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Structure and Enzymatic Properties of Genetically Truncated Forms of the Water-Insoluble Glucan-Synthesizing Glucosyltransferase from *Streptococcus sobrinus*[1]

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Glucosyltransferase-I (GTF-I: 175 kDa) of a cariogenic bacterium, *Streptococcus sobrinus* 6715, mediates the conversion of water-soluble dextran (α-1,6-glucan) into a water-insoluble form by making numerous α-1,3-glucan branches along the dextran chains with sucrose as the glucosyl donor. The structures and catalytic properties were compared for two GTF-I fragments, GTF-I′ (138 kDa) and GS (110 kDa). Both lack the N-terminal 84 residues of GTF-I. While GTF-I′ still contains four of the six C-terminal repeats characteristic of streptococcal glucosyltransferases, GS lacks all of them. Electron microscopy of negatively stained samples indicated a double-domain structure for GTF-I′, consisting of a spherical head with a smaller spherical tail, which was occasionally seen as a long extension. GS was seen just as the head portion of GTF-I′. In the absence of dextran, both fragments simply hydrolyzed sucrose with similar K_m and k_{cat} values at low concentrations (<5 mM). At higher sucrose concentrations (>10 mM), however, GTF-I′ exhibited glucosyl transfer activity to form insoluble α-1,3-glucans. So did GS, but less efficiently. Dextran increased the rate and efficiency of the glucosyl transfer by GTF-I′. On removal of the C-terminal repeats of GTF-I′ by mild trypsin treatment, this dextran-stimulated transfer was completely lost and the dextran-independent transfer became less efficient. These results indicate that the N-terminal two-thirds of the GTF-I sequence are organized as a structurally and functionally independent domain to catalyze not only sucrose hydrolysis but also glucosyl transfer to form α-1,3-glucan chains, although not efficiently; the C-terminal repeat increases the efficiency of the intrinsic glucosyl transfer by the N-terminal domain as well as rendering the whole molecule primer-dependent for far more efficient insoluble glucan synthesis.

Key words: C-terminal repeats, dextran, domain structure, α-1,3-glucan, glucosyltransferase.

The glucosyltransferases (GTFs) from oral streptococci belong to a group of enzymes (EC 2.4.1.-) that catalyze glucosyl transfer from sucrose to a glucan chain as described by a generalized scheme:

$$\text{sucrose} + (\text{glucose})_n \rightarrow \text{fructose} + (\text{glucose})_{n+1} \quad (1)$$

where $(\text{glucose})_n$ and $(\text{glucose})_{n+1}$ are growing glucan chains. The enzymes are commonly divided into two categories: those that synthesize water-insoluble, mainly α-1,3-linked D-glucans (GTF-I, I for insoluble), and those that synthesize water-soluble α-1,6-linked D-glucans (GTF-S, S for soluble). The combined action of these enzymes leads to the formation of water-insoluble, adherent glucans, which facilitate bacterial aggregation to form dental plaque on smooth tooth surfaces (*1*). Because of this etiological importance, these enzymes have been the subjects of intense investigations over the past two decades by biochemical and molecular genetic methods (*2, 3*). Several interesting structural features shared by these GTFs have been revealed, including large molecular sizes with highly homologous primary structures (1,300-1,700 residues), the participation of an aspartyl residue in catalysis (*4, 5*), the homology of the sequence surrounding this aspartate with that conserved in all members of the α-amylase family and related enzymes (*6, 7*), and the characteristic C-terminal repeats for acceptor-glucan binding (*8*). However, further details of the structure-function relationship

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Abbreviations: GTF, glucosyltransferase; GTF-I, glucosyltransferase that synthesizes water insoluble glucans; GTF-S, glucosyltransferase that synthesizes water soluble glucans.

of GTFs have not yet been provided, due mostly to the large sizes of the enzyme molecules which makes structural investigations difficult.

Since large proteins tend to form domains as structurally independent entities (*9*), one way to overcome this difficulty is to isolate such domains or fragments thereof retaining certain functions related to the GTF reaction given by Scheme 1, sucrose splitting, glucosyl transfer, and interaction with an acceptor glucan. Proteolytic digestion has so far been used successfully to isolate the C- terminal 55-60 kDa peptide from GTFs (*10-12*). This peptide covers the region comprising the C-terminal repeats and shows a high affinity for dextrans. To date, however, no fragment having a glucosyl transfer or sucrose hydrolytic activity has been obtained by proteolysis.

An alternative approach to obtaining functional domains is the utilization of molecular genetic methods. The dextran-binding domain of GTF-S of *Streptococcus mutans* can now be expressed as a separate peptide in *Escherichia coli*, and its strong affinity for dextrans has been demonstrated (*13*). We previously cloned the whole GTF-I gene and several of its fragments from *Streptococcus sobrinus* by colony immunoassay (*14*). One of these clones (pAB2) is of particular interest, because it expresses a protein (GTF-I′, 138 kDa) that lacks about 80 N-terminal amino acid residues and 260 C-terminal residues of GTF-I (175 kDa; see Fig. 1), but catalyzes the formation of insoluble glucans from sucrose and dextran.

In the present investigation, we constructed a subclone of pAB2 by inserting a stop codon into a region just upstream of the start of the nucleotide sequence encoding the C-terminal repeats, resulting in the expression of a protein that lacks the remaining 240 C-terminal residues so that it does not bind dextran but hydrolyzes sucrose (GS, 110 kDa). Since both GTF-I′ and GS are produced under the control of the *E. coli lac* promoter, these proteins are now available in quantity for biochemical studies. This paper describes the gross molecular shapes of these proteins observed by electron microscopy, their basic enzymatic properties together with a structural analysis of their reaction products, and changes in the enzymatic activities of GTF-I′ by tryptic digestion. Based on the results of these studies, the structural and functional correlation of the N-terminal two-thirds and the C-terminal one-third of the GTF-I molecule will be discussed.

