Generation of Amylosucrase Variants That Terminate Catalysis of Acceptor Elongation at the Di- or Trisaccharide Stage[†]

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An amylosucrase gene was subjected to high-rate segmental random mutagenesis, which was directed toward a segment encoding amino acids that influence the interaction with substrate molecules in subsites –1 to +3. A screen was used to identify enzyme variants with compromised glucan chain elongation. With an average mutation rate of about one mutation per targeted codon, a considerable fraction (82%) of the clones that retained catalytic activity were deficient in this trait. A detailed characterization of selected variants revealed that elongation terminated when chains reached lengths of only two or three glucose moieties. Sequencing showed that the amylosucrase derivatives had an average of no more than two amino acid substitutions and suggested that predominantly exchanges of Asp394 or Gly396 were crucial for the novel properties. Structural models of the variants indicated that steric interference between the amino acids introduced at these sites and the growing oligosaccharide chain are mainly responsible for the limitation of glucosyl transfers. The variants generated may serve as biocatalysts for limited addition of glucose moieties to acceptor molecules, using sucrose as a readily available donor substrate.

Amylosucrase (AS) is a glucosyltransferase (EC 2.4.1.4) that was first isolated from bacteria belonging to the genus *Neisseria* and that transfers a D-glucose (Glc) residue, typically obtained from sucrose (Suc) as the donor, to acceptor molecules such as Glc itself, D-fructose (Fru), or α -1,4-glycosidically linked Glc oligomers and polymers, particularly glycogen (9, 14, 19, 20, 21, 25, 36). While use of Fru as the acceptor gives rise to Suc isomers (or reformation of Suc), use of Glc as the acceptor leads to further elongation to form α -1,4-linked malto-oligosaccharides (designated G2, G3, etc.), which are extended to amylose-like glucans. The enzyme consists of a single polypeptide chain consisting of about 640 amino acid residues (22). It has been categorized as a member of family 13 of the glycoside hydrolases (10). Catalysis by AS involves a two-step mechanism (13, 30). The first step is a nucleophilic attack by the Asp286 side chain at Suc C-1 to displace Fru and form an AS-glucosyl intermediate. Subsequently, this activated ester is typically attacked by a hydroxy group at the nonreducing end of a growing glucan chain, resulting in chain elongation. It can also be attacked slowly by water. The latter reaction, yielding Glc, is an essential step for glucan formation in the presence of Suc as the sole substrate, as neither Suc itself nor Fru serves as a chain initiator (2).

Three-dimensional structures have been determined for AS, the AS-Glc intermediate, and various complexes consisting of AS or the inactive Glu328Gln variant and Glc, Suc, or G7 (13, 16, 32, 33). In combination with generation and characterization of AS variants, this has yielded a wealth of information about the reaction mechanism and the residues involved in catalysis and substrate binding (1, 30). These investigations also identified some residues that influence glycogen binding or chain elongation. Thus, replacement by Ala of Asp394, Arg446, or Arg415, which contact an active-site-bound maltoheptaose molecule at subsite +1 or +4 (33), increased Suc hydrolysis and the percentage of G2 and/or G3 formed at the expense of polymer synthesis (2). Furthermore, replacement by Ala of Arg226, which contacts G7 at subsite +2 (33), led to a larger fraction of insoluble glucans instead of short products (2). A twofold reduction in activation of the enzyme by glycogen was a consequence of the Phe417Ala change, an amino acid residue found to be located at the AS surface where binding of a second maltoheptaose molecule has been observed (4).

As glucansucrases like AS use Suc as an inexpensive donor substrate and have fairly broad acceptor ranges (3, 17, 24, 31), they are biotechnologically interesting as catalysts for the glucosylation of carbohydrate molecules, as well as noncarbohydrate molecules. Thus, suppression of the undesired formation of glucans and of the multiple addition of sugar moieties to acceptor molecules is of considerable interest. In order to obtain enzyme variants, we used a segmental random mutagenesis method and a screen to identify AS variants with deficiencies in polymer synthesis. For selection of the positive variants obtained, chain elongation properties were characterized, amino acid changes were identified, and structural modeling was used to interpret the findings.

