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Molecular Basis of the Amylose-like Polymer Formation Catalyzed by Neisseria polysaccharea Amylosucrase*

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Amylosucrase from Neisseria polysaccharea is a remarkable transglucosidase from family 13 of the glycoside-hydrolases that synthesizes an insoluble amylose-like polymer from sucrose in the absence of any primer. Amylosucrase shares strong structural similarities with α-amylases. Exactly how this enzyme catalyzes the formation of α-1,4-glucan and which structural features are involved in this unique functionality existing in family 13 are important questions still not fully answered. Here, we provide evidence that amylosucrase initializes polymer formation by releasing, through sucrose hydrolysis, a glucose molecule that is subsequently used as the first acceptor molecule. Maltoligosaccharides of increasing size were produced and successively elongated at their nonreducing ends until they reached a critical size and concentration, causing precipitation. The ability of amylosucrase to bind to and to elongate maltoligosaccharides is notably due to the presence of key residues at the OB1 acceptor binding site that contribute strongly to the guidance (Arg394, subsite +4) and the correct positioning (Asp384 and Arg446, subsite +1) of acceptor molecules. On the other hand, Arg226 (subsites +2/+3) limits the binding of maltoligosaccharides, resulting in the accumulation of small products (G to G3) in the medium. A remarkable mutant (R226A) activated by the products it forms, was generated. It yields twice as much insoluble glucan as the wild-type enzyme and leads to the production of lower quantities of by-products.

Amylosucrase (EC 2.4.1.4) is a glucansucrase belonging to glycoside-hydrolase (GH) family 13 (1, 2). This transglucosidase catalyzes the synthesis of an insoluble amylose-like polymer from sucrose (3), a cheap and easily available agroresource. This is in contrast to starch or glyogen synthases (4), which require nucleotide-activated sugar as a donor. Amylosucrase is thus attractive for the industrial synthesis of amylose-like polymers and for the modification of glucans (in particular to form nondigestible glucana) (5). Remarkably, amylosucrase is the only member of GH family 13 displaying polymerase activity and is clearly unique in this family that mainly contains starch-degrading enzymes. Amylosucrase was first isolated in the culture supernatant of Neisseria perflava (3) and later identified in various Neisseria strains (6, 7). Recently, data mining has revealed the presence of genes encoding putative amylosucrases in the genome of many other organisms such as Deinococcus radiodurans (8), Caulobacter crescentus (9), Xanthomonas campestris, Xanthomonas axonopodis (10), and Pirellula sp. (11). Recombinant amylosucrase from Neisseria polysaccharea (AS) has been the most extensively studied amylosucrase. The gene encoding AS (1) has been cloned, and its product has been purified to homogeneity. Characterization of the reaction products synthesized from sucrose substrate showed that sucrose isomers (turanose and trehalulose), glucose, maltose, and maltotriose were also produced besides the insoluble polymer containing only α-1,4-glucosidic linkages (1, 12).

No soluble maltoligosaccharides longer than maltotriose were detected in the reaction mixture, suggesting that the polymer chain remained bound to the enzyme and was elongated via a processive mechanism, as opposed to a nonprocessive mechanism during which the chain would be released after each glucosyl residue transfer. However, the initiation step, the direction of elongation, and its mechanism were not investigated further to absolutely confirm a processive elongation. More generally, the mechanism of polymer synthesis catalyzed by glucansucrases is still a subject of debate. According to Robyt et al. (13), dextran synthesis catalyzed by dextranu-
with maltoheptaose (26). This enabled the identification of key residues at the OB1 site involved in the polymerization process. Several variants with altered properties were obtained and opened the route to the rational design of AS with improved reaction specificity.

EXPERIMENTAL PROCEDURES

Plasmid and Bacterial Strains—The pGST-AS encoding glutathione S-transferase (GST; 26 kDa) fused to AS (70 kDa) (1) was used to express the fusion gene and for site-directed mutagenesis. Escherichia coli strain JM109 was used as the host of pGST-AS encoding wild-type or mutated AS.