MATERIALS AND METHODS

Chemicals—Sugars and dextrans were purchased from the following sources: sucrose for density gradient centrifugation, Nacalai Tesque (Kyoto); dextran T10 (10 kDa) for enzymatic studies, Pharmacia; dextrans for gel-filtration analysis (5-80 kDa), Fluka. Other reagents were of analytical grade.

Bacterial Strain and Plasmids—*E. coli* JM109 was routinely maintained and grown in 2×YT medium (*15*). Plasmids pAB2 for GTF-I′ (*14*) and pGS for GS were used; the latter was constructed by ligating the *Hin*dIII-digested segment of pAB2 with the *Hin*dIII-site of pUC 18 to insert a stop codon in a position corresponding to the C-terminal side of Ser1085 of GTF-I (Fig. 1).

Preparation of Proteins—The transformed *E. coli* cells were grown at 37°C in two 1,000-ml flasks, each containing 250 ml of 2×YT medium with ampicillin (100 μg/ml), and induced with 0.5 mM isopropyl-β-D-thiogalactoside when the culture density reached an absorbance of ~1.0 at 660 nm, and incubated 2 h further. Cells were collected by centrifugation, washed twice with 10 mM K-phosphate (pH 6.8), and stored at −20°C. Frozen cells (wet weight, *ca.* 2 g from two 250-ml cultures) were suspended in the same buffer with 1 mM phenymethylsulfonyl fluoride and sonicated for 2 min in an ice bath. After the cell debris was removed by high-speed centrifugation followed by ammonium sulfate fractionation (50% saturation), the extracts were chromatographed on a DEAE-Toyopearl 650M column (2.5 by 15 cm) using a linear 0-0.4 M NaCl gradient in 10 mM K-phosphate (pH 6.8). The activity of the protein fractions to release the reducing sugar from sucrose was monitored by a dinitrosalicylic acid method (*16*). Fractions with a high activity/protein ratio, usually eluting at NaCl concentrations around 0.125 M for GTF-I′ and 0.14 M for GS, were analyzed by SDS-PAGE (*17*), and those showing

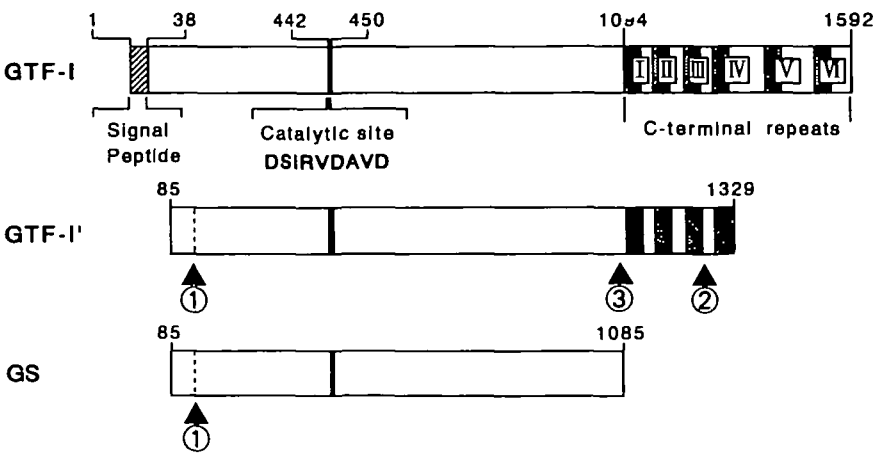

Fig. 1. **Primary structure of GTF-I and its genetically truncated fragments.** The major peptide segments with their start/end residue positions are indicated. The signal peptide segment was determined from the N-terminal analysis of GTF-I purified from the culture supernatant of *S. sobrinus* (Fukui, unpublished). The catalytic site was assigned by comparing the GTF-I sequence (*14*) with those reported (*14*). Note that the amino acid at 449 is not Glu as previously reported but Val, which had been deduced by a recent DNA sequence re-determination in this region (Kaseda and Fukui, unpublished). The C-terminal repeats are indicated by Roman numbers (I to VI), each of which consists of an A repeat (cross-hatched) designated by Ferretti *et al.* (*8*) and an intervening segment between neighboring A-repeats. The position of the N-terminal residues of GTF-I′ and GS were determined by N-terminal sequence analysis, whereas their C-terminal positions were deduced from the nucleotide sequences encoding the proteins. Numbered arrowheads indicate trypsin cleavage sites: ①, the unique N-terminal site (Lys134-Asn135); ②, the first cleavage site (near the C-terminal end of the third repeat); ③, the second cleavage site (around the start of the first C-terminal repeats, see text).

a single protein band were pooled and concentrated by ammonium sulfate precipitation. The yield was usually 20-25 mg for both GTF-I′ and GS from a single culture of conventional laboratory scale (2×250 ml). The products were stored at −80°C until use after dialysis against 10 mM K-phosphate (pH 6.8). Protein concentration was determined from the absorbance at 280 nm using a molar extinction coefficient of 2.0×10^5 $M^{-1}\cdot cm^{-1}$ for GTF-I′ and 1.5×10^5 $M^{-1}\cdot cm^{-1}$ for GS calculated from the deduced amino acid compositions (*14*).

Electron Microscopy—Negative staining of protein samples was carried out according to a high-resolution protocol described in detail by Katayama (*18*). Electron micrographs were taken as a stereo-pair (10°) by a goniometer equipped in a JEM-2000ES electron microscope (JEOL).

N-Terminal Analysis—A protein sequencer (Applied Biosystems 476a) was used according to the manufacturer's standard protocol.

