not maintain the activity because of an inappropriate position. The distance between OD1 of Asp286 and the C1 of the pyranosyl ring has to be optimal (3.1 Å in the native structure) to make nucleophilic attack possible. Similarly, in the native structure, OE1 of Glu328 hydrogen bonds to O1 of the glucosyl ring with a distance of 2.6 Å. The side chain of Gln328 occupies a position similar to that of Glu328 and the distance between OE1 and O1 of the glucose is 3.2 Å. The lack of the acidic group renders impossible the protonation of the glycosidic bond of the sucrose molecule aligned for optimal transfer via interactions with the enzyme.

In addition, replacement of residue Asp393 by Asn or Glu strongly affected the activity (Tab. 1). The activity of mutant D393N was not detectable, whereas D393E exhibited 0.15% of the wild-type AS activity when glycogen was added to the medium at a concentration of 30 g/L. This result confirms the essential role of the third residue of the well-known catalytic triad. This aspartic acid, which interacts both with glucose at subsite −1 and fructose at subsite +1, stabilizes the geometry of the catalytic groups. Indeed, it is involved in the alignment of the lone pair of O1 of sucrose with the acidic hydrogen of Glu328. The almost linear O1:lone pair H-O will minimize the proton-transfer energy barrier during catalysis and will therefore stabilize the transition state towards the covalent intermediate. It contributes to maintaining the general acid catalyst in the protonated form over the pH range for optimum activity.

Residues His187 and His392 were replaced by

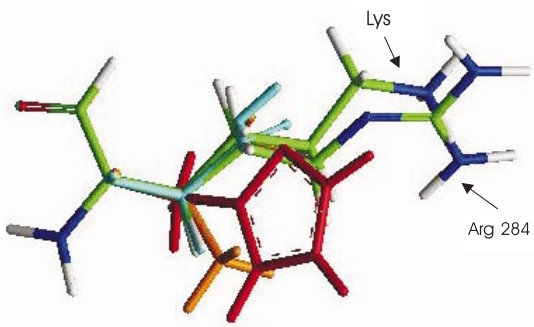


Fig. 3. Superimposition of amino acids corresponding to replacement of Arg284 (coloured by atom) by Val (orange), Asp (blue ), His (red) and Lys (coloured by atom).

Gln and Asn respectively. Mutants H187Q and H392N retained 2% and 3%, respectively, of the activity of the wild-type enzyme in the standard conditions. The initial activity increased 25 and 6 times for H187Q and H392N, respectively, when the glycogen concentration was increased from 0.1 to 30 g/L, while about a 33-fold increase was observed for the wild-type enzyme (Tab. 1). These two His interact by hydrogen bonding with the glucosyl ring in position −1: His187 with O6 and His392 with O2 and O3. In addition, His 392 forms a short hydrogen bond to the hydroxyl of Tyr147. This interaction is suggested to be pivotal for the correct positioning of the stacking platform (Brzozowski & Davies, 1997). These two residues make a large contribution to the transition–state

Table 1. Mutagenesis of highly conserved residues of AS active site.[a]

| Mutation | Relative activity[b] (%) | Relative activity[c] (%) |
|---|---|---|
| Wild type | 100 | 3300 |
| D286N | ND | ND |
| D286E | ND | ND |
| E328Q | ND | ND |
| D393N | ND | ND |
| D393E | ND | 0.15 |
| H187Q | 2 | 50 |
| H392N | 3 | 18 |
| R284V | ND | – |
| R284D | ND | – |
| R284H | ND | – |
| R284K | 11 | – |

[a]Activities of mutant ASs are expressed as a percentage of the activity of the wild-type enzyme in the standard conditions (50 g/L sucrose and 0.1 g/L glycogen). ND: not detectable; – : not measured.
[b]Activity in the presence of 50 g/L sucrose and 0.1 g/L glycogen.
[c]Activity in the presence of 50 g/L sucrose and 30 g/L glycogen.

stabilisation energy but are not involved directly in catalysis. The mutation of these residues weakened the hydrogen network at subsite −1, but the main effect is the loss of stability of the intermediate which results in a roughly 40-fold decrease of $k_{\mathrm{cat}}$.