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MATERIALS AND METHODS

Chemicals, including oligonucleotides. Chemicals were the highest purity available. Biochemical-grade glycogen with a molecular mass range of 2.7×10^5 to 3.5×10^6 Da was obtained from Merck (Darmstadt, Germany). Primers AS677+ (CCGACCAATACGACCGCACCTG), AS1434– (GAGTTTGATG CCGTCAACGGCG) (desalted), and AS1191– (GACGTAGTTGACCCAG GCG) (HYPUR purified) were purchased from MWG-Biotech AG (Ebersberg, Germany). Degenerate oligonucleotide ASM5High1181+ (TCAACTACGTC CGAGCCACGACGACATCGGCTGGACGTTTGC) was synthesized and purified by polyacrylamide gel electrophoresis (PAGE) by IBA GmbH (Göttingen, Germany); during synthesis, 70% of the wild-type (WT) nucleotide precursor and 10% of each non-WT nucleotide precursor were used at each degenerate position (underlined).

Bacterial strains and plasmids used. The *E. coli* strain used for construction of the library of mutant genes encoding the AS of *Neisseria polysaccharea* ATCC 43768 was ElectroTen-Blue (Stratagene, Amsterdam, The Netherlands). The plasmid used for expression of the genes was pJQA1000, which confers resistance to ampicillin. Details concerning its construction will be published elsewhere. Briefly, a translational fusion was generated between the AS gene and the His tag-encoding sequence of expression vector pQE-81L (Qiagen, Hilden, Germany). Transcription was controlled by fusion of a phage T5 promoter with the *E. coli lac* operator.

Generation of the mutant AS gene library. Primers AS677+ and AS1191– and primers ASM5High1181+ and AS1434– were used to generate overlapping WT and mutant DNA segments in separate PCRs (27). They were fused by overlap extension PCR (11) so that they included two restriction sites for cloning. Using previously described procedures (29), the WT XmaI/SacII restriction fragment was replaced by the corresponding PCR products. Competent cells of *E. coli* ElectroTen-Blue were transformed by using the corresponding ligation mixtures and were plated onto QTray plates (X6023; Genetix, Hampshire, United Kingdom) for automated colony picking using a QPix2 XT robot (Genetix). Colonies were transferred to 384-well low-profile X6001 microplates (Genetix) containing 50 μ l LB medium (29) supplemented with 50 μ g/ml kanamycin and 200 μ g/ml ampicillin. The plates were incubated for 24 h at 30°C on a GYO gyratory shaker (New Brunswick, Edison, NJ) at 225 rpm. For storage they were replicated using 384-well deep-well microplates (AB-1178; Thermo Fisher Scientific, Waltham, MA) and QReps X5053 384-pin disposable polypropylene stamps. Storage plates containing 175 μ l LB freeze medium (38) were incubated as described above and stored at –70°C.

Screening of the mutant library. Storage plates were replicated in type 781201 384-well microplates (Greiner Bio-One, Frickenhausen, Germany) containing 75 μ l LB medium supplemented with 50 μ g/ml kanamycin, 200 μ g/ml ampicillin, and 0.4 mM isopropyl- β -D-thiogalactopyranoside. They were incubated as described above. Subsequently, optical densities at 600 nm (OD_{600}) were determined with a μ Quant microplate spectrophotometer (BioTek Instruments, Bad Friedrichshall, Germany). To enhance the sensitivity of the activity assay, the procedure for cell lysis was improved (details will be described elsewhere). Briefly, a freeze-thaw procedure in the presence of Triton X-100 was used. Subsequently, the microplates were centrifuged for 45 min at $1,500 \times g$ in a Megafuge 2.0 RS (Thermo Fisher Scientific, Waltham, MA). Using an Evolution P3 precision pipetting platform (PerkinElmer, Waltham, MA), 5 μ l of supernatant was added to each well of 384-well microplates (781101; Greiner) containing 37 μ l of premixed reagents, which resulted in final concentrations of 33% (vol/vol) TRA buffer, 80 mU hexokinase, 40 mU glucose-6-phosphate dehydrogenase, and 200 mU phosphoglucose isomerase (all obtained from r-biopharm, Darmstadt, Germany), as well as 50 mM sucrose and 0.1 g/liter glycogen. This coupled the reduction of NAD to the liberation of Fru and Glc. Activity was determined by incubating the preparations at room temperature and monitoring the absorbance at 340 nm for up to 45 min. After 50 min, 60 μ l of an iodine solution (3 mM I_2 , 50 mM KI, 10 mM HCl) was added to each well, and 10 min later polymer formation was determined by measurement of the absorbance at 570 nm.

Preparation of cell extracts. Strains were grown using a 5-ml format essentially as described above. After harvesting and resuspension in water at a final OD_{600} of 10 to 15, lysis was carried out in Eppendorf tubes as described above, followed by centrifugation for 10 min at $12,000 \times g$ in a tabletop centrifuge.