Enzyme Assay—All measurements were made at 25°C. The reaction mixture in a total volume of 850 μl contained 10 mM buffer (see below), 100 mM NaCl and 0.01 to 200 mM sucrose. The buffers used for the pH ranges indicated were: pH 5.0-6.0, acetate; 5.5-6.5, MES; 6.5-7.5, MOPS; 7.5-8.5, EPPS; 8.5-9.5, borate. The reaction was started by adding 40 nM GTF-I′ or GS and quenched by mixing with 1/10 volume of SDS (0.5%, w/v)/Na_2CO_3 (800 mM) mixture and allowing them to stand for 10 min. The dodecylsulfate was removed as a precipitate together with denatured protein by adding 1/10 volume of saturated KCl and centrifuging. The supernatant was then subjected to enzymatic assay for glucose and fructose with a commercial assay kit (F-kit for glucose/fructose; Boehringer Mannheim). Since sucrose is formed from equimolar glucose and fructose residues, sucrose splitting was determined from the amount of fructose released, and the extent of glucosyl transfer was calculated by subtracting the free glucose concentration from the fructose concentration.

Trypsin Digestion—GTF-I′ and GS were digested in 10 mM MOPS (pH 7.0) with TPCK-trypsin (Sigma) at 25°C. The weight ratio for trypsin/protein was 1/250. Digestion was halted at intervals with soybean trypsin inhibitor (Sigma) in an amount twice that of trypsin. Digested samples were subjected to SDS-PAGE and the measurement of enzymatic activity.

^{13}C NMR Analysis—GTF-I′ or GS (40 nM) was incubated with 100 mM sucrose at 25°C for 8 to 10 h. The insoluble material was collected by centrifugation, washed first with distilled water and then ethanol, and dried under reduced pressure. Dextran T10 and the insoluble glucan thus prepared were dissolved at concentrations of 80 $mg\cdot ml^{-1}$ in 1.0 M NaOD in D_2O and analyzed at room temperature in a Varian VXR-500 spectrometer at the following settings: spectral width, 30 kHz; acquisition time, 0.5 s; number of transients, 8,000. ^{13}C chemical shifts were expressed in parts per million related to the resonance of external sodium 3-(trimethylsilyl)propansulfonate. The assignment of peaks was based on the data of Colson *et al.* (*19*).

Periodate Oxidation—Carbohydrate solutions of 0.5 ml each containing *ca.* 1 mg dry material were mixed with 1.0 ml of 50 mM acetate buffer (pH 4.0) and 0.5 ml of 0.01 M potassium periodate, and kept at 4°C. Absorbance changes of the mixtures at 290 nm were measured at various time intervals (*20*).

Analysis of Molecular Size—The dry insoluble glucan was dissolved in 0.5 M NaOH (dry material concentration, 3 mg/ml) and chromatographed on a Toyopearl HW-50F column (1.5 by 90 cm) equilibrated with 0.5 M NaOH. Small fractions were collected and their carbohydrate contents were determined by the phenol-sulfuric acid method (*21*). The column was calibrated with molecular-weight standard dextrans.

RESULTS

Primary Structure—Table I summarizes the chemical and enzymatic properties of GTF-I′ and GS. The N-terminal structures of both proteins are consistent with that deduced from the nucleotide sequence in the upstream proximity of the *Bam*HI site of pUC18 ligated with *Sau*3a1 digests of the GTF-I gene (*14*). Thus, a stretch of an extra 11 amino acids is attached to the N-terminal end of both GTF-I′ and GS and an extra amino acid (Leu) to the C-terminal end of the former. The molecular masses of GTF-I′ and GS estimated from SDS-PAGE agree well with those calculated from the deduced sequences, including these extra amino acids.

Gross Molecular Shape—GTF-I′ and GS were negatively stained and subjected to electron microscopy (Fig. 2). The fields of both molecules appear filled with numerous globular particles almost homogenous in size, although slight variations are seen in their shapes, probably because of some conformational flexibility and/or the orientation of

TABLE I. **Protein chemical data and enzymatic parameters of GTF-I′ fragments.**

		GTF-I′	GS
Protein chemical data			
Number of residues	Deduced	1257	1012
Molecular mass	SDSPAGE	140 kDa	110 kDa
N-terminal sequence[a]	Deduced	TMITNSSSVPG-*DQASAAE*.....	TMITNSSSVPG-*DQASAAE*.....
	Found	TMITNSSS...	TMITNSSS....
pI	Calculated	4.71	4.63
Kinetic parameters[b]			
	Optimum pH	6.5-7.0	6.5-7.0
−dextran[c]	K_m	0.42±0.07 mM (4)	0.34±0.05 mM (3)
	k_{cat}	9.4±0.8 s^{-1} (4)	9.8±0.3 s^{-1} (3)
+dextran[c]	K_m	1.6±0.1 mM (3) [3.3 mM][d]	0.45±0.04 mM (3)
	k_{cat}	22.0±0.5 s^{-1} (3) [40 s^{-1}][d]	10.2±0.7 s^{-1} (3)

[a]Residues in italics are those in GTF-I. [b]K_m and k_{cat} values are the mean±SE of different preparations, the numbers of which are given in parentheses. [c]Values are for low sucrose concentrations; those for high concentrations are not given because of their low accuracy. [d]Values were calculated from the data determined at 37°C for GTF-I purified from the culture supernatant of *S. sobrinus* 6715 (*22*).

the molecules within the stain-layer.

