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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. Maltooligosaccharides 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 and to elongate maltooligosaccharides is notably due to the presence of key residues at the OB1 acceptor binding site that contribute strongly to the guidance (Arg^{415}, subsite +4) and the correct positioning (Asp^{394} and Arg^{446}, subsite +1) of acceptor molecules. On the other hand, Arg^{226} (subsites +2/+3) limits the binding of maltooligosaccharides, 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)[1] family 13 (1, 2).[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 glycogen 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 glucans) (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 maltooligosaccharides 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 dextransucrase, a glucansucrase produced by *Leuconostoc mesenteroides* NRRL B-512F classified in GH family 70, follows a processive mechanism with an elongation by the reducing end involving two active sites. In contrast, a mode of polymer elongation by the nonreducing end is proposed by Mooser *et al.* (14, 15). To date, neither of these mechanisms has been elucidated at the molecular level because of the absence of any three-dimensional structure for glucansucrases.

The AS three-dimensional structure is the only glucansucrase structure to have been solved (16). It reveals the three common domains usually found in GH family 13: a catalytic

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[1] The abbreviations used are: GH, glycoside-hydrolase(s); AS, amylosucrase from *N. polysaccharea*; MOS, maltooligosaccharide(s); DP, degree of polymerization; G*n*, maltooligosaccharide with a degree of polymerization of *n*; SB, sucrose binding site; OB, oligosaccharide binding site; HPLC, high performance liquid chromatography; HPAEC, high performance anion exchange chromatography; PBS, phosphate-buffered saline; GST, glutathione *S*-transferase; MOPS, 4-morpholinepropanesulfonic acid.

[2] P. M. Coutinho and B. Henrissat (1999) Carbohydrate-Active Enzymes server on the World Wide Web at afmb.cnrs-mrs.fr/~cazy/CAZY/index.html.

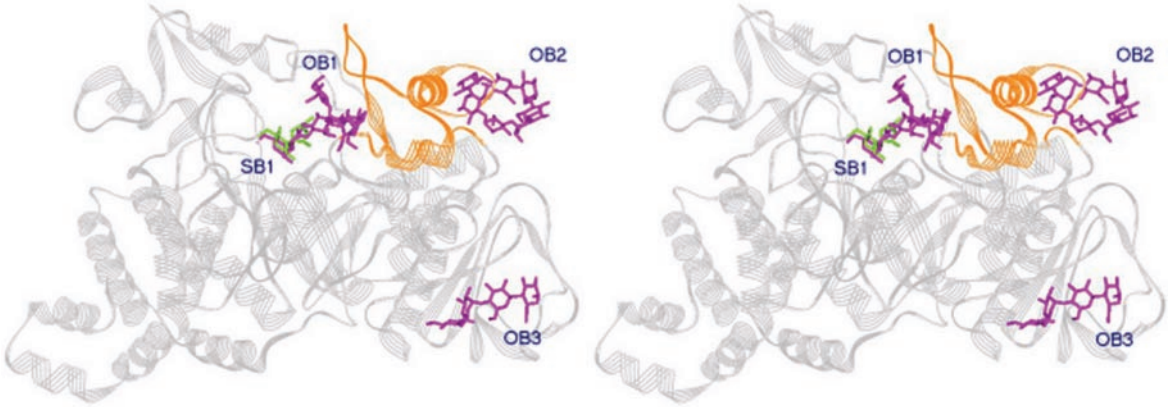

FIG. 1. **Stereo image illustrating the sucrose binding site SB1 (*green*) and the oligosaccharide binding sites OB1, OB2, and OB3 (*pink*) on AS.** The B′-domain is colored *orange*.

