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MECHANISM OF SYNTHESIS OF D-GLUCANS BY D-GLUCOSYLTRANSFERASES FROM *Streptococcus mutans* 6715*

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ABSTRACT

Two glucosyltransferases from *Streptococcus mutans* 6715 were purified and separated. One of the glucosyltransferases synthesized an insoluble glucan, and the other, a soluble glucan. The enzymes were immobilized on Bio-Gel P-2 beads, and the mechanism of glucan synthesis was studied by pulse and chase techniques with ^{14}C-sucrose. Label was associated with the immobilized enzymes. The label could be quantitatively released by heating at pH 2. Analysis of the labeled products from the pulse experiment showed labeled glucose and labeled glucan; the chase experiment showed labeled glucan and a significant decrease in labeled glucose. The glucans from the pulse and the chase experiments were separated from glucose by chromatography on Bio-Gel P-6. They were reduced with sodium borohydride, and the products hydrolyzed with acid. Analysis of the labeled products from the reduced and hydrolyzed, pulsed glucans showed labeled glucose and labeled glucitol; label in the glucitol was greatly decreased in the chase experiment. These experiments showed that glucose and glucan were covalently attached to the active site of the enzymes during synthesis, and that the glucose was being transferred to the reducing end of the glucan chain. A mechanism for the synthesis of the glucans is proposed in which there are two catalytic groups on each enzyme that holds glucosyl and glucanosyl units. During synthesis, the glucosyl and glucanosyl units alternate between the two sites, giving elongation of the glucans from the reducing end.

The addition of increasing amounts of B-512F dextran to the insoluble-glucan-forming glucosyltransferase produced a decrease in the proportion of insoluble glucan formed and a concomitant increase in a soluble glucan. The total amount of glucan synthesized (soluble plus insoluble) was increased 1.6 times over the amount of insoluble glucan formed when no exogenous dextran was added. It is shown that the addition of B-512F dextran affects the solubility of the synthesized α-(1→3)-glucan by accepting α-(1→3)-glucan chains at various positions along the dextran chain, to give a soluble, graft polymer.

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INTRODUCTION

Streptococcus mutans 6715 produces two types of extracellular polysaccharides from sucrose, namely, a water-soluble α-D-glucan and a water-insoluble α-D-glucan. The soluble glucan contains 64% of α-(1→6) linkages and 36% of α-(1→3) branch linkages, and is dextran-like[1]. The insoluble glucan contains 93% of α-(1→3) linkages and 7% of α-(1→6) branch-linkages[2].

Many reports on the purification of the glucosyltransferases (glucansucrases) that synthesize *S. mutans* glucans have appeared[3-10]. Of these, three[3,5,12] have clearly shown that one type of glucosyltransferase synthesizes the soluble glucan, and another type synthesizes the insoluble glucan. We have combined two of these methods[5,12] in order to obtain two distinct glucosyltransferases, one that synthesizes the soluble glucan exclusively, and the other that synthesizes the insoluble glucan exclusively. We attached these purified enzymes covalently to Bio-Gel P-2 beads, and studied the mechanism of biosynthesis of the glucans by using pulse and chase techniques with ^{14}C-labeled sucrose, as we had previously done[20] with the dextransucrase of *Leuconostoc mesenteroides* B-512F.

We found that, during synthesis of glucan, these enzymes form glucosyl and glucanosyl covalent complexes with the active site, and that the synthesis of the glucan occurs by the transfer of the glucosyl unit to the reducing end of the growing glucan chain, which remains attached to the active site.

We also found that the addition of increasing amounts of B-512F dextran to the insoluble-glucan-forming glucosyltransferase stimulates its action, but decreases the amount of insoluble glucan synthesized and produces, instead, soluble glucan. We have found that the α-(1→3)-glucan chains synthesized by the glucosyltransferase are transferred to various positions along the dextran chain, to give a soluble, branched, graft polymer.