In the image of the GTF-I′ sample (Fig. 2a), a substantial amount consists of two globular parts, whereas the remainder appears as a long extension (*ca.* 5 nm in length) from a single sphere. One of two domains of the former population seems somewhat larger (*ca.* 3.5 nm in diameter) than the other (*ca.* 2 nm) [N.B.: the diameter of the molecules mentioned here should be regarded as the minimum estimate because of the shrinkage of the negative stain-layer used to get highly contrasted images of the molecules]. Since the preparation was purified to homogeneity, the presence of two conformers can be interpreted as meaning the second domain is flexible enough to fold or sometimes unfold to make the long extension. On the other hand, the structure of GS appears simpler (Fig. 2b), mostly showing only one globular part, the size of which is the same as the larger domain of GTF-I′. Again, some GS appears to have the extension from the globular part (see insets), although it is much thinner than in the case of GTF-I′.

Enzymatic Properties—The initial velocity of sucrose splitting and glucosyl transfer in the absence of added dextran was measured for GTF-I′ and GS over a wide range of sucrose concentrations (Fig. 3, a and b). At lower sucrose concentrations (<5 mM), both fragments released stoichiometric amounts of fructose and glucose from sucrose (hence, glucosyl transfer was not significant), obeying simple Michaelis-Menten kinetics with similar K_m and k_{cat} values (Table I). The velocity of sucrose splitting rose at higher concentrations (>10 mM) with a concomitant acceleration in glucosyl transfer. GTF-I′ showed much more efficient glucosyl transfer than GS. These results suggest that the N-terminal two-thirds of the GTF-I protein residues are organized into a catalytic domain hydrolyzing sucrose with an intrinsic glucosyl transfer activity. Also suggested is a dual role of sucrose in the GTF-I reaction in the absence of dextran, a glucosyl donor and a glucosyl acceptor, although not a good one (see "DISCUSSION"). Alternatively, sucrose would be a weak activator of the glucosyl transfer reaction.

Both sucrose splitting and glucosyl transfer by GTF-I′ were activated by dextran T10 with Michaelis-Menten type kinetics (Fig. 3c). The dextran-increased portion of sucrose splitting was just equal to the accelerated glucosyl transfer, which suggests that glucosyl transfer is the rate-limiting step in the GTF-I reaction cycle. Thus, GTF-I′ catalyzes both dextran-independent and dextran-dependent glucosyl transfer. On the other hand, dextran T10 neither stimulated nor inhibited sucrose splitting by GS but inhibited the glucosyl transfer, although not strongly (Fig. 3d). This inhibition was also observed with dextrans of different molecular sizes. Apart from this rather unexpected finding (not pursued further in the present investigation), the