$(\beta/\alpha)_8$-barrel fold called the A-domain, a B-domain between β-strand 3 and α-helix 3, and a C-terminal Greek key domain (16). Unlike glucansucrases of GH family 70 from lactic acid bacteria (dextransucrase, alternansucrase, and mutansucrase) (17), for which a circularly permutated $(\beta/\alpha)_8$-barrel-fold is predicted (18), the catalytic A-domain of AS adopts a nonpermutated $(\beta/\alpha)_8$-barrel fold like all of the enzymes of GH family 13 (19). Structural analyses supported by single-site mutational experiments enabled the assignment of the nucleophile (Asp^{286}) and of the acid/base catalyst (Glu^{328}) (20). The importance of three additional residues (Asp^{393}, His^{187}, and His^{392}), also conserved in GH family 13 and known to assist in catalysis, has been demonstrated. As a member of GH family 13, it has been assumed that AS catalysis proceeds through an α-retaining mechanism involving the formation of a covalent glucosyl-enzyme intermediate (21–23). The AS structure also reveals two novel domains of unknown function: an N-terminal helical domain and a B′-domain corresponding to an extended loop between β-strand 7 and α-helix 7 of the barrel. The structure of the inactive mutant E328Q complexed with a sucrose molecule (24) gave a detailed description of the interaction between the amino acid residues in the active site named SB1 (situated at the bottom of a narrow pocket) and the natural substrate. Four residues not conserved in GH family 13 were found to occupy key positions; Asp^{144} and Arg^{509} form a salt bridge that blocks the bottom of the active site, and Asp^{394} and Arg^{446} interact with the fructosyl ring at subsite +1. These amino acids are thought to be responsible for the specificity of AS toward sucrose (24, 25). Finally, the E328Q-maltoheptaose complex (26) revealed the presence of three oligosaccharide binding sites named OB1, OB2, and OB3 (Fig. 1). OB1 spans the −1 and +1 binding sites (the active site) and five additional acceptor subsites (+2, +3, +4, +5, and +6), which were mapped out and numbered according to the nomenclature defined by Davies *et al.* (27). The nonreducing end of the maltoheptaose (G7) molecule bound in OB1 occupies the −1 subsite. OB2 is more distant from the active site and is exposed at the surface of the protein in the B′-domain. OB3 is situated in the C-domain, and its function has not been attributed. Of the 57 residues of the B′-domain, 6 have contacts to G7 at OB2, and 8 have contacts to G7 at OB1. Consequently, this domain is thought to play a pivotal role in structural changes and in the polymerase activity (26).

Here, we propose to unequivocally establish the mechanism of polymer formation by amylosucrase from *Neisseria polysaccharea*. First, this required a detailed biochemical investigation of the kinetics of polymer synthesis from sucrose using sensitive analytical methods. Biochemical data were then examined with respect to the structural information of AS in complex 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′-GACCGGACCCTG**GCC**GAAATCTTCCCCGACCAGCACCCG-3′ AvaII; R226A_rev:5′-CGGGTGCTGGTCGGGGAAGATTTC**GGC**CAGGGTCCGGTC-3′ AvaII; D394A_for, 5′-CGCAGCCACGAC**GCC**ATCGGCTGGACCTTTGCC-3′ AvaII; D394A_rev, 5′-GGCAAAGGTCCAGCCGAT**GGC**GTCGTGGCTGCG-3′ AvaII; R415A_for, 5′-GCATACCTAGGCATAAGCGGCTACGACCAC**GCC**CAATTCCTC-3′ AvrII; R451A_rev, 5′-GAGGAATTG**GGC**GTGGTCGTAGCCGCTTATGCCTAGGTATGC-3′ AvrII; R446A_for, 5′-GGCGACTGC**GCT**GTCAGTGGTACAGCGGCGGC-3′ SacII*; R446A_rev, 5′-GCCGCCGCTGTACCACTGAC**AGC**GCAGTCGCC-3′ SacII*. The mutations were confirmed by DNA sequencing (Genome Express, Grenoble, France).

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 $\mu g \cdot ml^{-1}$) and isopropyl-β-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 A_{600} 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 (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 dithiothreitol). The protein content was determined by the micro-Bradford 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 (Am-

ersham Biosciences) ready made gels under denaturing conditions. Staining with 0.5% (w/v) $AgNO_3$ led to a single-band profile.

Kinetics of Soluble Compound Formation from 100 mM Sucrose Using Wild-type AS—Reaction in the presence of 100 mM sucrose was carried out at 30 °C in PBS buffer at pH 7.3, using pure wild-type AS at 375 mg/liter. During sucrose consumption, a white precipitate was observed in the medium, corresponding to the insoluble polymer. The soluble and insoluble fractions were separated by centrifugation (5 min, 10,000 × *g*). During the reaction (2 h), samples from the soluble fraction were collected at regular intervals from 5 min and analyzed using HPLC on Dionex, C18, and Aminex columns for quantification. At the end of the reaction, the insoluble fraction was solubilized and analyzed by HPAEC (see "Carbohydrate Analysis").

Effect of Sucrose Concentration on the Distribution of the Products Synthesized by Wild-type AS—Reactions with the wild-type AS at 375 mg/liter were performed at 30 °C in PBS buffer at pH 7.3 in the presence of sucrose concentrations ranging from 100 to 900 mM. The sucrose was totally consumed except from 900 mM. At the end of the reaction, the soluble and insoluble (when formed) fractions were separated by centrifugation (5 min, 10,000 × *g*) and analyzed by HPAEC. The yields of the soluble compounds were determined by HPLC analyses (see "Carbohydrate Analysis") and were expressed as a percentage of the sucrose consumed. The quantity of insoluble glucan synthesized was determined as the difference between the amount of sucrose consumed and the amount of glucosyl residues released by hydrolysis or in the form of soluble oligosaccharides.