Site-directed Mutagenesis—Site-directed mutagenesis of the AS gene was carried out with the QuikChange™ site-directed mutagenesis kit (Stratagene), as previously described (20). The procedure utilized the pGST-AS double-stranded DNA vector and two synthetic oligonucleotide primers, each complementary to opposite strands of the vector. Primers contained the desired mutation (boldface type in the following sequences) and were designed to create or remove (asterisks) a restriction site (underlined in the following sequences and identified after each), which was used to screen the correct mutation. The following primers were used to construct the mutant enzymes:

- R226A_for, 5'-GACGGACCCCTGGCCAGAACGAGGCCACCG-3' (AvrII)
- R226A_rev, 5'-GGGCTTCCGCGAAGACGCGCAGGGTTGCTGGTCGGGGGAAGATTTC-3' (SacII*)
- R446A_for, 5'-GGCGACTGCACGACCCGGCCGACAGGGTCACTAGGCA-3' (AvaII)
- R446A_rev, 5'-GGCGACTGCACGACCCGGCCGACAGGGTCACTAGGCA-3' (AvaII)
- D394A_for, 5'-GCCGCCGCTGTACCACTGACGGCAGCTGGTGGTCGTAGCCGCTTATGCCTAGGTATGC-3' (SacII*)
- R415A_rev, 5'-GCCGCCGCTGTACCACTGACGGCAGCTGGTGGTCGTAGCCGCTTATGCCTAGGTATGC-3'

The mutations were confirmed by DNA sequencing.

Enzyme Extraction Methods—E. coli carrying the recombinant pGST-AS plasmid encoding the wild-type and mutated AS gene was grown on LB medium containing ampicillin (100 µg·mL⁻¹) and isopropyl-β-D-thiogalactopyranoside (1 mM) for 10 h at 30 °C. The cells were harvested by centrifugation (8000 × g, 10 min, 4 °C), resuspended, and concentrated to an A₂₆₀ of 80 in PBS buffer (140 mM NaCl, 2.7 mM KCl, 10 mM Na₂HPO₄, 1.8 mM KH₂PO₄, 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 (10,000 × g, 10 min, 4 °C), the supernatant was used as the source for enzyme purification.

Purification of Wild-type and Mutated AS—Amylosucrase was purified by affinity chromatography of the GST/AS fusion protein on glutathione-Sepharose 4B (Amersham Biosciences) as previously described (1). Since pure GST/AS fusion protein possesses the same function and the same efficiency as pure AS (data not shown), enzymes were purified simply to the GST/AS fusion protein stage (96 kDa).

The enzymes (wild-type or mutated) were obtained and stored in elution buffer (50 mM Tris-HCl, pH 7.0, 150 mM NaCl, 1 mM EDTA, 1 mM diithiothreitol). The protein content was determined by the microBradford method, using bovine serum albumin as a standard (28).

Electrophoresis of pure enzymes was carried out with the Phast system (Amersham Biosciences), using PhastGel™ gradient 8–25.
Mechanism of Polymerization Catalyzed by Amylosucrase

The chromatogram of the soluble fraction (Fig. 2A, Table I) shows the presence of glucose (G), maltose (G2), maltotriose (G3), and sucrose isomers (turanose and trehalulose) that were previously described by Potocki de Montalk et al. (12). However, it also reveals the presence of maltooligosaccharides (MOS) longer than G3 that were not detected in the initial studies. In fact, all species between G4 and G25 were found in the soluble fraction. However, MOS from G4 to G25 were detected at very low concentrations (ranging from 0.03 to 0.05 mM) compared with G, G2, and G3 (from 2 to 5 mM) (Fig. 3). Thus, only 12% of the glucosyl units were incorporated from sucrose into these MOS, whereas the yield of G to G3 reached 26% (Table I).

The chromatogram of the insoluble fraction (totally dissolved in KOH) was superposed on that of the soluble fraction (Fig. 2B). It also shows the presence of MOS from G4 to at least G35. The yield of the insoluble fraction was calculated from the difference between the amount of sucrose consumed and the glucosyl units incorporated into soluble saccharides and reached 45%.

**Kinetic Study of the Formation of Soluble Oligosaccharides Synthesized by Wild-type AS from 100 mM Sucrose**—The production of glucose, sucrose isomers, and soluble MOS from G2 to G25 was followed versus time and is shown in Fig. 3, A and B. For clarity, we only report in Fig. 3B the concentrations of MOS from G4 to G13.