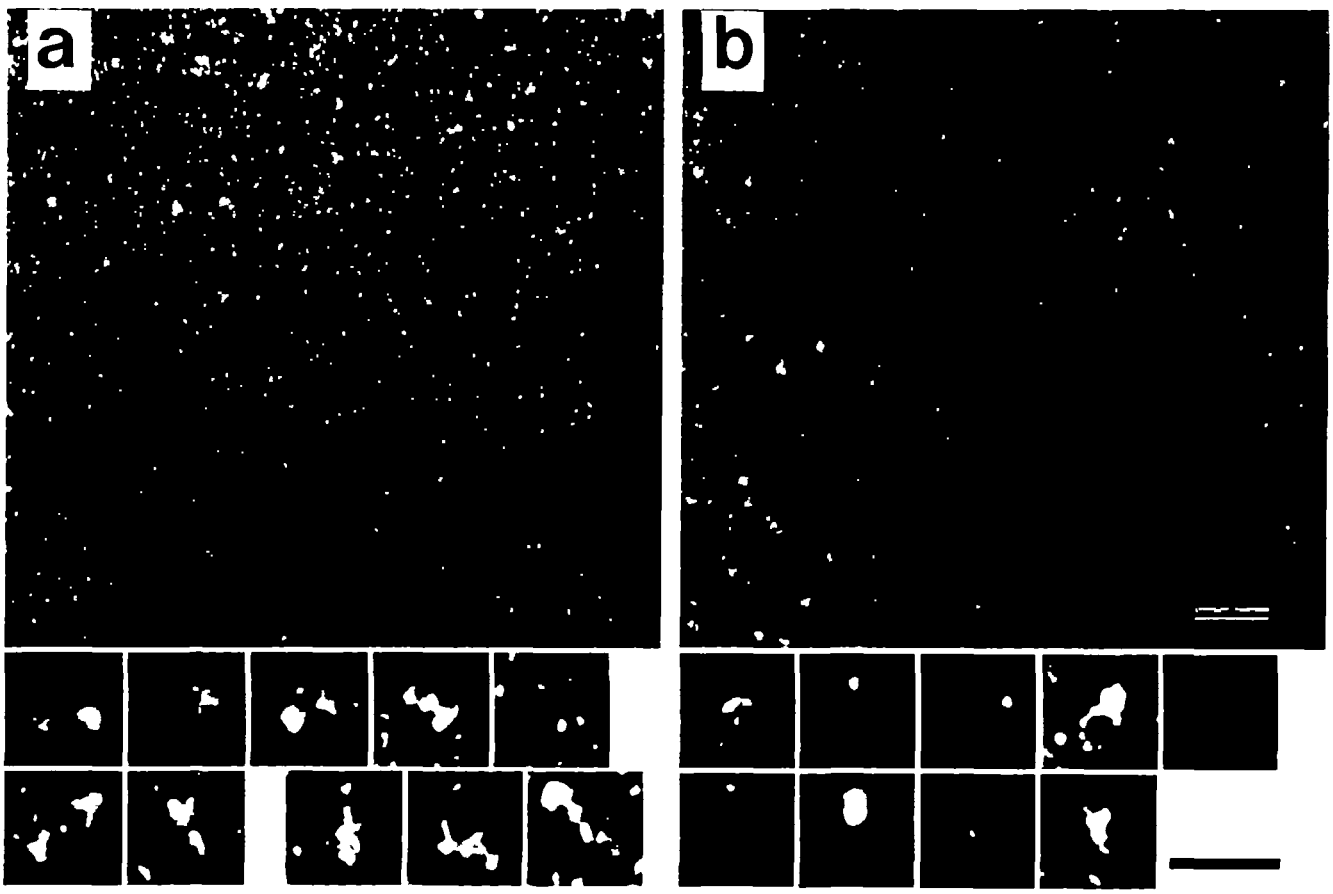

Fig. 2. **Electron micrographs of negatively stained GTF-I′ (a) and GS (b).** Top panels indicate general views (scale bars, 20 nm) and the insets show some selected particles (scale bars, 10 nm). Some of the GTF-1′ molecules consist of two globular domains (see 7 insets) and the others show one globular domain with a long extension (see 3 insets). The majority of GS consists of a single globular domain, but some particles have a thin extension.

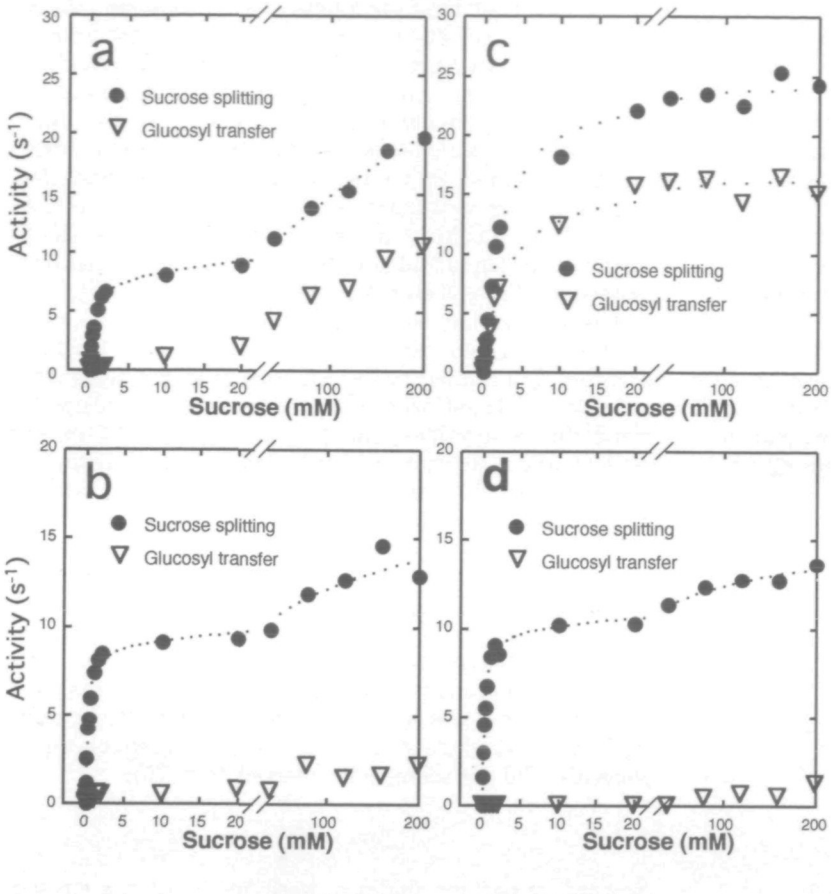

Fig. 3. **Enzymatic activities of GTF-I′ and GS as a function of sucrose concentration.** Initial velocities of fructose and glucose release, v_f and v_g, were measured for the first 4 min of the reaction period at 25°C. Sucrose splitting velocity is equal to v_f, and glucosyl transfer velocity to $v_f - v_g$: both are expressed in the catalytic center activity (s^{-1}). a and b, activities for GTF-I′ and GS, respectively, in the absence of added dextran. c and d, activities for GTF-I′ and GS, respectively, in the presence of dextran T10 (10 μg/ml).

results for GTF-I′ and GS are consistent with the dextran-binding properties of these fragments. When mixed with Sephadex or Sephacryl beads (cross-linked dextrans), GTF-I′ was mostly recovered in the precipitate after low-speed centrifugation, while all the GS remained in the supernatant, indicating a strong binding of GTF-I′ to dextran in contrast with GS, which lacks an affinity for dextran. Thus, it is clear that the C-terminal repeat structure is essential to render the enzyme dextran-dependent in glucosyl transfer activity.

Structure of Dextran-Independent Glucosyl Transfer Products—When set aside after kinetic measurement, the reaction mixtures containing high concentrations of sucrose but without dextran became more or less opaque within an hour or so at room temperature. This change was reproducibly observed for both GTF-I′ and GS, indicating insoluble glucan synthesis as a result of dextran-independent glucosyl transfer by these fragments. The anomeric configuration and position of glucosidic linkages were then analyzed by ^{13}C-NMR spectroscopy (Fig. 4 and Table II). It must be noted that there is a systematic deviation in the chemical shifts of all carbons (C1 to C6) of dextran between the present and earlier studies (*19*). Thus, our results show an upfield shift of 1.8 ppm on average. Taking this into consideration, large differences in the chemical shifts between the insoluble glucan and dextran occur at C1, C3, and C6, and the chemical shifts of carbons of the insoluble glucan are all in good agreement with those of nigerose (α-1,3-linked disaccharide). No resonance characteristic of the β-configuration of C1 was detected, which should be seen at a higher field (*ca.* 103 ppm) as in the case of laminarin (*19*).