Enzyme purification. Strains were grown in 300 ml LB medium with antibiotics at 37°C and 150 rpm to an OD_{600} of 0.7 to 0.9. Subsequently, transcription was induced by addition of 0.4 mM isopropyl- β -D-thiogalactopyranoside, and incubation was continued for 4 h at 30°C. Cells were harvested and resuspended in native binding buffer containing 20 mM sodium phosphate, 0.5 M NaCl, 0.1% (vol/vol) Triton X-100, and 10 mM imidazole (pH 8.0) at an OD_{600} of 100. After

addition of 750 μ g/ml hen egg white lysozyme (92.7 U/ μ g [1 U decreases the absorbance at 450 nm by 0.001 per min at pH 7.0 and 25°C with *Micrococcus luteus* ATCC 4698 as the substrate]; Fluka, Buchs, Switzerland), 4 μ g/ml DNase I, and 8 μ g/ml RNase A (final concentrations), cells were incubated for 30 min on ice and subsequently disrupted by one passage through a French press (Aminco, Silver Spring, MD) at 138 MPa. Undissolved material was separated at 4°C by centrifugation for 45 min at $15,000 \times g$. Extracts were stored at –70°C. ASs were purified with a nickel chelate column, using the ProBond purification system (Invitrogen, Karlsruhe, Germany) according to the protocol of the supplier, with the following slight modifications. After protein binding, the column was washed twice with wash buffer containing 20 mM imidazole and twice with wash buffer containing 40 mM imidazole. Using Centriprep Ultracel YM-50 columns (Millipore, Schwalbach, Germany), the elution buffer was exchanged with phosphate-buffered saline consisting of 8 mM Na_2HPO_4 , 140 mM NaCl, 2.7 mM KCl, and 1.5 mM KH_2PO_4 (pH 7.3), which was supplemented with 1 mM dithiothreitol and 1 mM EDTA. The purity of the preparations was determined to be about 90% by sodium dodecyl sulfate-PAGE.

DNA sequencing. DNA sequencing was carried out as previously described (6).

Protein gel electrophoresis and quantitation. Samples were mixed with the same volume of $2\times$ denaturing buffer (35), and the proteins were separated by 0.1% sodium dodecyl sulfate-12.5% PAGE (12). Gels were stained with Coomassie blue (29). Band intensities in gel images were evaluated with the AIDA 4.15.025 software (Raytest, Straubenhardt, Germany), using bovine serum albumin as the standard.

Determination of protein concentration. Protein contents were determined by the Bradford method (7), using bovine serum albumin as the standard.

Determination of AS activities under standard conditions. AS activity was determined enzymatically with a reagent kit from r-biopharm. The protocol is based on the procedure of Mayer (15), modified to separately quantify Fru liberation and Glc liberation. Total activity was determined by measurement of Fru formation, hydrolytic activity was determined by measurement of Glc formation, and transfer activity was determined by calculation of “net Fru” formation, which is the difference between Fru formation and Glc formation. One unit of activity was defined as the formation of 1 μ mol of the respective compound per min under the assay conditions.

For standard activity measurement, aliquots of cell extracts or purified enzymes were incubated at 30°C for 15 min in phosphate-buffered saline supplemented with 150 mM Suc. When primers were present, their concentrations were 0.1 g/liter for glycogen and 150 mM for α -methyl-Glc (α -Me-Glc). Other details of the assay have been described previously (34).

Characterization of product patterns with and without potential acceptors added. The reaction conditions were the same as those described above for the standard assay. The concentrations of (potential) acceptors were as follows: α -Me-Glc, G2, and G3, 150 mM; α -para-nitrophenyl-G6 (α -pNP-G6), 30 or 150 mM; α -pNP-G2, 30 or 45 mM; and α -pNP-Glc, 15 mM. In experiments with cell extracts, the extracts constituted 20% (vol/vol) of the total volume, yielding final AS concentrations of approximately 30 μ g/ml (determined as described above for protein gel electrophoresis and quantitation). In experiments with purified enzymes, AS concentrations were increased to 200 μ g/ml to obtain complete turnover of Suc. Reaction mixtures were incubated for 20 or 24 h. Aliquots were analyzed by thin-layer chromatography (TLC) and/or high performance liquid chromatography (HPLC) as indicated below.