Comparison of Wild-type and Mutated AS—To compare wild-type and mutated enzymes, reactions were carried out at 30 °C in PBS buffer at pH 7.3 in the presence of 100 mM sucrose alone or supplemented with 30 g/liter glycogen. Purified wild-type or mutated GST/AS was employed at the following concentrations of AS (calculated to approach the same activity for each reaction): wild type, 100 mg/liter; R226A, 72 mg/liter; D394A, 247 mg/liter; R415A, 272 mg/liter; R446A, 120 mg/liter.

At the end of the reaction in the presence of sucrose alone, the soluble and insoluble (when formed) fractions were separated and analyzed as described above for the wild-type AS assay. When glycogen was present in the medium, only the soluble fraction was analyzed by HPAEC.

The specific activities were determined by measuring the initial rate of sucrose consumption, in the assay conditions. The specific activity of the variants was expressed relative to the specific activity of the wild-type AS.

Carbohydrate Analysis—Soluble and insoluble oligosaccharides and sucrose isomers produced during the reaction were identified by HPAEC using a Dionex Carbo-Pack PA100 column at 25 °C. Before analysis, the insoluble fraction was first washed three times with one volume of water and solubilized in one volume of 1 M KOH at 4 °C for 15 h. Mobile phase (150 mM NaOH) was used at a 1 ml/min flow rate with a sodium acetate gradient (from 6 to 600 mM in 60 min). Detection was performed using a Dionex ED40 module with a gold working electrode and an Ag/AgCl pH reference. HPAEC allowed quantification of sucrose isomers.

Sucrose, glucose, and fructose concentrations were measured by ion exchange chromatography at 25 °C using an Aminex HP87H column (Bio-Rad), with 8.5 mM H_2SO_4 at 0.5 ml/min as eluant. Detection was performed by refractometry.

Maltooligosaccharide quantification was performed at 25 °C with an octadecyl reverse-phase chromatography column (C18) (Bischoff Chromatography), eluted with water at 0.5 ml/min. Detection was performed by refractometry.

Fluorimetry—The fluorimetry experiments were performed using the inactive mutant E328Q purified as described previously (20). The spectrum of denaturation was obtained using purified E328Q variant at 50 mg/liter in fluorimetry buffer (30 mM MOPS, 125 mM NaCl, pH 7.0) without any substrate or supplemented with 100 mM sucrose, 20 mM maltoheptaose or 10 g/liter glycogen. Thermal denaturation was followed at the rate of 1 °C/min at 334 nm.

RESULTS

Characterization of the Soluble and Insoluble Products Synthesized by Wild-type AS in the Presence of 100 mM Sucrose—To investigate the mode of polymer formation, reaction in the presence of 100 mM sucrose was first performed using wild-type AS. The soluble and insoluble fractions formed were both analyzed by HPAEC.

The chromatogram of the soluble fraction (Fig. 2*A*, 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. 2*B*). 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. 3*B* 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. 3*B*). Sucrose isomer formation increased with the accumulation of fructose in the reaction medium (Fig. 3*A*). 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 glucan 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.