EXPERIMENTAL

Purification of glucosyltransferases. — *S. mutans* 6715 was grown on a liquid medium, as described by Ciardi *et al.*[12]. A Microferm laboratory fermentator (New Brunswick Scientific Co.) was used to obtain four 10-L batches (40 L). Growth was allowed to proceed for 12 h at 37° from a 10% inoculum, with 150-r.p.m. stirring and no aeration. The cells were centrifuged off, and the 40 L of fermentation broth was concentrated to 350 mL with a Bio-Fiber 80 miniplant (Bio-Rad Laboratories, Richmond, CA). The purification scheme of Ciardi *et al.*[12] was used, up to the stage of obtaining the unfractionated enzyme (UFE). A modified procedure of Walker and Hare[14] was then used to obtain the two purified glucosyltransferases by adding 12 g of hydroxylapatite (Bio-Gel HTP, Bio-Rad Laboratories) to UFE (25 mL). This mixture was stirred for 15 min at 4°, centrifuged, the supernatant liquor decanted, and the HTP washed with 25 mL of 10mM imidazole · HCl buffer pH 6.5. The soluble-glucan-producing glucosyltransferase (GTF-S) was obtained by elution

from the HTP with 25 mL of 0.07M K_2HPO_4 buffer, pH 6.5; the HTP was centrifuged, the GTF-S supernatant liquor removed, and the HTP washed twice with 10mM imidazole · HCl buffer. The insoluble-glucan-producing glucosyltransferase (GTF-I) was eluted from the HTP with 25 mL of 0.4M K_2HPO_4 buffer, pH 6.5. The enzymes were concentrated in dialysis bags with poly(ethylene glycol) 20,000.

Glucosyltransferase activity was measured by the method of Germaine *et al.*[21]. One unit of enzyme produced 1 μmol of glucose incorporated into glucan per min at pH 6.5 and 37°.

Protein was determined by the Lowry procedure[22]. Carbohydrate was determined by the phenol–sulfuric acid method[23]. Poly(acrylamide)-gel electrophoresis was conducted as previously described[24].

Preparation and characterization of the glucans formed by the purified enzymes. — The soluble glucan was prepared by adding 2 mL of GTF-S (0.1 U/mL) to 100 mL of 100mM sucrose buffered at pH 6.5 with 10mM imidazole · HCl, and allowing the reaction to proceed for 24 h at 37°. The polysaccharide was precipitated with ethanol (3 vol.), centrifuged off, and dried *in vacuo* for 12 h at 40°. The insoluble glucan was prepared similarly by using GTF-I, with omission of the ethanol-precipitation step. The insoluble glucan was centrifuged off, washed with water, and dried.

The polysaccharides (10 mg) were dissolved in M sodium hydroxide (10 mL), the solutions diluted to 250 μg/mL, and made neutral. The insoluble glucan will remain in solution as long as the concentration is 250 μg/mL, or less.

The glucans (100–250 μg) in 4 mL of 50mM acetate buffer, pH 4, were oxidized with 3.5mM sodium periodate. The periodate consumed was determined[25] at 290 nm.

Immobilization of the enzymes. — The enzymes were covalently linked to Bio-Gel P-2 beads. Bio-Gel P-2 (100–200 mesh; 1 g) was heated in ethylenediamine (20 mL), with stirring, for 7 h at 90°. The gel was filtered off, and washed with water (1 L). It was then treated with 20 mL of 2.5% glutaraldehyde for 30 min at 1°, filtered off, and washed with water at 4°. The enzymes were coupled to the activated Bio-Gel by the addition of gel (1 g) to 4 mL of enzyme (7.5 U GTF-I/mL and 10.7 U GTF-S/mL) which was stirred for 12 h at 4°. The gel was removed by decantation, and washed with water (6 × 10 mL). The gel was then incubated for 1 h at 22° in 50mM L-lysine (4 mL). The supernatant liquor was decanted and the gel was washed with water (6 × 10 mL). The resulting GTF-I gel had an activity of 40 mU/100 mg of gel, and the GTF-S gel had an activity of 80 mU/100 mg of gel.

Pulsing and chasing of the immobilized enzymes with ^{14}C-sucrose. — The two immobilized enzymes were first charged by incubating 1 g of gel in 2 mL of mM sucrose (pH 6.5) for 30 min. The gel was washed by decantation with water (6 × 10 mL). It was pulsed by incubating in 2 mL of buffer containing 1 μCi of [^{14}C-(U)]sucrose (3 μM) for 12 h at 20°, and then washed with water (6 × 10 mL). The radioactivity in the last wash was reduced to background. The gel was divided into halves. One half was chased by incubating with mM sucrose for 10 min, after which the gel was washed with water (6 × 10 mL).