Enzymatic digestion of oligosaccharides. Incubation with β -amylase from sweet potato (1 U/10 μ l [1 U liberates 333 μ g or ~ 1 μ mol of G2 per min from starch under the conditions used]; Sigma, Munich, Germany) was carried out at room temperature in 40 mM sodium acetate (pH 4.8). Incubation with α -glucosidase from *Saccharomyces cerevisiae* (1 U/10 μ l [1 U liberates 1 μ mol of Glc per min from α -pNP-Glc under the conditions used]; Sigma) was performed at 37°C in 285 mM potassium phosphate (pH 6.8). Negative controls (without enzyme) and positive controls (containing 10 mM G3 for β -amylase or 10 mM G2 for α -glucosidase) were run in parallel. Reaction mixtures were analyzed by TLC as described below.

TLC. The plates used were Silica Gel 60 F₂₅₄ plates with a concentration zone (Merck, Darmstadt, Germany). The mobile phases were ethyl acetate–2-propanol–water (50/40/10, vol/vol/vol) (eluent A) and acetonitrile–ethyl acetate–1-propanol–water (85/20/50/60, vol/vol/vol/vol) (eluent B) (26). One or two ascents were carried out. Carbohydrates were detected as previously described (18).

HPLC. Analyses were performed with a Shimadzu LC10AD instrument (Shimadzu Corp., Kyoto, Japan) equipped with an SC Lichrosphere 100 RP8 5 μ m column (length, 125 mm; inside diameter, 4.6 mm; Bischoff, Leonberg, Germany) and a diode array detector. Products of α -pNP-Glc and α -pNP-malto-oligosaccharides were eluted isocratically with 10% methanol at a flow rate of 1 ml/min. Products were quantitated using HPLC peak areas.

TABLE 1. Amino acid sequences at positions 390 to 396 of the mutagenized region in selected AS variants

AS	No. of substitutions	Sequence ^a
WT	NA ^b	RSHDDIG
V16	2	RCHDDI E
V17	2	R K HDDIA
V18	1	RSHDDID
V19	2	RSHDEIA
V20	1	RSHDDID
V21	3	CSHDDY S
V23	3	R I HDEIS
V24	2	RSHDEIV
V25.1 ^c	1	RSHDDID
V25.2 ^c	3	RSHD TLD
V27	4	RCHDEVS
V28	1	RSHDDIA
V29	1	RSHDDID
V30	2	RSHDEIS
Consensus sequence	NA	*C***** ^d S

^a Underlined amino acids were not subjected to mutagenesis. Italics indicates amino acids introduced by one nucleotide substitution, and bold type indicates amino acids introduced by two nucleotide substitutions.

^b NA, not applicable.

^c Clone 25 was resolved into two different clones.

^d An asterisk indicates an invariant residue. At position 391, one of two amino acids (C or S [$\begin{smallmatrix} C \\ S \end{smallmatrix}$]) was found; C was the more frequent residue (19/24). The data are data for alignment of 24 amino acid sequences that have been shown to possess or, due to sequence similarity, are likely to possess AS activity. Their accession codes in the NCBI protein database (<http://www.ncbi.nlm.nih.gov/protein/>) are AAM51153, CAA09772, ZP_00413542, ZP_00768599, CAD78342, ABA56748, ZP_01092731, ZP_00775239, ZP_01111447, ZP_01116521, ABB42389, ZP_01083586, EAN28930, ZP_00564554, ZP_01120231, AAF10510, ZP_00396018, AAM38333, AAQ93678, CAJ25349, YP_199737, BAE67751, and M42629.

In silico simulation of AS modifications. Structural differences of AS variants were modeled based on the crystal structures of the WT molecule with no ligand (1G5A) and the inactive Glu328Gln variant containing a maltoheptaose molecule bound at the active site (1MW0). Computations were done with version 6.1 of Modeler (5, 28) in the full refinement mode, combined with CCP4i (23) as a graphic interface. The figures were drawn with Pymol (8).

RESULTS

Generation and screening of AS variants. According to AS crystal structures (13, 16, 32, 33), residues 390 to 396 (numbering of the crystal structures is used throughout this paper) either directly line substrate molecules in active site subsites -1 to +3 or contribute to the positioning of such amino acids. Therefore, changes at these positions might influence the binding and/or translocation of acceptor molecules inside the sub-

strate access tunnel. All residues in this region except Ser391 appear to be invariant based on DNA sequence data (Table 1). Random mutagenesis of the corresponding gene segment was carried out by incorporating degenerate oligodeoxynucleotides via PCR as described in Materials and Methods. The codons for His392 and Asp393 were omitted from mutagenesis because of the importance of these amino acids for catalytic activity (30). In the family 13 enzyme cyclodextrin glycosyltransferase these residues appear to contribute to formation of the enzyme-glycosyl intermediate by distortion of the Glc ring in subsite -1 and formation of favorable hydrogen bonds (37). The quality of mutagenesis was verified by sequencing a random selection of clones. The average substitution (4.9 nucleotides per gene) was in good agreement with the theoretically expected value (4.5 nucleotides per gene).