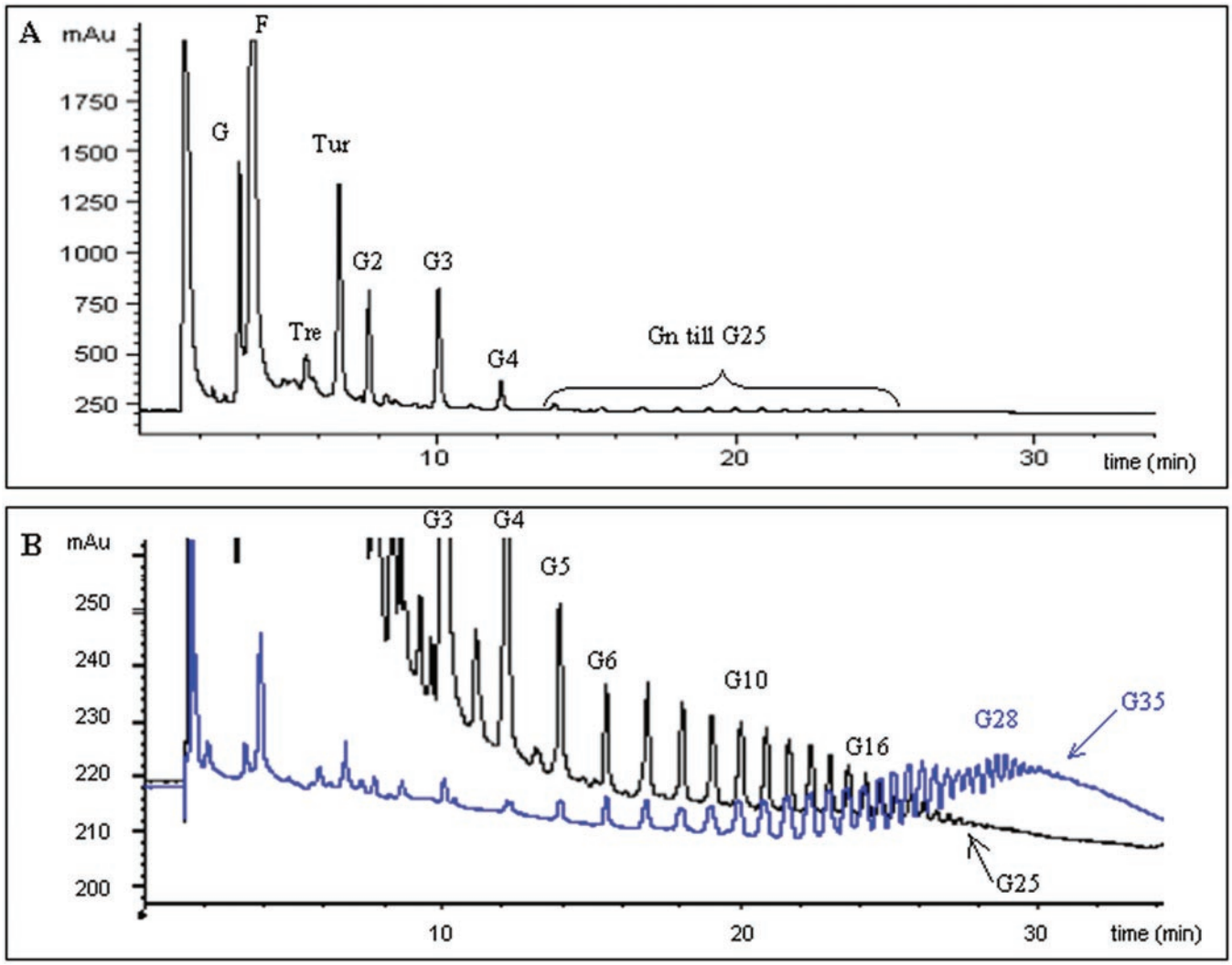

FIG. 2. **Dionex HPAEC profile of the soluble and insoluble fractions, obtained at the end of the reaction (t = 2 h) using wild-type AS in the presence of 100 mM sucrose.** *A*, full scale of the profile of the soluble fraction; *B*, superposition of the profile of the soluble fraction (*black*) (*enlarged scale*) with the profile of the insoluble fraction (*blue*). *G*, glucose; *F*, fructose; *Tre*, trehalulose; *Tur*, turanose; *Gn*, maltooligosaccharides of ΔPn.

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 +2 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 amylosucrases (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

Mutant	Subsite, domain	Relative activity	Sucrose consumed	Concentrations and reaction yields						
				Glucose	Turanose	Trehalulose	G2	G3	Soluble Gn, $n > 3$	Insoluble glucan
		%	%				*mM*, %			
Wild type		100 (1000 units/g)	100	5 mM, 5 %	14 mM, 14 %	3 mM, 3 %	2.3 mM, 5 %	5.3 mM, 16 %	0.03-0.05 mM, 12 %	ND,[a] 45 %
D394A	+1, B′	23.5	60	38 mM, 63 %	5 mM, 8.5 %	1 mM, 1.5 %	4.2 mM, 14 %	1.4 mM, 7 %	<0.02 mM, 6 %	0 %
R446A	+1, B′	15	16	11 mM, 69 %	0.6 mM, 4 %	0.2 mM, 1 %	1.2 mM, 15 %	0.5 mM, 10 %	<0.01 mM, 1 %	0 %
R415A	+4, B′	4.3	20	5 mM, 25 %	1 mM, 5 %	0.2 mM, 1 %	1.8 mM, 18 %	3.2 mM, 48 %	<0.01 mM, 3 %	0 %
R226A	+2/+3, B	30	100	3 mM, 3 %	2.5 mM, 2.5 %	0.5 mM, 0.5 %	0.5 mM, 1 %	1.5 mM, 4.5 %	<0.01 mM, 3.5 %	ND 85 %

[a] ND, not determined.

wild-type activity. Despite this apparently lower activity, this variant was 1.5 times more efficient in consuming all of the sucrose (Table I, Fig. 7). The kinetic profile of sucrose consumption clearly shows that the enzyme was increasingly active during the reaction course, suggesting that mutant R226A was activated by the reaction products. Even more noticeable was the yield of soluble products synthesized (glucose, sucrose isomers, and MOS), which decreased to only 15% (compared with the 55% yield obtained with the wild-type enzyme) (Table I). This was to the benefit of the insoluble glucan yield, which reached 85%. This result demonstrates that this variant was particularly efficient for catalyzing the elongation of MOS and polymer synthesis, at the cost of the hydrolysis reaction and the sucrose isomer formation.