Quantitative release of the labeled products from the pulsed and chased gels

was obtained by adjusting the pH to 2, and heating for 15 min at 98 . Aliquots of the labeled products were chromatographed on Whatman No. 3 MM paper by three ascending irrigations with 46:35:18 (v/v/v) 95% ethanol–nitromethane–water. The rest was chromatographed on a column (1 × 47 cm) of Bio-Gel P-6 (see Fig. 3). The glucan fractions were pooled, and concentrated to 1 mL.

Reduction of the pulsed and chased glucans, and acid hydrolysis. — The pulsed and chased glucans, which were separated from glucose (see Fig. 3) were reduced by adding 10 mg of sodium borohydride/mL and heating for 15 min at 98 . The glucans were hydrolyzed with 4M trifluoroacetic acid in sealed ampoules for 2 h at 121 . The samples were evaporated with methanol (5 × 2 mL) to remove borate, and dissolved in water (250 μL), and aliquots (100 μL) were added to Whatman No. 3 MM paper (20 × 50 cm) for descending chromatography for 12 h with 8:1:1:1 (v/v/v/v) nitromethane–acetic acid–ethanol–water saturated with boric acid[20] at 37 . Labeled glucose and glucitol, located with standards, were cut (2 × 2 cm) from the paper, and counted by liquid scintillation with a toluene cocktail[20,27].

Addition of B-512F dextran to GTF-I digests. — Several reaction-mixtures consisting of 100 μL of 100mM [^{14}C-(U)]sucrose (1 μCi), 1 mL of various amounts (0–20 mg/mL) of B-512F Sigma dextran (mol. wt. 2 × 10^6), and 0.4 mL of GTF-I (0.3 U/mL) were incubated at 37 ; 0.4-mL aliquots were taken at 10, 20, and 30 min. The aliquots were filtered through Whatman No. 3 MM paper, to remove insoluble glucan formed, and the papers were washed with methanol (3 × 1 mL). Soluble glucan was precipitated from the original filtrate with ethanol (1.5 mL). The precipitates were centrifuged off, and dissolved in water (0.5 mL); 50 μL was added to Whatman No. 3 MM papers (1.5 × 1.5 cm), which were washed with methanol (3 × 100 mL), dried, and counted in a toluene cocktail[27]. The radioactivity of the glucan *vs.* time of reaction was plotted, and the slope of the resulting line was plotted against the amount of B-512F dextran added to the reaction mixture.

The same reactions were repeated with [^{3}H-(U)]sucrose (1 μCi/digest) and [^{14}C-(U)]B-512F dextran (45 nCi/digest). In this experiment, the reaction was allowed to proceed to equilibrium (~12 h). The suspension of insoluble glucan was centrifuged, and the supernatant liquor decanted. The insoluble glucan was washed three times with water, an aliquot was filtered on Whatman No. 3 MM paper, and ^{3}H and ^{14}C were counted. The soluble glucan was precipitated from the supernatant liquor with ethanol (3 vol.), the suspension centrifuged, and the solid washed with methanol (3 × 2 mL), and dissolved in water (0.5 mL): 25-μL aliquots were added to Whatman No. 3 MM papers (1.5 × 1.5 cm), and ^{3}H and ^{14}C were counted. *Penicillium funiculosum* endodextranase (Sigma Chem. Co.; 1 unit) was added to 200 μL of the ethanol-precipitated glucan obtained from the GTF-I digests containing 0, 2, and 20 mg of ^{14}C-dextran/mL and to ^{14}C-dextran alone; 25-μL aliquots were removed at various times (0–630 min) from each dextranase digest, and placed on Whatman No. 3 MM papers (1.5 × 1.5 cm). The papers were washed with methanol (3 × 200 mL), and dried, and the ^{3}H and ^{14}C were counted in a toluene cocktail with a liquid scintillation spectrometer.