Arg284 is an additional conserved residue which is involved in the hydrogen network between the enzyme and the substrate in subsite −1. It forms a salt-bridge to the OD1 of the nucleophile and it interacts with the O2 of the glucosyl ring. This residue was replaced by four different amino acids in order to modify the steric hindrance and the functional group at this position. A small neutral residue (Val), an acidic residue (Asp) and two basic residues (His and Lys) were introduced. Mutants R284V, R284D and R284H had an undetectable activity under the standard assay conditions. R284K presents 11% of the wild-type activity. This result suggests that a basic group has to be at the right distance from the O2 of the glucosyl ring to maintain activity. Indeed, the Lys residue mimics Arg because of its long chain and its basic group. Superimposition of these 5 amino acids shows that Lys is the only one which can substitute for Arg and maintain some activity (Fig. 3). Besides its involvement in the hydrogen network at −1, Arg284 stands out because of its key position in the structure. Indeed, Arg284 is situated in the fourth $\beta$-strand of the barrel, just before the nucleophile Asp286 and may be crucial for the overall structure. Consequently, it appears to be essential for both the structural integrity and the correct positioning of the nucleophile. The loss of activity observed when Arg284 is mutated is expected to be mainly due to changes induced in the overall structure rather than to a modification of the binding of the substrate.

The results from all these mutations and from structural analysis enabled us to unequivocally assign the catalytic residues and to propose a mechanism similar to that described for other $\alpha$-amylases, at least for the first part of the reaction (Mac Carter & Withers, 1994; Davies & Henrissat, 1995; Uitdehaag et al., 1999; Mirza et al., 2001). The nucleophile Asp286, correctly positioned with the help of Arg284, attacks C1 of the glucosyl ring at position −1. This nucleophilic attack is assisted by the general acid Glu328 which first functions as an acid, protonating the glycosidic bond of the sucrose molecule aligned for optimal transfer. Glu328 gives its proton to O2 of the fructosyl leaving group at subsite +1. This leads to the formation of the covalently-linked glucosyl-enzyme intermediate involving Asp286. In a second step, fructose may be released leaving free access for subsequent attack by the incoming nucleophile. Glu328 now serves as general base to activate the incoming nucleophile by attacking C1. The glucosyl unit is then transferred onto an acceptor which can be either a water molecule or another saccharide. The first step of the disproportionation reaction is expected to be similar.

Stacking residues

The formation of the complex is likely to be associated with hydrophobic interactions in addition to hydrogen bonding. The glucose ring stacks on Tyr147 and Phe250 (Fig. 4) which sandwich the glucose ring at position −1. Tyr147 is always conserved in the $\alpha$-amylase family and belongs to loop 2 of the barrel. It seems to be the main hydrophobic contact. Phe250, which belongs to the B domain (loop 3 of the barrel), stacks on the opposite side and interacts mainly with C6. This Phe is not so highly conserved. Such stacking is also found at an identical position in oligo-1,6-glucosidase (Watanabe et al., 1997) but not in TAKA-amylase (Brzozowski & Davies, 1997).

Each of the two stacking residues was replaced by three amino acids: two non-aromatic residues (Ala, Asn) and one aromatic residue

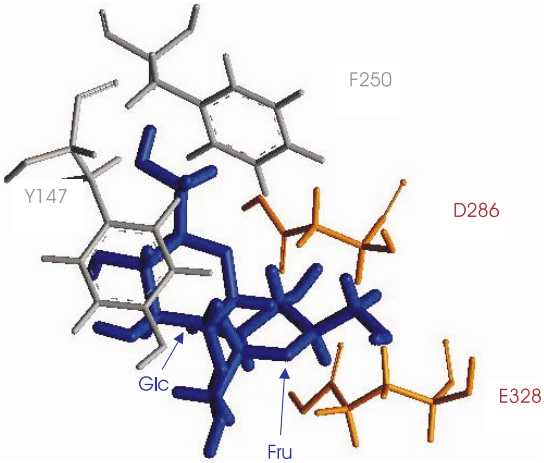


Fig. 4. Stacking of the glucosyl ring at subsite −1.

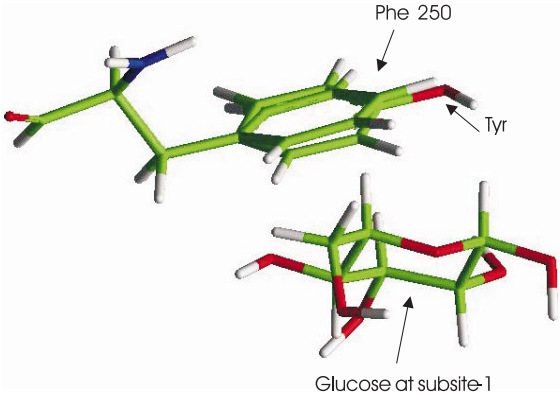


Fig. 5. Superimposition of amino acids corresponding to replacement of Phe250 (coloured by atom and stacked with the glucose ring at subsite −1) by Tyr (coloured by atom).