Characterization of the Mutants at Site OB1 in the Presence of 100 mM Sucrose and Glycogen (30 g/liter)—To further characterize the activity of the mutants, reactions were carried out in the presence of sucrose (100 mM) and glycogen (30 g/liter). In these conditions, the wild-type AS was very efficiently activated (the k_{cat} was increased 100-fold) and transferred the glucosyl residues exclusively onto glycogen branches, which act as acceptor (5). In contrast, the variants D394A, R446A, and R415A were very poorly or even not activated by glycogen as shown in Table II. A qualitative HPAEC analysis revealed that, unlike the wild-type enzyme, these mutants still catalyzed the synthesis of soluble saccharides, like in the absence of glycogen (data not shown). The transfer of glucosyl moieties from sucrose onto this acceptor was 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 amylose-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

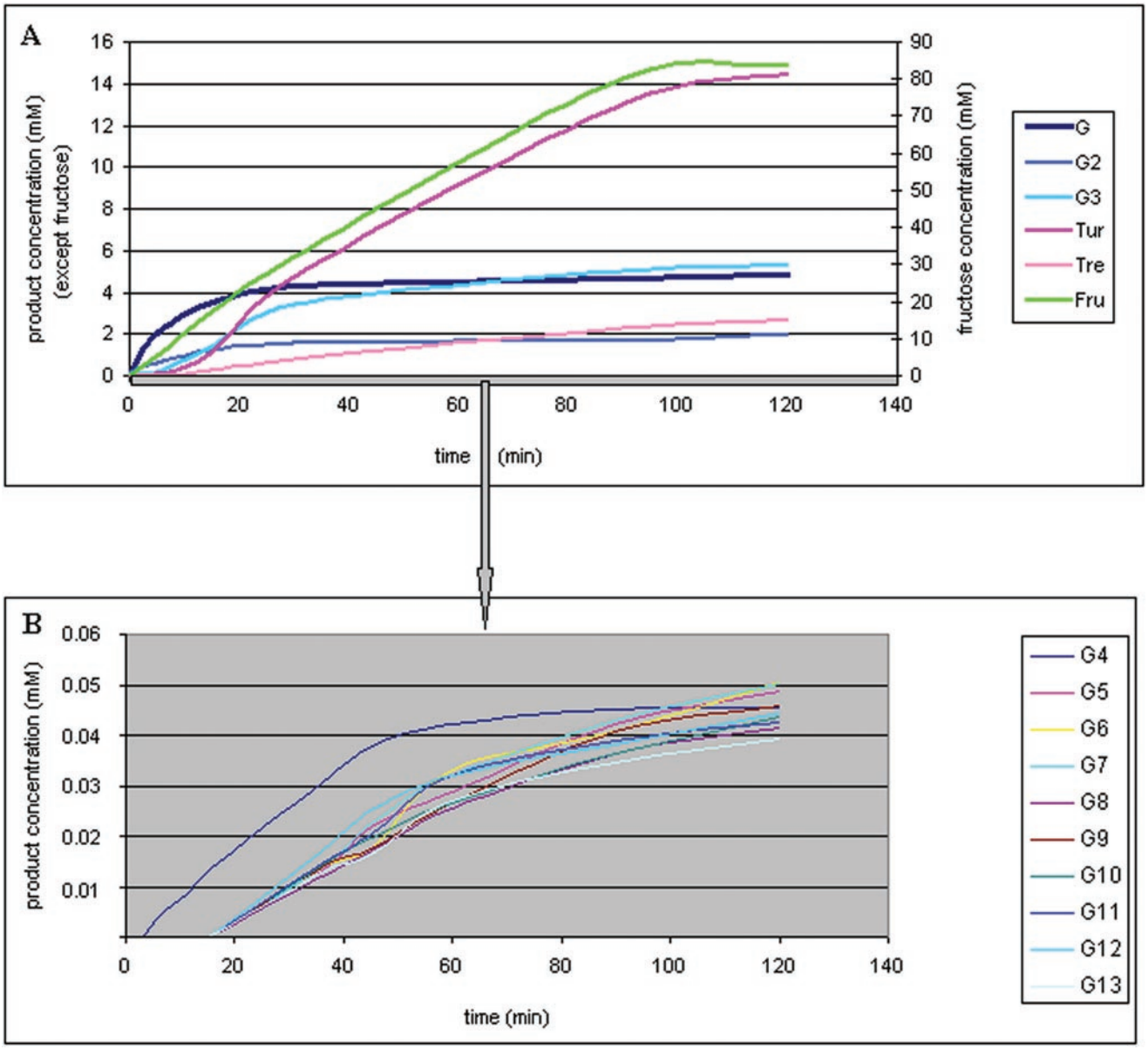

FIG. 3. **Kinetics of glucose and soluble oligosaccharide synthesis using wild-type AS in the presence of 100 mM sucrose.** *A*, fructose, glucose, and oligosaccharides having a degree of polymerization lower than 4; *B*, maltooligosaccharides from G4 to G13.