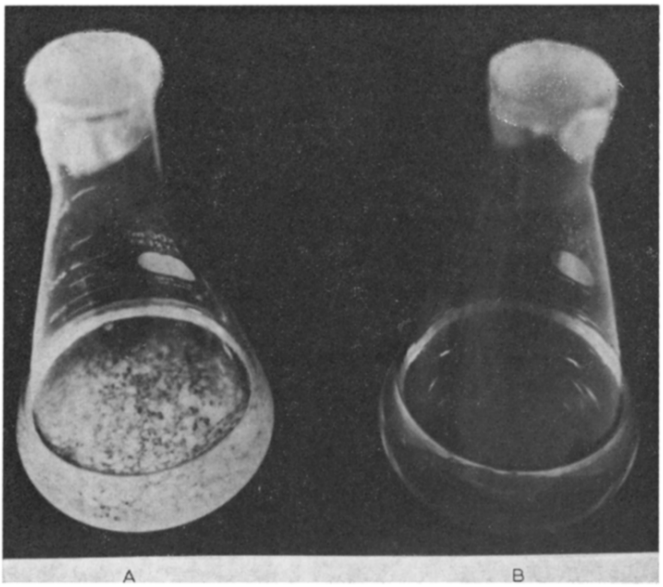

Fig. 1. Appearance of products produced by the action of GTF-I (A) and GTF-S (B) on sucrose.

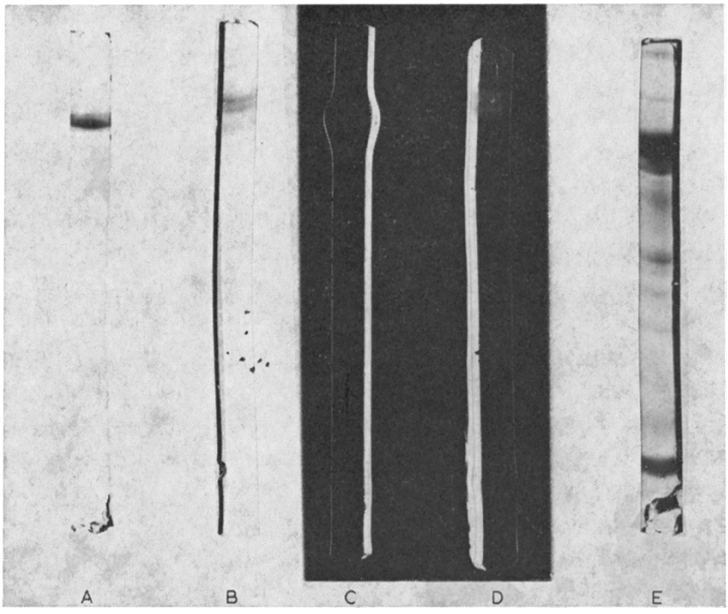

Fig. 2. Poly(acrylamide)-gel electrophoresis of separated and purified glucosyltransferase from *Streptococcus mutans* 6715. [(A) Purified GTF-S, protein stain; (B) purified GTF-I, protein stain; (C) purified GTF-S, incubated with sucrose; (D) purified GTF-I, incubated with sucrose; (E) crude, unfractionated enzyme (UFE).]

RESULTS

Characterization of the purified glucosyltransferases. - Two enzymes, GTF-I and GTF-S, were obtained. They respectively synthesized an insoluble and a soluble glucan (see Fig. 1). The insoluble glucan formed by GTF-I was removed by filtration, and 3 volumes of ethanol were added to the filtrate. No precipitate was formed, indicating that GTF-I exclusively synthesized an insoluble glucan. The GTF-S digest was completely clear, with no observable glucan (see Fig. 1), indicating that GTF-S exclusively synthesized a soluble glucan, which could be precipitated by adding 2 volumes of ethanol.

Poly(acrylamide)-gel electrophoresis of the enzymes (see Fig. 2) showed a single, protein band for GTF-S. When the gel was incubated with sucrose, a clear band having an index of refraction different from that of the gel was obtained. In many experiments, this band expanded, to give a bulge to the gel (see Fig. 2C). Two closely migrating, protein bands, different from GTF-S, were obtained for GTF-I, and, on incubation with sucrose, these gave white precipitates (see Fig. 2D). GTF-I appears to be composed of two isozymes. McCabe and Smith[13] and Figures and Edwards[19] separated, from *S. mutans*, two glucosyltransferases that synthesized insoluble glucans. Both groups found that one of the glucosyltransferases was stimulated by exogenous, soluble B-512F dextran, and the other was not. Perhaps the difference between these two glucosyltransferases is that the one not stimulated by soluble dextran is already saturated with glucan, whereas the one that is stimulated is not saturated with glucan.