In the periodate-oxidation analysis, insoluble glucan was found to consume very little periodate per glucose unit (<0.01). This can be explained by assuming that no glycol structure susceptible to periodate oxidation is present other than in the reducing and non-reducing ends. This structural requirement can only be met by glucans with α or β-1,3-glycosidic linkages. The amount of periodate consumed by the insoluble glucan suggests a minimum number of glucose units in the glucan chain >33, corresponding to a molecular mass >5 kDa. In fact, on alkaline gel-filtration chromatography, the insoluble glucans eluted over a range of fractions corresponding to those of dextran standards (8 to 12 kDa). Thus, the insoluble glucans formed by GTF-I′ and GS from sucrose in the absence of dextran consist mainly of non-branched α-1,3-linked residues.

Tryptic Digestion—When GTF-I′ (138 kDa) was subjected to mild tryptic digestion in the absence of added dextran, smaller fragments with molecular masses of 127, 120, and 110 kDa were successively produced (Fig. 5a). The initial step to the 120 kDa fragment was rapid and completed within a few minutes, but the following step was much slower. N-terminal analysis of the 5 and 180 min digests indicated that the N-terminal amino acid of the 120 and 110 kDa fragments is Asn135 of the GTF-I sequence. This suggests that the rapid cleavage occurs to an N-terminal stretch consisting of 61 residues from Asp85 to Lys134 and the preceding 11 additional residues derived originally from the multi-linker site of the plasmid vector (pUC18,

cf. Ref *14*). On the other hand, the C-terminal cleavage occurs stepwise, rapid deletion of *ca.* 40 and another 70 residues in turn, followed by a slower deletion involving a further 120-130 residues, resulting in the formation of a fragment with a size slightly smaller than that of GS.

Changes in sucrose splitting and glucosyl transfer activity without added dextran corresponding to the time-course of tryptic digestion are shown in Fig. 5b, indicating no change in sucrose splitting activity throughout the period of digestion but a gradual decrease in the efficiency of glucosyl transfer. In contrast, the dextran-dependent activity did not change during the first 20 min of the digestion period, and then decreased gradually (Fig. 5c), nearly in parallel with the decrease in the 120 kDa fragment. These results suggest that a trypsin-generated GTF-I′ subfragment of 120 kDa retains both the dextran-independent and dextran-dependent GTF-I activities almost completely. Further removal of the C-terminal stretch yielding the 105 kDa fragment results in a complete loss of dextran-dependent activity with a concomitant decrease in the efficiency of dextran-independent glucosyl transfer. Electron micrographs of a negatively stained sample of a 10 min digest (not shown), which contained mainly the 120 kDa fragments, indicated that most of the fragments had clear double-domain structures as observed for GTF-I′ (Fig. 3). Thus, there should be a certain critical length of the C-terminal stretch to fold into an active fragment for dextran-dependent glucosyl transfer.

When digestion was carried out in the presence of dextran, fragmentation to form the 120 kDa fragment occurred, but did not proceed further (Fig. 6a), suggesting a dextran-induced protection of the upstream region toward the N-terminus due to a change in the protein conformation. No change was observed for dextran-dependent glucosyl transfer accompanied by a slight increase in sucrose splitting activity (Fig. 6b).

GS (110 kDa) was also digested by trypsin, yielding 105 and 97 kDa fragments in turn (Fig. 7a). The N-terminal sequence of these fragment was identical to that of tryptic subfragments of GTF-I′, indicating the removal of the N-terminal stretch as in the case of GTF-I′ together with the C-terminal stretch consisting of about 70 residues. However, there was no decrease in sucrose splitting activity and a weak glucosyl transfer at high sucrose concentrations also did not seem to be affected (Fig. 7b).

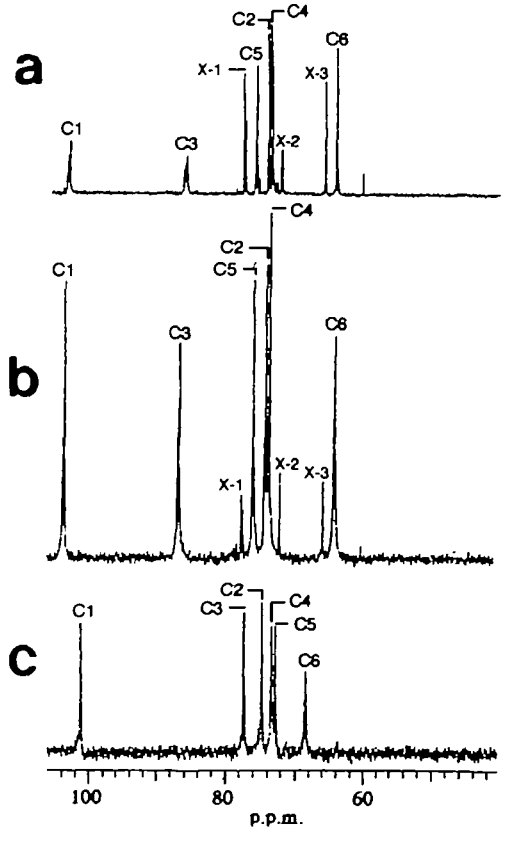

Fig. 4. **^{13}C-NMR spectrum of insoluble glucans produced by GTF-I′ and GS.** The peak assignments were made according to Colson *et al.* (*19*). a, GTF-I′-produced insoluble glucan; b, GS-produced insoluble glucan; c, dextran T10. Minor peaks indicated by ×1 to ×3 in a and b were attributed to artefacts produced by the 0.5 M NaOH used to dissolve the insoluble glucans.

DISCUSSION

Several important findings were made in the present investigations using a genetically truncated form of GTF-I of *S. sobrinus* (GTF-I′) and its subfragment (GS). The latter was designed on the basis of our previous deletion study of the GTF-I gene showing that the expressed proteins retain sucrose hydrolyzing activity, even if the 3′-end deletion proceeds just long enough to abolish the dextran binding ability (*14*).