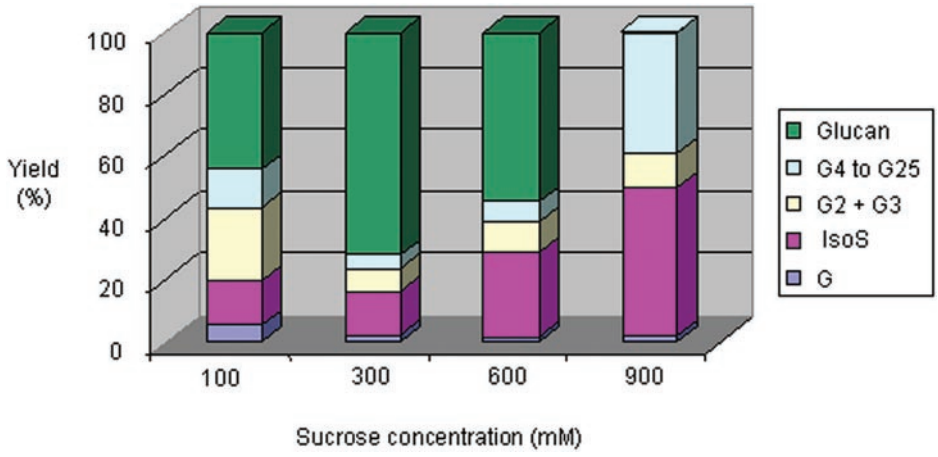

FIG. 4. **Distribution of the products synthesized by wild-type AS from various sucrose concentrations.** The yields are expressed as a percentage of the sucrose consumed. *G*, glucose; *IsoS*, sucrose isomers; *Gn*, maltooligosaccharides of DP *n*; *Glucan*, insoluble fraction.

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 accu-

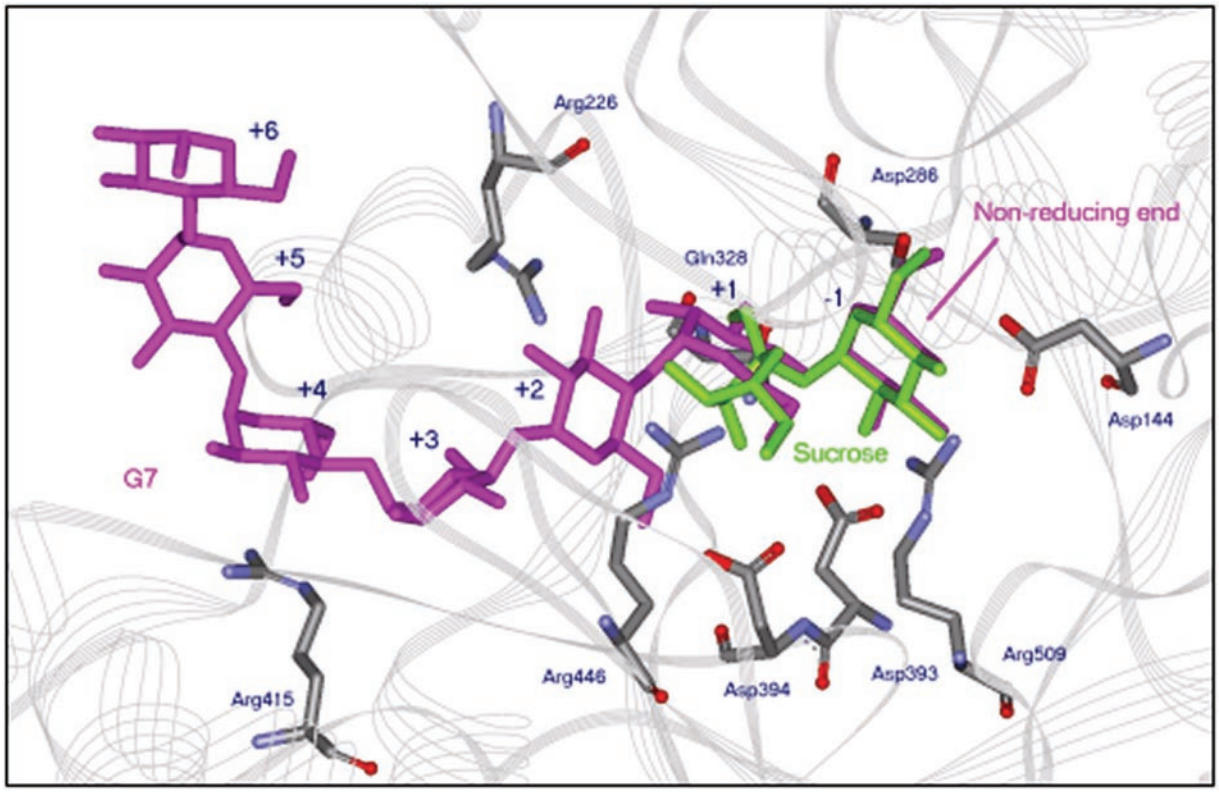

FIG. 5. **Superposition of the sucrose (*green*) and the maltoheptaose (*pink*) molecules occupying SB1 and OB1 binding sites, respectively.**