About 1,000 clones of the mutant library were subjected to two types of screening. The first type determined catalytic activity. The second type indicated loss of the ability to convert, under the conditions used, glycogen primers into iodine-stainable products. The fraction of active clones was 6.8%, and 82% of these clones were unable to form iodine-stainable products. A total of 15 such clones that showed comparatively high activities with Suc were chosen for further characterization.

Verification of screening results. Cell extracts of the selected clones were prepared. AS activity was confirmed in all but one case. TLC analysis of the products formed in the presence of Suc as the sole substrate showed that the product patterns of all variants differed significantly from that of the WT (Fig. 1). The enzymes not only were deficient in polymer formation but were affected in the formation of short malto-oligomers. Roughly, three types of variants could be distinguished. The first type (V19, V21, V23, V24, V27, and V30) showed mainly hydrolysis of Suc with no or very little formation of G2. The second type (V17, V18, V20, V25, V26, V28, and V29) synthesized G2 (which is hardly formed by the WT) as the major product, together with minor amounts of G3. The third type, represented only by V16, synthesized major amounts of G2 but essentially no longer oligomers.

Determination of amino acid substitutions in AS variants selected from the screen. The whole restriction fragments that had been subjected to mutagenesis were sequenced. This analysis showed that no nucleotide substitutions had been introduced outside the five mutagenized codons. Amino acid substitutions were found at all mutagenized positions in the

FIG. 1. Product patterns formed by WT AS and selected variants in the presence of sucrose as the sole substrate. Enzymes selected by the screen were used in the form of cellular extracts. V22 was omitted, as its catalytic activity was not confirmed. For experimental details, see Materials and Methods. The analysis was carried out by performing TLC with eluent A. Carbohydrates in the marker lanes (lanes M) are indicated on the left.

TABLE 2. Statistics for amino acid substitutions in random and selected AS variants

Wild-type amino acid	Fraction of substitutions in random variants		Selected variants			
			Fraction of substitutions		No. of individual substitutions ^a	
	Mean	SD	Mean	SD		
R390	0.58	0.14	0.07	0.07	1 C	
S391	0.46	0.14	0.29	0.12	2 C, 1 I, 1 K	
D394	0.67	0.14	0.43	0.13	5 E, 1 T	
I395	0.75	0.13	0.21	0.11	1 L, 1 V, 1 Y	
G396	0.92	0.08	1.00	0.08	5 D, 4 S, 3 A, 1 V, 1 E	

^a A total of 14 variants were examined.

selected clones. Their frequencies, however, differed in a position-specific manner from those of the random clones (Table 2). Compared to the latter, the numbers of amino acid replacements in selected clones were reduced most substantially at positions 390 and 395 and were decreased moderately at positions 391 and 394, but they were not decreased at all at position 396. The amino acid sequences of the mutagenized segments of the selected clones are shown in Table 1. A summary of the frequencies of occurrence of the newly introduced amino acids is shown in Table 2. Two results appear to be remarkable. First, a broad range of amino acids at position 396 (not significantly different from the range found in the random clones) is able to fulfill the selection criteria for catalytic activity and elongation deficiency. Second, five of the six changes at position 394 introduced a Glu residue.

Characterization of selected AS variants. Based on the results described above, five of the variants, V16, V17, V19, V20, and V28, representing all three types described above, were selected for purification and more detailed characterization.

Significant differences in specific activities between the WT and most of the variants were observed under standard assay conditions, either with Suc as the sole substrate or in the presence of additional potential acceptor molecules. We determined total activities, as well as transfer and hydrolytic activities, as described in Materials and Methods (Table 3). With Suc as the sole substrate, over 90% of the total activity of the WT and four of the five variants was hydrolytic; for V17 the value was somewhat lower (78%). When absolute values were examined, V16 and V17 showed nine- and fourfold-faster hydrolysis than the WT, respectively. For glucosyl transfer, the

FIG. 2. Product patterns for WT AS and purified variants in the presence of Suc and malto-oligosaccharides. (A) G2 as the potential acceptor. (B) G3 as the potential acceptor. For experimental details, see Materials and Methods. The analysis was carried out by performing TLC with eluent A (A) or eluent B (B). Lane Bg, background incubation without enzyme; lanes M1 and M2, markers. The positions of carbohydrates are indicated on the right. Abbreviations: Tur, turanose; Tre, trehalulose.

same variants exhibited >10- and >30-fold-higher activity than the WT, respectively. As Glc is the initial primer for glucan formation when Suc is the only substrate present (2), faster Suc hydrolysis by V16 and V17 may be a major reason for the higher transfer activities of these variants. No transglucosylation was detected for V19, in spite of higher hydrolytic activity than the WT. This appears to be due to the very low acceptor efficiency of Glc and related molecules with this variant (see below).