Table 2. Mutagenesis of stacking residues at subsite −1.[a]

| Mutation | Relative activity[b] (%) |
|---|---|
| Wild type | 100 |
| Y147A | 11 |
| Y147N | 7 |
| Y147F | 67 |
| F250A | 10 |
| F250N | 22 |
| F250Y | 25 |

[a]Activities of mutant ASs are expressed as a percentage of the activity of the wild-type enzyme in the standard conditions (50 g/L sucrose and 0.1 g/L glycogen).
[b]Activity in the presence of 50 g/L sucrose and 0.1 g/L glycogen.

(Phe/Tyr). The activities were measured from crude supernatants under standard conditions by measuring the initial rates of the release of reducing sugars (such as fructose and glucose). They are expressed as a percentage of the activity of the wild-type enzyme. Individual replacement of the stacking residues by Ala and Asn led to mutants with 7% to 22% of the wild-type AS activity under standard conditions (Tab. 2). The mutation of these residues is less critical for activity than the mutation of the five amino acids previously described (the catalytic triad and the two stabilising His). Indeed, they are not involved directly in the catalysis but in the binding of the glucose ring at subsite −1. The study of a double mutant in which both aromatic residues were replaced by non-aromatic residues would demonstrate if at least one stacking interaction is necessary for activity. Besides, mutants F250Y and Y147F retained 25% and 67% of the activity, respectively (Tab. 2). These results suggest that the replacement of one aromatic residue by another maintains activity better than when a non-aromatic residue is introduced. Nevertheless, position 250 does not leave enough room for a Tyr. The additional hydroxyl group prevents the correct positioning of the aromatic ring for optimal stacking (Fig. 5).

In conclusion, all these mutant enzymes have reduced activity compared to that of the wild-type enzyme, confirming that both Tyr147 and Phe250 are important and contribute to the activity of AS by strengthening the binding of a glucose ring at subsite −1.

However, the purification of these mutant ASs is now essential to fully characterise the remaining activity. Is the pattern of the products synthesised strictly identical to that of the wild-type enzyme? The replacement of hydrophobic residues by non-hydrophobic residues reduces the hydrophobicity of the active site. It will be important to evaluate the effect of these mutations on the ratio of hydrolysis versus transglycosylation.

*Site-directed mutagenesis of unconserved residues*
In addition to the conserved residues of the $\alpha$-amylase family described above which are known to interact with the substrate, unconserved residues are found in key positions in the active site of AS. These residues are unique in AS and are thought to participate in the specificity of AS for

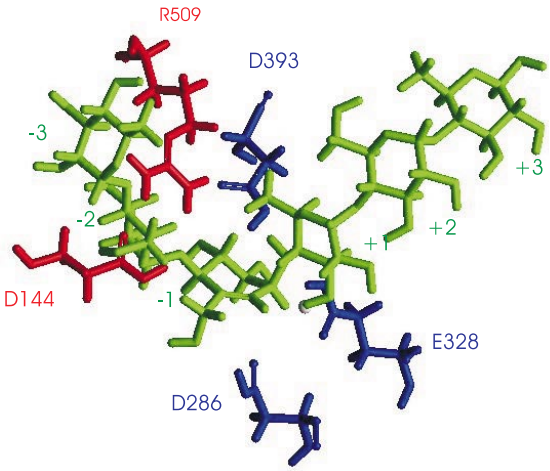


Fig. 6. Superimposition of the residues of the active site of AS:sucrose with transglycosylated acarbose from the TAKA-amylase:acarbose complex (acarbose in green).

sucrose. Among the unconserved residues, Asp144 and Arg509 are involved in a salt bridge that blocks the bottom of the active site, and Asp394 and Arg446 interact with the fructose in position +1. As before, the residual activities of the mutant enzymes were measured from crude supernatants. The activities are expressed as a percentage of the activity of the wild-type enzyme.

Salt bridge

Superimposition of the active sites of AS:sucrose and TAKA-amylase:acarbose complexes revealed that the active site of AS, compared to the TAKA-amylase active site, is blocked by Asp144 and Arg509 which form a salt bridge (Fig. 6). These two residues belong to loop 2 and loop 8, respectively, and they interact with the substrate by hydrogen bonds with the O4 of the glucosyl ring in position −1. The subsites corresponding to subsites −2 and −3 of TAKA-amylase are therefore disrupted in the AS structure, leaving space only for the positioning of one glucosyl moiety at subsite −1. Without this blockage, the active site topology would be a tunnel. A similar salt bridge is observed in the exo-acting oligo-1,6-glucosidase which possesses an Asp and an Arg at the positions corresponding to Asp144 and Arg509 of AS. An Ala and an Asp are found in these positions in TAKA-amylase.