N.poly	225_LREIFPDQHP	388_YVRSHDDI	414_HRQFLNRFF	443_GDCRVSGTA
X.axo	LGQVFPHTAP	YVRCHDDI	SLRDVARFY	GVHGTNGMA
X.camp	LVQVFPQTAP	YVRCHDDI	SLREVAQFY	GVHGTNGMS
C.cresc	LIDVFPDTAP	YVRCHDDL	DLRRWSNAY	GVPSTNGMA
D.radio	LPEIFPDFAP	YVRCHDDI	HRHFLSDFY	GDRRISGTA
Piru	LREIFPTVRR	YLRCHDDI	HRQFLNAFY	GDMRISGTL

FIG. 6. **Sequence alignments of putative amylosucrases.** The regions aligned contain the residues mutated in the present study. *N. poly*, *N. polysaccharea*; *X. axo*, *X. axonopodis*; *X. camp*, *X. campestris*; *C. cresc*, *C. crescentus*; *D. radio*, *D. radiodurans; Piru*, *Pirullela* sp.

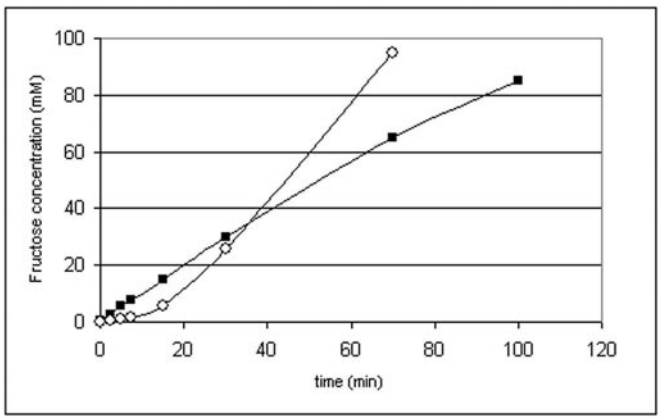

FIG. 7. **Kinetics of fructose release using wild-type AS (■) and R226A mutant (○) in the presence of 100 mM sucrose.**

TABLE II

Relative specific activity of wild type and mutant AS and insoluble polymer formation obtained at the end of the reaction in the presence of 100 mM sucrose and 30 g/liter glycogen

Mutant	Relative activity	Activation factor of glycogen[a]	Sucrose consumed	Insoluble glucan[b]
	%		%	
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.