In order to make the transfer activity largely independent of the rate of Suc hydrolysis, α -Me-Glc, which had been found previously to be an efficient acceptor with the WT (our unpublished results), was included in the standard assay mixture. This increased the transglucosylation activity of the WT about 500-

TABLE 3. Specific total, transfer, and hydrolytic activities of WT AS and selected variants in the presence of different substrates^a

AS	Sp act (U/g)																	
	Sucrose						Sucrose and α -Me-Glc						Sucrose and glycogen					
	Hydrolytic		Transfer		Total		Hydrolytic		Transfer		Total		Hydrolytic		Transfer		Total	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD
WT	407	10	14	5	421	9	380	5	7,110	70	7,490	70	421	8	8,460	130	8,890	140
V16	3,650	170	199	22	3,850	150	3,160	130	8,350	310	11,500	500	4,050	240	247	6	4,290	240
V17	1,720	160	477	30	2,200	190	572	9	16,100	400	16,700	400	1,660	70	1,510	80	3,170	150
V19	832	16	0	8	825	20	730	11	1,050	10	1,780	30	787	20	30	7	818	19
V20	868	13	30	4	898	16	690	60	14,100	100	14,800	100	854	31	38	7	892	24
V28	518	8	41	2	559	9	475	7	19,000	400	19,500	400	527	8	949	25	1,480	30

^a The substrate concentrations were 150 mM Suc, 150 mM α -Me-Glc, and 0.1 g/liter glycogen.

TABLE 4. Quantitation of products formed by WT AS and selected variants in the presence of Suc and α -pNP-G2 as potential acceptors^a

AS	Fraction of product (%)						
	pNP-OH	α -pNP-Glc	α -pNP-G2	α -pNP-G3	α -pNP-G4	α -pNP-G5	α -pNP-G $\geq$ 6
WT	3.3	0.8	15.7	16.3	11.8	9.3	44.0
V16	4.5	ND	95.5	ND	ND	ND	ND
V17	0.0	ND	45.3	52.0	2.7	ND	ND
V19	4.1	0.8	92.1	3.1	ND	ND	ND
V20	4.1	0.1	93.5	2.3	ND	ND	ND
V28	4.4	2.5	25.4	60.4	6.6	0.7	ND

^a The substrate concentrations were 150 mM Suc and 45 mM α -pNP-G2. Preparations were incubated for 20 h at 30°C with 200 μ g/ml of enzyme. The analysis was carried out by using HPLC. The standard deviations were $\pm 15\%$. Abbreviations: ND, not detected; pNP-OH, *para*-nitrophenol.

fold. The hydrolytic activity remained essentially unchanged. α -Me-Glc also stimulated Glc transfer by the variants. When absolute values were examined, all of the variants except V19 possessed transfer activity that was similar to or higher than that of the WT. V19 showed roughly 10-fold-lower activity, suggesting that the acceptor efficiency of α -Me-Glc was low.

Assays of standard activity in the presence of glycogen revealed drastically reduced stimulation of transfer activity for the variants compared to the WT, in agreement with the selection carried out. While the activity of the WT was increased about 600-fold, the activities of the variants were increased only between 1.2- and 23-fold.

The patterns of elongation products formed by the purified enzymes were characterized under different conditions.

Incubation with Suc as the sole substrate (data not shown) confirmed the deficiencies in chain elongation described above for the cell extracts (Fig. 1). In addition to short malto-oligomers, two bands, comigrating with turanose and leucrose, were typically observed, particularly with V16 and V20. As shown previously (21), these bands represent the Suc isomers turanose and trehalulose arising from glucosylation of liberated Fru at carbons 3 and 1, respectively.