Characterization of the glucans synthesized by GTF-S and GTF-I. The two glucans synthesized by GTF-S and GTF-I respectively consumed 1.8 and 0.15 mol of periodate per mol of glucosyl residue. This indicates that the soluble glucan has a dextran-like structure, with appreciable, contiguous α-(1→6) linkages. The insol-

TABLE I

CHROMATOGRAPHIC ANALYSIS OF THE LABELED PRODUCTS RELEASED FROM PULSED AND CHASED BIO-GEL GTF-I AND GTF-S

Compound	*Pulse*		*Chase*	
	C.p.m.	M_f[a]	*C.p.m.*	M_f[a]
GTF-I				
Glucose	5,150	0.34	2,710	0.15
Glucan	10,100	0.66	15,020	0.85
GTF-S				
Glucose	4,420	0.26	740	0.12
Glucan	13,030	0.74	5,430	0.88

[a] M_f = mol fraction = (c.p.m. of the compound)/(total c.p.m.).

uble glucan consumed much less periodate per mol of glucosyl residue, indicative of a glucan having appreciable, contiguous α-(1→3) linkages.

Analysis of the labeled products released from the immobilized enzymes when pulsed with ^{14}C-sucrose and chased. — The labeled products attached to the immobilized enzymes could be released by heating at pH 2. The products released from the pulse and chase experiments were analyzed by paper chromatography, and are given in Table I. In the pulse experiments, for both GTF-I and GTF-S, only two labeled products were observed, namely, glucose and glucan. In the chase experiments, labeled glucan was observed, and there was a decrease in the label in glucose from 34 to 15 mol percent for GTF-I, and from 26 to 12 mol percent for GTF-S.

The labeled products were tightly attached to the immobilized enzyme-gel, and were not released when the system was extensively washed with buffer. The products were, however, quantitatively released when the system was heated at pH 2 for 15 min at 98°. Other treatments, *e.g.*, pH 10.5, 5M urea, and 2% sodium dodecyl

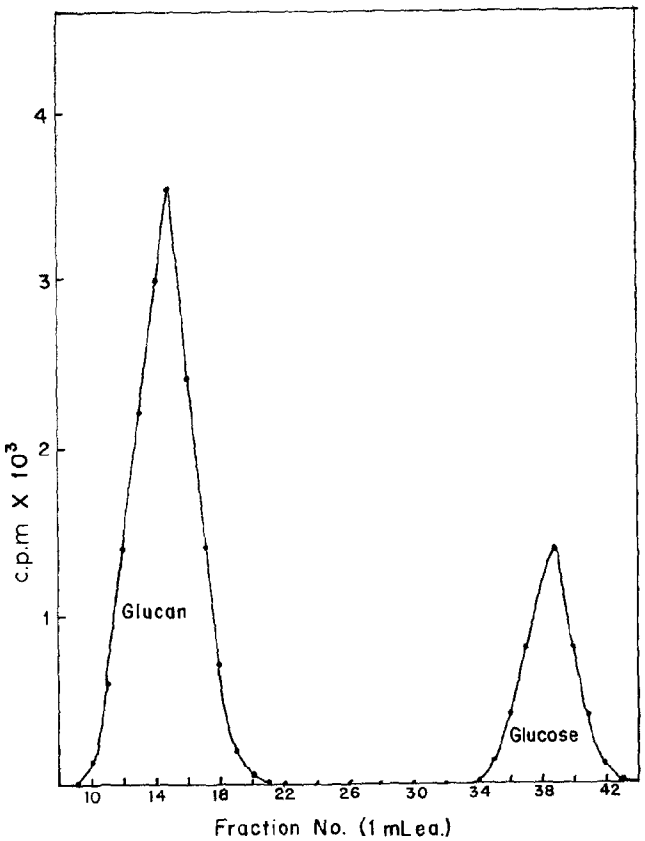

Fig. 3. Separation of labeled glucan from glucose by gel filtration on Bio-Gel P-6. [A typical experiment: separation of ^{14}C-glucose and ^{14}C-glucan that were released from pulsed, Bio-Gel P-2-immobilized GTF-I.]