GTF-I′, GTF-I with deletions of the N-terminal 84 residues and the C-terminal 264 residues, catalyzes active insoluble-glucan synthesis in the presence of primer dextran. Taking into account the difference in assay conditions between the present study and our previous study on GTF-I purified from the culture supernatant of *S. sobrinus* (*22*), the enzymatic activities of GTF-I′ are comparable to those of the mother GTF-I as shown in Table I. Thus, GTF-I′ would be a good substitute for GTF-I in biochemical studies. It contains three of the six C-terminal repeats (I to III) and approximately half of the fourth (see Fig. 1). Removal of the latter incomplete repeat together with the N-terminal sixty residues by tryptic digestion does not affect the dextran-dependent activity. Tryptic removal of these segments occurs even in the presence of dextran

TABLE II. **^{13}C-NMR chemical shifts of glucose polymers.**

Compounds	C-1	C-2	C-3	C-4	C-5	C-6	References
Insoluble glucan	103.5	74.1	86.1	73.6	75.8	64.1	This study
Dextran T-10	101.3	74.9	77.3	73.5	72.9	68.6	*ibid.*
Dextran	99.4	73.1	75.4	71.8	71.1	66.8	Colson *et al.* (*19*)
Nigerose (α-1,3 linked)	101.3	72.2	83.2	71.7	73.7	62.2	*ibid.*
Laminaribiose (β-1,3 linked)	104.7	74.9	75.4	71.8	71.1	66.8	*ibid.*

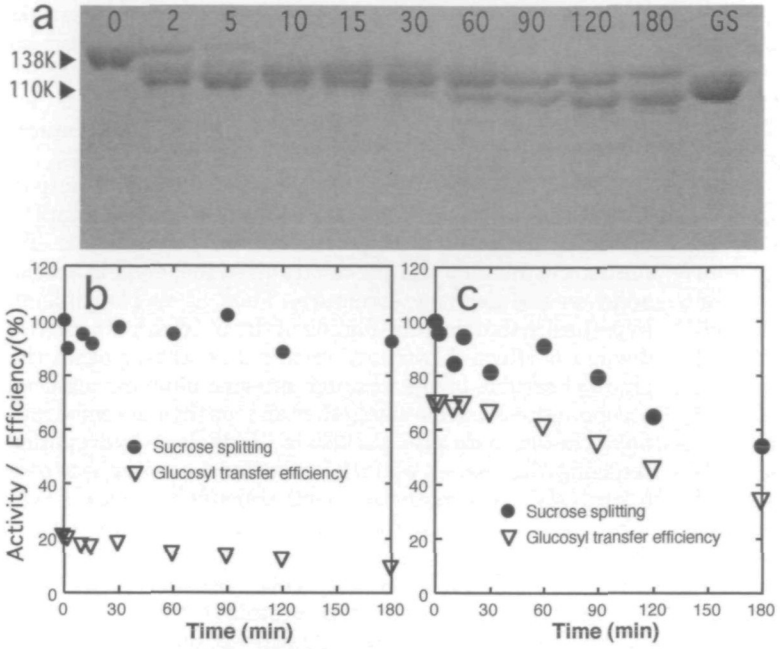

Fig. 5. **Effects of tryptic digestion on enzymatic activities of GTF-I′.** GTF-I′ was digested with TPCK-trypsin for 2 to 180 min at 25°C. Aliquots were withdrawn from the reaction mixture at intervals and mixed with soybean trypsin inhibitor. a, SDS-PAGE of the digests and GS (molecular mass marker); b, the remaining sucrose splitting activity and glucosyl transfer efficiency at 40 mM sucrose; c, those at 40 mM sucrose + 9 μg/ml dextran T10.

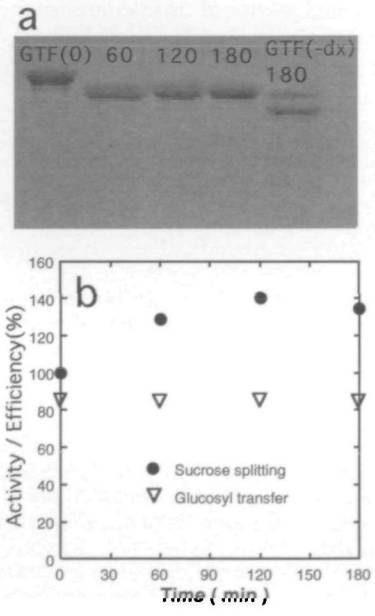

Fig. 6. **Dextran protection of tryptic digestion of GTF-I′.** GTF-I′ was digested with TPCK-trypsin in the presence of dextran T10 (100 μg/m). a, SDS-PAGE patterns of the digests and GTF-I′ digested for 180 min in the absence of T10 as was shown in Fig. 5a; b, the remaining sucrose splitting activity and glucosyl transfer efficiency at 40 mM sucrose. Since the carry-over of dextran T10 from the tryptic digestion mixture into enzyme assay solutions was not negligible, only dextran-dependent activity was measured. Experimental conditions and data treatment are the same as those in Fig. 5.

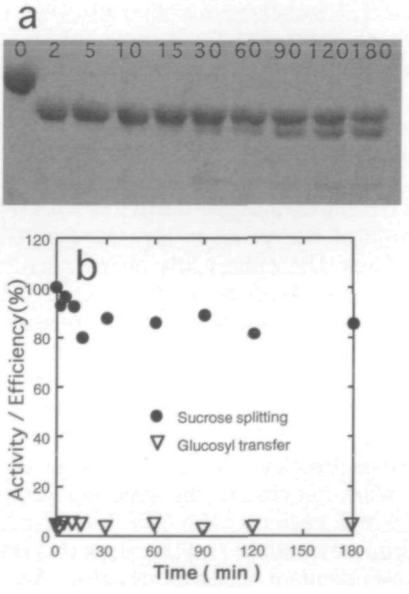

Fig. 7. **Effects of tryptic digestion on enzymatic activities of GS.** GS was digested with TPCK-trypsin at 25°C as in Fig. 5. a, SDS-PAGE analysis; b, activities at 40 mM sucrose.