Glucose appeared first in the medium followed by maltose and maltotriose. Maltotetraose was detected 5 min after the start of the reaction, whereas MOS having a degree of polymerization (DP) higher than 4 appeared after a delay of 20 min and were synthesized at almost the same rate (Fig. 3B). Sucrose isomer formation increased with the accumulation of fructose in the reaction medium (Fig. 3A). During the first 30 min of the reaction, all of the glucosyl moieties consumed were found to be present as glucose and soluble oligosaccharides from DP2 to DP25 (data not shown). Notably, no insoluble fraction was formed. After a 30-min reaction, the glucose incorporated into the soluble products no longer accounted for the sucrose consumed. The deficit observed is due to the formation of the insoluble fraction. This demonstrates that, during their elongation, maltooligosaccharides precipitate once they reach a critical length and concentration.

**Influence of Initial Sucrose Concentration on the Distribution of the Products Synthesized by Wild-type AS**—In order to examine the influence of the initial sucrose concentration on the polymerization reaction, the products synthesized by wild-type AS in the presence of sucrose ranging from 100 to 900 mM were analyzed. The production of insoluble glaucan was optimal at 300 mM sucrose with a remarkably high yield (72%) (Fig. 4). This was correlated with a very low accumulation of soluble MOS from G2 to G25, compared with the reaction in the presence of 100 mM sucrose. At 600 mM sucrose, the formation of insoluble product decreased to 55%. This mainly occurred in favor of sucrose isomer synthesis, for which the yield reached 27%. This phenomenon was even more pronounced when starting with 900 mM sucrose, where 51% of the glucose released from sucrose was transferred onto fructose. However, in such conditions, a 30% inhibition of the enzyme activity (data not shown) was observed; only 57% of the sucrose initially introduced (i.e., 513 mM) was consumed. However, soluble MOS from G4 to G25 then accumulated in far greater proportions, indicating that, under these conditions, more hydrolysis occurred, resulting in larger amounts of smaller compound. Consequently, the chains were not elongated enough, and no insoluble fraction was formed.
Molecular Features Possibly Involved in the Polymerization Reaction—Special attention was paid to the interactions at binding site OB1 situated in the unique access channel to the active site (26) (Fig. 1). Several residues were identified in the aglycon site occupying critical positions (Fig. 5). In particular, Asp^{394} and Arg^{446} are hydrogen-bonded with the ring found at subsite +1 (fructosyl in the case of sucrose binding or glucosyl for maltooligosaccharide binding), and Arg^{226} makes two hydrogen bonds to the glucosyl moiety but could also move to subsite +3, whereas Arg^{415} provides a hydrophobic platform for the sugar ring at subsite +4, at the entrance of the channel to the active site. Interestingly, three of these four residues (Asp^{394}, Arg^{415}, and Arg^{446}) belong to the B-domain (loop 7), which is specific to AS (16). Arginine 226 is situated in the B-domain (loop 3). Among these residues, Asp^{394} is the only residue to be strictly conserved in putative amylomaltases (Fig. 6). It is situated just after His^{392} and Asp^{393}, which are always conserved in GH family 13 and are known to stabilize the glucosyl-enzyme intermediate (20, 23). The role of these four residues (Asp^{394}, Arg^{446}, Arg^{226}, and Arg^{415}) was investigated by site-directed mutagenesis. They were individually changed to alanine in order to prevent any hydrogen bonding or hydrophobic contacts with a maltooligosaccharide substrate.

Characterization of the Mutants at the OB1 Site in the Presence of 100 mM Sucrose—The relative specific activity of variants D394A and R446A was 23.5 and 15% of the wild-type activity, respectively, according to the initial rate of sucrose consumption. Although sucrose was not totally consumed in the conditions of the assay, the distribution of the products synthesized by these two mutants clearly showed that hydrolysis activity was predominant (Table I). The ratio [G]/([G2] + [G3]) was about 10 times higher in the case of D394A and R446A variants than for wild-type AS. Besides, the elongation of MOS of higher DP, and consequently polymer synthesis, were limited.

Mutant R415A had a very low but still appreciable activity compared with the wild-type enzyme (4.3%; Table I), indicating that this residue, distant from the active site, was very important for activity. Consequently, only 20% of initial sucrose was consumed in the assay conditions. Very few MOS with a DP higher than 4, and thus no insoluble fraction, were synthesized (Table I). This product distribution resembles that observed for the wild type at this stage of the reaction (data not shown). Unfortunately, the limited activity of the R415A mutant prevented total sucrose depletion from being reached in the reaction conditions used.