TABLE II

ANALYSIS OF ACID-HYDROLYZED, REDUCED, PULSED AND CHASED GLUCANS PRODUCED BY GTF-I AND GTF-S

Compound	*Pulse*			*Chase*		
	C.p.m.	R[a]	M_1[b]	*C.p.m.*	R	M_1
GTF-I						
Glucose	4,420	6.6	0.87	4,530	40	0.98
Glucitol	670	1	0.13	110	1	0.02
GTF-S						
Glucose	3,840	3.9	0.80	2,520	21	0.96
Glucitol	980	1	0.20	120	1	0.04

[a] R = ratio of D-glucose to D-glucitol [b] M_1 = mol fraction (c.p.m. of the compound)/(total c.p.m.).

sulfate, failed to release a significant amount of label. The latter conditions indicate that multiple hydrogen-bonding or hydrophobic interactions are probably not the cause of the association of glucan and glucose with the immobilized enzyme. The quantitative release of glucose and glucan at pH 2 and 98° suggests, however, a labile, covalent linkage with the enzyme.

These experiments show that, during synthesis, two types of carbohydrate, glucose and glucan, are covalently attached to the enzymes, and that these products may be released from the enzymes by heating at pH 2. The decrease of the label in glucose in the chase experiment further shows that the glucose, which is covalently linked to the enzyme, is being incorporated (chased) into the glucan fraction.

Analysis of the pulse and chase label in the GTF-I and GTF-S glucans. - The released glucans from the pulse and chase experiments were separated from the released glucose by gel filtration on a column of Bio-Gel P-6 (see Fig. 3). The isolated glucans were reduced with sodium borohydride, the products hydrolyzed with acid, and the products analyzed by paper chromatography. The results are given in Table II. In the pulse experiments, the ratio of labeled glucose to labeled glucitol was 6.6:1 for GTF-I, and 3.9:1 for GTF-S, and, for the chase experiments, 40:1 for GTF-I, and 21:1 for GTF-S. These results show that the glucitol was labeled in the pulse experiments and, in the chase experiments, was significantly decreased, to one-seventh for GTF-I, and one-fifth for GTF-S. Because glucitol is produced by the reduction of the (reducing) glucose residue of the glucans, the formation of labeled glucitol from the pulsed glucans, and the decrease of label in glucitol from the chased glucans, demonstrate that both of the glucans are synthesized by the transfer of covalently linked glucose to the reducing end of the glucan. Furthermore, because the glucans that were analyzed were released from immobilized enzyme, the synthesis occurs while both the glucose and the glucan are covalently attached to the enzyme.

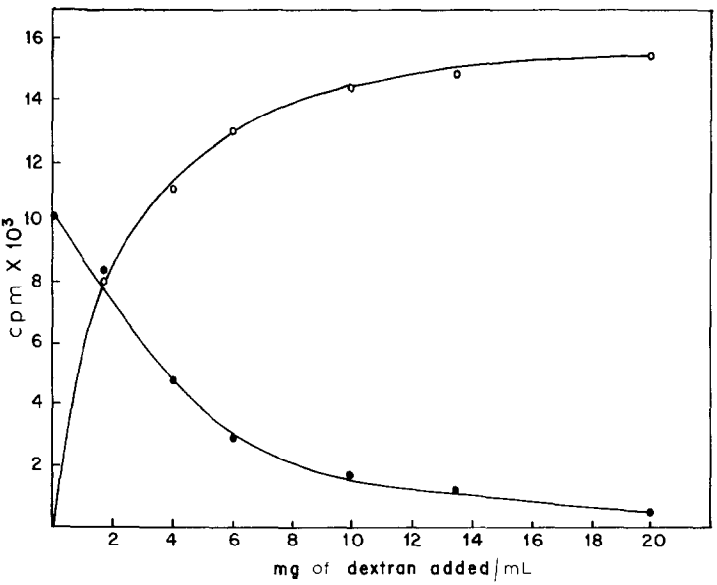

Fig. 4. Reaction of GTF-I with 100mM ^{14}C-sucrose in the presence of various amounts of B-512F dextran. [Key: —●—, insoluble glucan; —○—, soluble glucan.]

Analysis of ^{14}C-glucans from GTF-I in the presence of various concentrations of B-512F dextran. — GTF-I was incubated with ^{14}C-sucrose and various concentrations of B-512F dextran. The ^{14}C-labeled, insoluble and soluble glucans were measured as a function of the amount of added B-512F dextran. The results are given in Fig. 4. As the amount of dextran is increased, there is a concomitant increase in the soluble glucan formed, and a decrease in the insoluble glucan formed. When the concentration of dextran was 1.7 mg/mL, the amount of soluble glucan equaled the amount of insoluble glucan formed, and the total amount of glucan formed (soluble plus insoluble) was 1.6 times the initial amount of insoluble glucan formed when no B-512F dextran was added. Furthermore, this increase is relatively constant within the range of added dextran studied, indicating that B-512F dextran activates GTF-I at relatively low concentrations, as well as affecting the ratio of insoluble and soluble glucans formed.