(formation of the 120 kDa fragment). A smaller fragment (105 kDa) formed by further C-terminal removal no longer shows the dextran-dependent activity. These results indicate that for the tight binding of dextran as the glucosyl acceptor in the GTF-I reaction, the minimum structural requirement is three of the C-terminal repeats attached to the N-terminal large catalytic domain, confirming our earlier observations (*14*). It is worth mentioning here that the C-terminal domain consisting of these repeats is much more liable to trypsin attack than the N-terminal domain. The distribution of potential cleavage sites along the primary structure is approximately one per ten residues on average for both domains calculated on the basis of the deduced amino acid sequence of GTF-I (*14*). Hence, the protein-folding patterns of these domains may be quite different.

GS is probably the first N-terminal fragment prepared from GTFs that retains functions somehow related to GTF

catalysis, such as sucrose hydrolysis (invertase activity) and glucosyl transfer, which has long been pursued by proteolytic and genetic methods. Of particular significance is the demonstration of its measurable dextran-independent glucosyl transfer activity. GTF-I′ also has this activity, but it is much more efficient (see below). This primer-independent activity cannot be ascribed to a functional change in the protein caused by the deletion of part (GTF-I′) or all (GS) of the C-terminal dextran-binding domain and a stretch of N-terminal residues. Thus, judging from the data in earlier papers or GTF-Is without proteolytic or genetic modification, many authors should have noticed insoluble glucan synthesis in the absence of primer, but did not pay much attention to the phenomenon except in a few cases (*23, 24*). This is partly because the affinity of GTF-I for dextran is commonly so high that trace glucan contamination in sucrose could be sufficient to initiate glucan synthesis in the absence of an exogenous glucan acceptor. Of course, this interpretation cannot be applied to the insoluble glucan synthesis by GS, which cannot bind dextran at all. In addition, the protein expressed by the whole gene (*14*) was also found to catalyze insoluble glucan synthesis in the absence of added dextran (Matsuno and Kodama, unpublished). Since structural analysis indicated that the insoluble glucan thus synthesized by GTF-I′ and GS contains only α-1,3 linkages, we conclude that the primer-independent glucosyl transfer leading to the formation of insoluble α-1,3 glucan is a genuine function of the N-terminal catalytic domain of GTF-I. In other words, the domain on its own is sucrose: α-1,3-D-glucan glucosyltransferase in a strict sense.

Electron micrographs of negatively stained samples of GTF-I′ and GS can be interpreted in line with the functional differentiation of the primary structure of GTF-I so far described. Thus, the spherically shaped structure of GS undoubtedly corresponds to the larger spherical portion of GTF-I′, which corresponds to the N-terminal catalytic domain of GTF-I. On the other hand, the smaller portion of GTF-I′, which is occasionally seen as a long extension, corresponds to the C-terminal dextran-binding domain.

Returning to self-initiated glucosyl transfer, there arise three questions directly related to the reaction mechanism of GTF-I: what serves as the glucosyl acceptor in the beginning; which region of the GTF-I sequence is responsible for acceptor binding; and how does this correlate with the dextran-dependent glucosyl transfer. As for the first question, our preliminary experiments suggest maltose and isomaltose as effective acceptors, consistent with a well-known broad acceptor specificity in marked contrast with a rather limited donor specificity (*2*). In the absence of any exogenously added acceptors, however, it is likely that the glucosyl residues are initially transferred to glucose or sucrose, and then to the oligosaccharides thus the formed, which finally results in the formation of insoluble α-1,3-linked glucans. This self-initiation hypothesis, although not contradicting any known observations, must be tested by kinetic experiments.

Little is known in answer to the second and third questions. However, one clue obtained in this study is that GTF-I′ and GS show little kinetic differences in sucrose hydrolysis at low substrate concentrations (<5 mM), while glucosyl transfer at high concentrations is much more efficient in the former than in the latter. With an increase in the C-terminal deletion of GTF-I′ by tryptic digestion, its transfer efficiency falls to the level of GS without an accompanying change in sucrose splitting activity. These results indicate that the C-terminal region, which is not essential for sucrose splitting, somehow affects chemical processes in the catalytic site.

It is now generally accepted that the key intermediate in GTF reactions is an unstable glucosyl-enzyme complex involving the catalytic Asp residue (*4*). The catalytic site containing this Asp and the dextran-binding domain are far apart in the primary structure. Thus, it is plausible to hypothesize that protein-folding of the N-terminal catalytic domain is affected by the C-terminal repeats, so that the glucosyl residue of the unstable intermediate and a glucan acceptor site come close together and/or the microenvironment in the catalytic pocket is made less hydrophilic, reducing the extent of futile transfer to water (sucrose hydrolysis). Alternatively, such transfer-favoring structural reorganization in the catalytic site and nearby is more easily induced by the substrate sucrose in GTF-I′ than in GS. Thus, examining the differences between GTF-I′ and GS in molecular shape and protein conformation and changes induced by substrates (sucrose, dextran, *etc.*) and comparing their kinetic parameters for the intermediate steps (sucrose and dextran binding, enzyme glucosylation, and glucosyl transfer) are of immediate importance to our understanding of the molecular mechanism of the GTF-I reaction.

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