These two residues were replaced by an Ala by site-directed mutagenesis in order to weaken the salt bridge and convert the pocket topology into a tunnel. The mutant enzymes D144A and R509A were almost inactive, with only 0.2% and 0.1% of residual activity in the presence of 50 g/L sucrose supplemented with 0.1 g/L and 30 g/L glycogen respectively. Activity was not detectable in the presence of sucrose alone (Tab. 3). These results demonstrate that the hydrogen bonds formed with O4 of the glucosyl ring are crucial for the binding of the sucrose in the active site. Besides, the overall architecture of the active site is probably supported to a great extent by the salt bridge. However, the mutant D144A which mimics the TAKA-amylase may have gained an endo-$\alpha$-amylase activity. Maltooligosaccharide substrates may therefore bind at subsites −2 and −3. Further investigations are in progress to check this hypothesis.

Hydrogen network in subsite +1

The residues in enzymes of the $\alpha$-amylase family known to be involved in the contacts between the enzyme and the substrate at subsites +1 and +2 are not structurally conserved in AS (Mirza et al., 2001). This suggests that the +1 subsite of AS is modelled to accommodate the furanosyl

Table 3. Mutagenesis of the two unconserved residues forming a salt bridge at the bottom of AS active site.[a]

| Mutation | Relative activity[b] (%) | Relative activity[c] (%) | Relative activity[d] (%) |
|---|---|---|---|
| Wild type | 100 | 3300 | 33 |
| D144A | 0.2 | 0.1 | ND |
| R509A | 0.2 | 0.1 | ND |

[a]Activities of mutant ASs are expressed as a percentage of the activity of the wild-type enzyme in the standard conditions (50 g/L sucrose and 0.1 g/L glycogen). ND: not detectable.
[b]Activity in the presence of 50 g/L sucrose and 0.1 g/L glycogen.
[c]Activity in the presence of 50 g/L sucrose and 30 g/L glycogen.
[d]Activity in the presence of 50 g/L sucrose.

Table 4. Mutagenesis of the two unconserved residues interacting with the fructosyl ring at subsite +1.[a]

| Mutation | Relative activity[b] (%) | Relative activity[c] (%) | Relative activity[d] (%) |
|---|---|---|---|
| Wild-type | 100 | 3300 | 33 |
| D394A | 2 | 1 | 9 |
| R446A | 1.2 | 0.3 | 6 |

[a]Activities of mutant ASs are expressed as a percentage of the activity of the wild-type enzyme in the standard conditions (50 g/L sucrose and 0.1 g/L glycogen).
[b]Activity in the presence of 50 g/L sucrose and 0.1 g/L glycogen.
[c]Activity in the presence of 50 g/L sucrose and 30 g/L glycogen.
[d]Activity in the presence of 50 g/L sucrose.

ring of sucrose. The interactions with the fructosyl moiety at subsite +1 involve hydrogen bonds with residues Asp393, Asp394 and Arg446. These residues are responsible for a distortion of the torsion angles of the sucrose which is involved in the alignment of the lone pair of sucrose O1 with Glu328. Interestingly, Asp394 and Arg446 belong to loop 7 (B' domain) and are not conserved in the family. These two residues were replaced by Ala to weaken the hydrogen bond network at subsite +1. Interestingly, mutant enzymes D394A and R446A have 9% and 6% activity, respectively, in the presence of sucrose alone compared to the native enzyme activity in the standard conditions (50 g/L sucrose and 0.1 g/L glycogen) (Tab. 4). This indicates that the specificity for sucrose is maintained even when the hydrogen network in subsite +1 is modified. However, as shown in Table 4, their relative activities decrease to 2% and 1.2%, respectively, when glycogen is added to the medium at 0.1 g/L (standard conditions) and to 1% and 0.3%, respectively, when glycogen is added to the medium at 30 g/L. Unlike the wild-type enzyme, which is activated 3-fold in the presence of 0.1 g/L glycogen, the mutants D394A and R446A are not activated by glycogen. Remarkably, they show a reduced activity when glycogen is added. Consequently, it seems that residues Asp394 and Arg446 are involved in the acceptor reaction, in particular in the positioning either of the glucosyl unit transferred or of the acceptor molecule. However, it will be important to characterise the pattern of the products synthesised by these two mutant enzymes.

Full characterisation of the various mutant enzymes will allow the precise assignment of the effect of the mutations on the specificity, and the identification of the structural features that control the different reactions catalysed by AS: hydrolysis of sucrose, oligosaccharide and polymer synthesis, glucosylation of acceptor and disproportionation of maltooligosaccharides. Such an approach will give new insights into the structure/function relationships of amylosucrase from *Neisseria polysaccharea*.

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