Distribution of ^{3}H and ^{14}C in insoluble and soluble glucans formed by GTF-I from ^{3}H-sucrose and ^{14}C-B-512F dextran. — GTF-I was incubated with [^{3}H]sucrose and various concentrations of B-512F [^{14}C]dextran. After reaction for 12 h, the insoluble glucan formed was centrifuged off, and ^{3}H and ^{14}C were counted. Soluble glucan was precipitated with ethanol, the precipitate was dissolved in water, and ^{3}H and ^{14}C were counted. The results are given in Table III. As in Fig. 4, there is a decrease in the amount of insoluble glucan formed, and an increase in the amount of soluble glucan formed, as the concentration of dextran is increased, as seen by the incorporation of ^{3}H into the two glucans. A relatively small proportion (15%) of

TABLE III

INCORPORATION OF ^{3}H AND ^{14}C INTO INSOLUBLE AND SOLUBLE GLUCANS SYNTHESIZED BY GTF-I FROM [^{3}H]SUCROSE IN THE PRESENCE OF VARIOUS CONCENTRATIONS OF B-512F[^{14}C] DEXTRAN

B-512F dextran added (mg)	*Mol fractions*[a]			
	Insoluble glucan		*Soluble glucan*	
	^{3}H	^{14}C	^{3}H	^{14}C
0	0.850	0	0.150	0
2	0.130	0.030	0.870	0.970
4	0.120	0.020	0.880	0.980
7	0.040	0.015	0.960	0.985
10	0.030	0.014	0.970	0.986
15	0.027	0.010	0.973	0.990
20	0.005	0.005	0.995	0.995

[a]Mol fraction = (fraction of label incorporated into glucan)/(total label in soluble plus insoluble glucan).

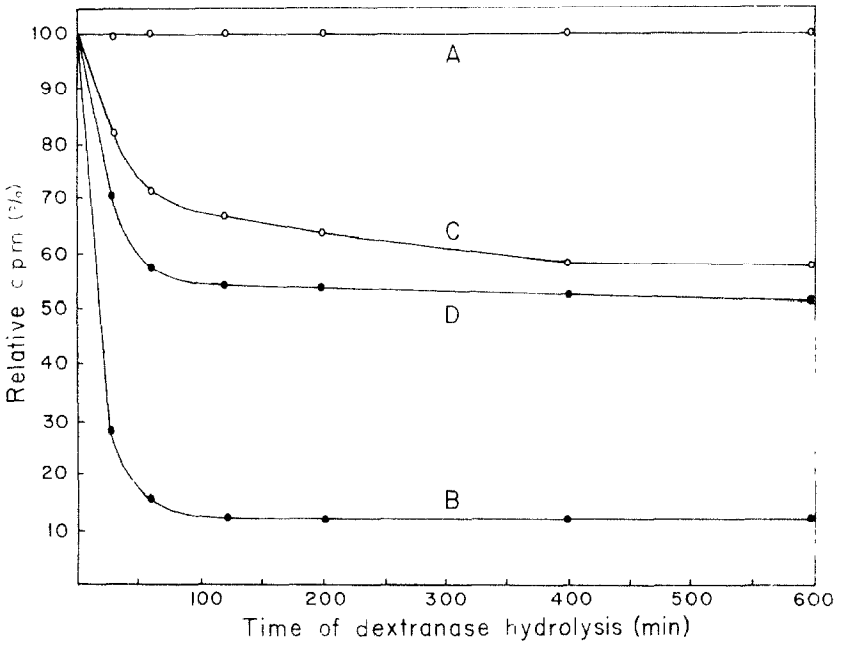

Fig. 5. Endodextranase hydrolysis of ^{3}H- and ^{14}C-glucans. (Key: —○—○—, ^{3}H-label; —●—●—, ^{14}C-label. A is ^{3}H-labeled glucan synthesized by GTF-I in the absence of dextran. B is B-512F ^{14}C-dextran. C and D are, respectively, ^{3}H and ^{14}C released from the soluble glucan synthesized by GTF-I from [^{3}H]sucrose and 2 mg of [^{14}C]dextran per mL.)