Particularly noteworthy is mutant R226A. The initial activity of this mutant was found to correspond to 30% of the
TABLE I

| Relative specific activity of wild-type and mutant AS and concentrations and reaction yields of the products obtained at the end of the reaction from 100 mM sucrose | Concentrations and reaction yields | Insoluble glucan | Soluble Glc, n > 3 | Soluble Glc, n > 3 | Soluble Glc, n > 3 | Soluble Glc, n > 3 |
|---|---|---|---|---|---|---|
| | | | | | | |
| | | | | | | |

Wild type

| Subsite, domain | Relative activity | Sucrose consumed | Glucose | Sucrose | Trehalose | Table II | Glucosyl moieties from sucrose onto this acceptor were limited, particularly in the case of variants R446A and R415A, which did not synthesize any insoluble modified glycogen. In contrast, variant R226A was strongly activated by glycogen. Its initial activity was similar to that of the wild-type enzyme (Table II). No soluble oligosaccharides were synthesized (data not shown), indicating that the glucosyl residues were exclusively transferred from sucrose onto glycogen branches.

Fluorimetry—In order to determine the influence of sucrose and acceptor (maltoheptaose and glycogen) binding on the overall conformation of the enzyme, fluorimetry experiments were performed on the enzyme alone and in the presence of these substrates. The inactive mutant E328Q was used for the assay to avoid interference due to reaction catalysis. The plots of thermal denaturation presented in Fig. 8 clearly show the appearance of an additional transition near 40 °C, becoming increasingly pronounced upon the addition of sucrose, maltoheptaose, and glycogen to the reaction medium. This demonstrates that these molecules modify the conformation of the enzyme, resulting in a local destabilization of the structure, probably necessary for activity.

DISCUSSION

Biochemical characterization, structural analyses, and site-directed mutagenesis experiments were combined to gain new insights into the molecular basis of the polymer synthesis catalyzed by AS in the presence of sucrose as sole substrate.

Elucidation of the Initiation and Elongation Steps—Biochemical analyses of the kinetics of polymer synthesis demonstrated that the insoluble amylase-like polymer is formed by elongation of the soluble maltooligosaccharides produced by AS. At the beginning of the reaction, there is only sucrose in the medium, and once the glucosyl-enzyme intermediate has been formed, transfer onto water occurs (no transfer onto sucrose itself or fructose being detected during the first stage of the reaction). The glucose released is subsequently used as an acceptor to form maltose that is released and at some point glucosylated to form maltotriose, and so forth. The orientation of the maltoheptaose molecule bound in the acceptor binding
site OB1 (26) reveals that the transfer occurs at the nonreducing end of the chain (Fig. 5). The MOS produced are elongated until they reach a critical size and concentration responsible for chain precipitation. This corresponds to the formation of the insoluble fraction. Aggregation is thought to displace the equilibrium toward insoluble chain formation, preventing the accumu-
mulation of soluble maltooligosaccharides. Thus, unlike in the previously suggested processive mechanism (12), this study, using more sensitive analytical methods, reveals that amylose-like polymer formation is nonprocessive. In addition, we clearly showed that the insoluble fraction contains polydisperse maltooligosaccharides. However, determination of the mean size and of the polydispersity of the insoluble products will now be necessary to compare our values with those previously reported (12).

Furthermore, this study illuminates the controversial question of the polymer synthesis mechanism of glucansucrases. In contrast to the mechanism proposed by Robyt et al. (13) that consists of an elongation at the reducing end involving two catalytic sites, our data rather support the theory of Mooser et al. (14, 15), except as concerns the requirement for a primer. Besides, the demonstration of a nonprocessive mechanism is provocative, in regard to the fact that a processive mechanism.

**TABLE II**

| Mutant   | Relative activity | Activation factor of glycogen | Sucrose consumed | Insoluble glucan |
|----------|-------------------|-------------------------------|------------------|------------------|
| Wild type| 100 (100,000 units/g) | 100                           | 100              | +++              |
| D394A    | 0.28              | 1.2                           | 79               | +                |
| R446A    | 0.12              | 0.8                           | 14               | –                |
| R415A    | 0.38              | 8.8                           | 55               | –                |
| R226A    | 108               | 360                           | 100              | +++              |

* a The activation factor of glycogen corresponds to the increase of activity observed when glycogen at 30 g/liter is added to 100 mM sucrose compared with the activity in the presence of 100 mM sucrose alone.

b Insoluble polymer formation is estimated visually.
has always been suggested for glucansucrases. However, like AS, glucansucrases from GH family 70 may be able to glucosylate long compounds as efficiently as shorter ones. Kinetic analyses of polymer formation would be very informative to deepen the understanding of the mechanism of family 70 glucansucrases and to more accurately compare it with the AS mode of action.