The inability of the variants to form products longer than G2 or G3 was examined under more stringent conditions by adding a relatively high concentration (150 mM) of these oligomers to the reaction mixtures to potentially act as primers (Fig. 2). Both G2 and G3 significantly enhanced the formation of longer malto-oligomers by the WT. The experiment performed with the high concentration of G2 revealed that V19 was also able to elongate the dimer. The single product, however, migrated slightly slower than G3 (Fig. 2A), indicating that it represented a trimer with an altered linkage type. In agreement with this conclusion, the product was not cleaved by

β -amylase, which rapidly digested G3. From a steric point of view, attack at O-3, vicinal to the normally attacked O-4 residue, appears to be the most likely attack, as this would require only a relatively small deviation from the normal orientation of G2 at the acceptor binding site. The formation of an α -1,3 linkage was in agreement with cleavage of the elongation product by α -glucosidase, which readily digested maltose and nigerose but neither kojibiose nor isomaltose. In contrast to all other variants, V16 did not catalyze detectable formation of any trimer. Inclusion of G3 in the reaction mixture confirmed that none of the variants formed more than trace amounts of G4 (Fig. 2B).

In order to mimic a situation where an acceptor carried an aglycon, the behavior of α -pNP-Glc and -G2 was investigated. The WT carried out virtually unlimited rounds of chain elongation with these substrates and in addition generated small amounts of α -pNP-Glc and/or *para*-nitrophenol through the use of α -pNP-G2 and -Glc as donors (3; our unpublished results). With the variants, however, the longest oligomers in the reactions were the dimer (V16, V19, and V20) or the trimer (V17 and V28), as shown in Table 4 for α -pNP-G2 as the substrate. The results for V16, V17, and V28 were similar to those obtained with the unmodified primers, whereas the presence of the aglycon appeared to prevent elongation to the G3 molecule by V19 and V20.

In order to examine if an oligomer that the variants were unable to form was inert in their presence, α -pNP-G6 was probed in the presence of Suc. The WT and all variants formed minor amounts of smaller oligomers and *para*-nitrophenol (Table 5), indicating that the modified enzymes showed a low level of productive binding of α -pNP-G6 and its shorter analogues at the donor position. However, only the WT efficiently used the hexamer as an acceptor. Absolutely no elongation was

TABLE 5. Quantitation of products formed by WT AS and selected variants in the presence of Suc and α -pNP-G6 as potential acceptors^a

AS	Fraction of product (%)										
	pNP-OH	α -pNP-Glc	α -pNP-G2	α -pNP-G3	α -pNP-G4	α -pNP-G5	α -pNP-G6	α -pNP-G7	α -pNP-G8	α -pNP-G9	α -pNP-G $\geq$ 10
WT	2.5	0.5	0.5	0.7	0.5	0.4	4.2	5.0	11.2	14.0	60.5
V16	7.2	1.3	0.9	0.7	0.5	0.4	89.0	ND	ND	ND	ND
V17	6.0	0.4	1.1	1.0	0.6	0.4	65.1	19.2	5.3	0.8	0.1
V19	5.1	1.8	1.2	0.6	0.4	0.4	90.5	ND	ND	ND	ND
V20	7.1	0.7	2.1	0.9	0.9	1.7	85.9	ND	0.2	0.1	0.4
V28	7.0	1.0	1.2	1.0	0.5	0.3	65.7	16.8	5.5	0.8	0.1

^a The substrate concentrations were 150 mM Suc and 30 mM α -pNP-G6. Preparations were incubated for 20 h at 30°C with 200 μ g/ml of enzyme. The analysis was carried out by using HPLC. The standard deviations were $\pm 15\%$. Abbreviations: ND, not detected; pNP-OH, *para*-nitrophenol.

detected with V16, V19, and V20. V17 and V28, however, were able to elongate a minor fraction of the oligomer. This result agrees with some stimulation of the transfer activity of these variants by glycogen (Table 3) and indicates that longer saccharide chains are able to partially overcome the almost total block of elongation observed for dimers or trimers (Fig. 2). Similar results were obtained with unsubstituted maltohexaose (not shown).

DISCUSSION

The usefulness of glucansucrases as biocatalysts for limited glucosylation of carbohydrate and noncarbohydrate acceptors is reduced by high levels of undesired glucan synthesis (particularly with low-efficiency acceptors) and by multiple attachment of sugar moieties to many acceptors and their glucosylation products, respectively (3, 17, 24, 31). The results described in this paper show that this shortcoming can be eliminated by appropriate modification of the enzyme. To this end, segmental random mutagenesis has been used successfully to introduce amino acid changes that lead to AS variants that retain catalytic activity but do not catalyze unlimited cycles of glucosyl transfer to acceptor molecules.