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

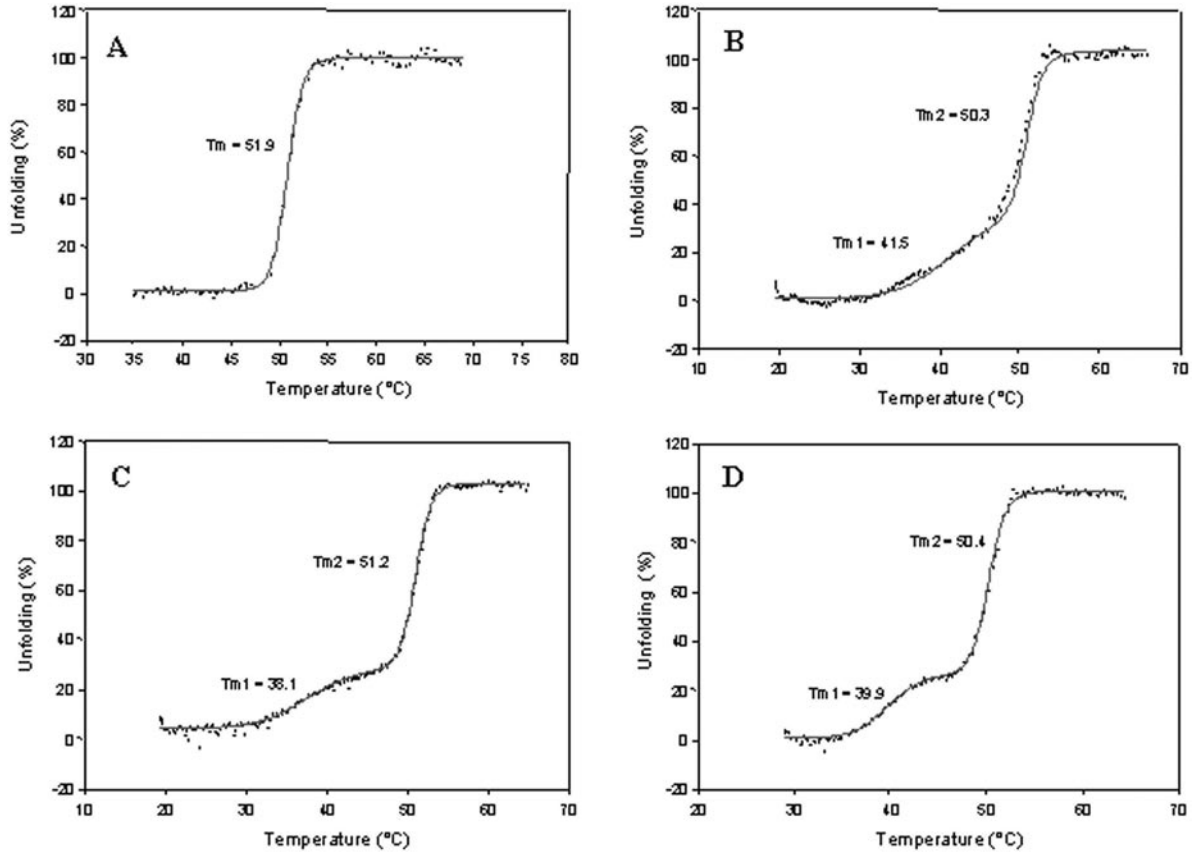

FIG. 8. **Thermal unfolding of the inactive mutant E328Q analyzed by fluorimetry.** Spectra were realized with the enzyme alone (*A*) and in the presence of 100 mM sucrose (*B*), 20 mM maltoheptaose (*C*), or 10 g/liter glycogen (*D*).

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 +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 +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 +2 to subsite +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

amylosucrases (Fig. 6). This suggests that each putative amylosucrase may have distinct features for their acceptor binding site and, consequently, different affinities toward maltooligosaccharides. It can be speculated that amylosucrases having a small residue at the position corresponding to Arg^{226} (such as amylosucrase from *X. axonopodis*, which possesses a glycine at this position) may be polymerases far more efficient than amylosucrase from *N. polysaccharea*.

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 enzyme, revealing a certain flexibility. This conformational change is even more pronounced upon maltoheptaose and glycogen binding. Since oligosaccharide binding sites OB1 and OB2 contain numerous amino acids belonging to the B′-domain (14 of the 57 residues of the B′-domain are involved in the binding of maltoheptaose at OB1 and OB2), we can assume that the conformational changes detected by fluorimetry analysis are due to B′-domain movement. Small but appreciable B′-domain displacement has already been observed through the structural analysis of the E328Q-G7 complex (26). The B′-domain, constituted by loop 7 specific for AS, is expected to be particularly flexible during polymer synthesis. These allosteric changes are thought to facilitate the transglucosylation reaction. They could be correlated to the increase of activity observed in the presence of glycogen (5). To deepen our understanding of polymer synthesis, dynamic modeling experiments would be very informative. They would help to assess the amplitude of the suspected movement and to describe the trajectories followed by the substrates and the products when they enter and leave the active site.

Rational Control of the Reactions—Our biochemical and mutagenesis results provide informative data to rationally improve or modify the activity of the enzyme, in particular for a better control of the polymerization reaction. First, since polymer synthesis results from the nonprocessive elongation of maltooligosaccharides, amylose-like chain length can be controlled by the initial substrate conditions employed, such as the sucrose concentration or the addition of MOS acceptors. Besides, rational or combinatorial mutagenesis approaches can also be applied to modify the affinity of AS toward MOS. In particular, the modulation of the binding strength at sites OB2 and OB3 may limit chain elongation, leading to an amylose-like product of controlled size. Finally, AS was successfully changed into an improved polymerase by replacing a key residue of site OB1. The R226A mutant constructed has remarkable properties (low yield of side products, high efficiency) that may further be improved by combining additional positive mutations.

Conclusion—The role of key residues of the acceptor binding site OB1 in the transglucosylation reaction (Asp^{394} and Arg^{446}) and in the anchoring and guidance of the chains to be elongated (Arg^{415}) was demonstrated in this study. These residues, all belonging to the B′-domain, ensure the elongation of the maltooligosaccharides initially produced, preventing the hydrolysis reaction. Their action is probably strengthened by other amino acids, especially of the B′-domain that is undoubtedly the structural determinant endowing the amylosucrase with a polymerase activity. The elucidation of the mechanism of polymer synthesis by a 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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