The Importance of Key Residues at the Acceptor Binding Site OB1—The enzymatic behavior of the variants D394A and R446A revealed that both mutations resulted in increased accumulation of glucose, showing a decreased affinity for this acceptor. Furthermore, no stimulating effect of glycogen was observed, emphasizing the role of these residues in the correct positioning of the glucosyl residue at subsite \( \text{H}^{+1} \) and consequently in the transglucosylation reaction. The modification of the acceptor binding site induced by mutations D394A and R446A facilitates the access of water to the active site, enhancing the hydrolysis reaction. Besides, the residual activity measured for the D394A and R446A variants revealed that sucrose binding and catalysis was still possible without a full contribution of the hydrogen bonding network at subsite \( \text{H}^{+1} \), indicating that amino acids Asp\(^{394} \) and Arg\(^{446} \) are not crucial for sucrose specificity.

Particularly noticeable is the drastically reduced activity of the R415A mutant in the presence of sucrose alone or supplemented with glycogen. We propose that the side chain of Arg\(^{415} \) provides a hydrophobic interaction at subsite +4 that is essential for the binding and the guidance of MOS acceptors. Above subsite +4, strong binding subsites may also exist, so the newly formed MOS (having a DP higher than 3) are efficiently glucosylated, approximately at the same rate. Strong subsites at +4 and above prevent MOS having a DP lower than 4 from being good acceptors.

In addition, this phenomenon is accentuated by the presence of Arg\(^{226} \) at subsites +2/+3. Indeed, we have shown that the mutant R226A has a marked ability to elongate MOS and to synthesize an insoluble fraction. This variant has a higher affinity than wild-type enzyme toward the MOS produced, in particular the smaller ones (maltose and maltotriose), which are much more efficient acceptors and, consequently, activate the enzyme. Arg\(^{226} \) probably causes steric hindrance at the acceptor binding site OB1. The side chain of Arg\(^{226} \), which can move from subsite \( \text{H}^{+2} \) to subsite \( \text{H}^{+3} \) of OB1, may interfere with MOS binding. This is the case especially for maltose and maltotriose, the binding of which cannot be strengthened by anchoring at subsite +4. Consequently, once formed, maltose and maltotriose are poorly glucosylated and accumulate in the medium. Replacing Arg\(^{226} \) with a small residue such as an alanine, we improved the polymerase activity of the AS and drastically reduced side reactions. The mutant R226A is consequently a very promising enzyme for the industrial synthesis of amylose-like polymers.

To sum up, two critical arginine residues at binding site OB1, Arg\(^{226} \) (subsites +2 and +3) and Arg\(^{415} \) (subsite +4) are mainly responsible for the remarkable difference of accumulation observed in this biochemical process between maltose and maltotriose and MOS of higher DP. This perfectly corroborates the results of Becker et al. (29), who reported that glucosylation of maltotriose by AS is minor when the enzyme is in the presence of sucrose and MOS acceptors ranging from G3 to G6. In addition, these data are also in accordance with the size selectivity previously demonstrated in the case of the disproportionation of maltooligosaccharides. MOS must be composed of at least five glucosyl units to be efficient glucosyl donors in the absence of sucrose (2). Interestingly, it is noteworthy that Arg\(^{226} \) and Arg\(^{415} \) are not conserved in the sequence of putative
The Importance of the B'-Domain: Possible Allosteric Phenomena—Fluorimetry analyses provided the first biophysical evidence that sucrose binding creates a local destabilization of the structural analysis of the E328Q-G7 complex (26). The conformational changes detected by fluorimetry analyses are due to B'-domain movement. First, since polymerisation results provide informative data to rationally improve the structural determinant endowing the amylosucrase with a polymerase activity. The elucidation of the mechanism of polymer synthesis by glucansucrase, which was for the first time conclusively shown to be nonprocessive, makes a strong contribution to the emergent field of the biosynthesis of carbohydrate polymers.

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amylosucrases (Fig. 6). This suggests that each putative amylosucrase may have distinct features for their acceptor binding site and, consequently, different affinities toward maltoligosaccharides. It can be speculated that amylosucrases having a small residue at the position corresponding to Arg299 (such as amylosucrase from X. axonopodis, which possesses a glycine at this position) may be polymerases far more efficient than amylosucrase from N. polysaccharea.