In all variants selected in the library screen Gly396 is replaced by a variety of other residues (Tables 1 and 2). Variants containing Asp (V20), Ala (V17, V19, V28), or Glu (V16) at this position were characterized in detail and are discussed here. The novel features of V20 and V28 must originate entirely from the Gly396 changes, as these variants have no other sequence alterations. V16, V17, and V19 each contain one additional substitution. Ser391 is replaced in the V16 and V17 variants. V16 harbors a Cys at this position. Ser and Cys differ solely by change from a hydroxy group to a sulfhydryl group, and the great majority of sequenced ASs have a Cys at this position (Table 1). It therefore appears very likely that the elongation deficiency of V16 originates from the substitution at position 396. The properties of V17 and V28 are very similar, indicating that the Ser391Lys substitution has only a minor effect. In contrast, the catalytic behavior of V19 differs strongly from that of V28, indicating that the additional Asp394Glu replacement in V19 strongly influences chain elongation. The properties of V19 greatly resembled those of V23, V24, V27, and V30, which also have the Asp394Glu substitution but different residues at position 396. This suggests that the unique properties of all these variants originate from the change at Asp394. Structural modeling confirmed this view.

The amino acid substitutions of the variants were modeled based on the crystal structures of the WT enzyme without a ligand (1G5A) (32) and the inactive Glu328Gln variant containing a maltoheptaose molecule at the active site (1MW0) (33). A stereo view of the relevant part of the latter structure is shown in Fig. 3A.

The models yielded very similar results and suggest that the altered properties of V16, V17, V20, and V28 are predominantly due to steric interference at subsites +2 and +3 between the novel amino acid residues at position 396 and the growing amylose chain. Thus, the normal positioning of G2 and even more the normal positioning of G3, when these residues act as acceptors, are affected. The steric clashes are greatest for V16, which has Glu at this position. This agrees

A

B

C

FIG. 3. Stereo views of malto-oligosaccharide binding at the active site of WT AS and modeled variants. (A) WT AS. (B) V16 AS. (C) V19 AS. Four nonreducing-end residues of a G7 molecule bound at subsites -1 to $+3$ of the active site of the WT (PDB structure 1MW0) are shown. They are shown using CPK representation, and the carbon atoms are violet (subsite -1), turquoise (subsite $+1$), green (subsite $+2$), and yellow (subsite $+3$). Amino acid residues within 4 Å of these Glc moieties are indicated by sticks, except for those changed in the variants and their counterparts in the WT structure, which are shown using CPK representation. Steric clashes between Glc moieties and changed amino acids are apparent.

with the total inability of this variant to use G2 as an acceptor. A stereo view of the interference between the glucan chain and Glu396, as indicated by modeling, is shown in Fig. 3B.

In five of the six variants of the V19 type (see above), Asp394 was replaced by the larger amino acid Glu. The WT residue hydrogen bonds acceptors and the growing chain at subsites +1 and +2, respectively (33). Modeling of the Glu394 variant (with or without the simultaneous replacement Gly396Ala [V19], Gly396Val [V24], or Gly396Ser [V30]) suggested that there was a loss of both bonds and, additionally, that there were steric clashes with acceptors in cavities +1 and +2 (Fig. 3C). Interestingly, it has been reported that a sterically converse substitution, the replacement of Asp394 by the much smaller amino acid Ala, also leads to preferential hydrolysis and to an increase in the fraction of G2 formed at the cost of polymer synthesis (2). This suggests that an increase in cavity size also leads to less productive binding of Glc and G2 at the acceptor site.

V21, the sixth variant of the V19 type, has three changes, Arg390Cys, Ile395Tyr, and Gly396Ser. In this case, modeling gave no clear indication of how structural changes led to the new phenotype. Tyr395 in the variant model, like Ile395 in the WT structure, points away from the glucan chain. However, accommodation of the bulky Tyr side chain, together with the simultaneous Arg390Cys change, which appears to cause loss of hydrogen bonds with Asp393 and Gly449, may lead to a shift of the adjacent Asp394, resulting in effects similar to those of the Asp394Glu replacement.

Suc hydrolysis by AS requires attack of a water molecule at C-1 of the glucosyl-enzyme intermediate. Wat800 in the crystal structure of this intermediate has been proposed to be the most likely candidate for this reaction (13). When a Glc molecule binds at subsite +1, Wat800 is displaced. However, interference of Glu394 (or a shifted Asp394 in V21) with the binding of Glc at this site would reduce this displacement and provide a rationale for increased Suc hydrolysis by V19-type enzymes.

The amino acid changes described in the current work terminate acceptor elongation after synthesis of a di- or trisaccharide, but they neither block dissociation of the reaction products nor otherwise inhibit the catalytic activity. This makes the variants generated interesting candidates for catalysts for the limited glucosylation of acceptor molecules. Further improvement of the properties of the variants by combinatorial saturation mutagenesis at the critical positions may be envisaged.

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