soluble [^{3}H]glucan was observed in this experiment when no dextran was added. This glucan was totally resistant to dextranase hydrolysis (see Fig. 5), and most probably constitutes a precursor of insoluble glucan. It was also observed that a small proportion (3%) of ^{14}C-dextran is initially incorporated into insoluble glucan, and that the amount of incorporation decreases as the concentration of the added dextran is increased. At relatively high concentrations (20 mg) of added dextran, the insoluble glucan formed is lessened to a very low proportion (0.5%).

The glucan synthesized by GTF-I from [^{3}H]sucrose is completely resistant to endodextranase hydrolysis. The B-512F [^{14}C]dextran, however, is rapidly hydrolyzed (see Fig. 5). When the soluble glucan obtained from the GTF-I digest with [^{3}H]-sucrose and [^{14}C]dextran (2 mg/mL) was treated with dextranase, the [^{14}C]dextran was much less susceptible to hydrolysis (49 *vs.* 88% conversion into methanol-soluble products), whereas the [^{3}H]glucan went from totally resistant to 40% converted into methanol-soluble, dextranase products. A similar type of result was obtained when the soluble glucan obtained from GTF-I, [^{3}H]sucrose, and [^{14}C]dextran (20 mg/mL) was treated with dextranase (data not shown).

The decrease in the endodextranase susceptibility of the ^{14}C-label, and the increase in the susceptibility of the ^{3}H-label, in the soluble glucans obtained when [^{14}C]dextran is added to GTF-I and [^{3}H]sucrose indicate that the GTF-I synthesized α-(1→3)-[^{3}H]glucan chains are added to [^{14}C]dextran at various positions along the dextran chain. Had the α-(1→3)-glucan chains been exclusively added to the nonreducing ends of the [^{14}C]dextran, the dextranase susceptibility of the ^{14}C- and ^{3}H-labels would not have been affected, and would have been identical to those of the [^{14}C]dextran and [^{3}H]glucan controls, *i.e.*, ^{14}C would have been converted into 88% methanol-soluble products, and ^{3}H would not have been converted into any methanol-soluble products.

The attachment of the α-(1→3)-glucan chains at multiple positions along the dextran chain must be by branch linkages. This attachment is most probably an α-(1→3) branch-linkage, which is the type of branch linkage found in B-512F dextran.

DISCUSSION

We have isolated from *Streptococcus mutans* 6715 the two glucosyltransferases, GTF-I and GTF-S, that respectively synthesize an α-(1→3)-linked insoluble, and an α-(1→6)-linked soluble, glucan. These enzymes were immobilized on Bio-Gel P-2 beads, and the mechanism of glucan synthesis was studied by pulse and chase techniques with ^{14}C-sucrose. We found that glucose and glucan were covalently attached to the active site of the enzymes, and that synthesis occurred by the transfer of a glucosyl group to the reducing end of the glucanosyl chain, which remained covalently attached to the active site. This is identical to what was found[20] for the synthesis of dextran by dextransucrase from *Leuconostoc mesenteroides* B-512F.

The mechanism of synthesis that we proposed[20] for dextransucrase involved two equivalent, nucleophilic, catalytic groups at the active site to which a glucosyl

group and a dextranosyl unit were covalently linked. During synthesis, the primary hydroxyl group of the glucosyl group makes a nucleophilic attack on C-1 of the reducing-end glucosyl unit of the dextranosyl chain, forming an α-(1→6) bond. This releases the nucleophile that was attached to the dextran, and it subsequently attacks C-1 of the D-glucosyl group of sucrose, forming a new D-glucosyl-enzyme complex. The primary hydroxyl group of this new D-glucosyl unit then attacks C-1 of the reducing glucosyl unit of the dextranosyl chain, forming another α-(1→6) bond. This process continues, with the two catalytic nucleophiles alternately forming covalent intermediates with D-glucose and with dextran. The growth of the chain is terminated when it is released from the active site by acceptor reactions[28,29]. This mechanism has been shown[30] to be stereochemically feasible by a three-dimensional study with models.

A mechanism for the synthesis of the soluble α-(1→6)-glucan synthesized by GTF-S may be offered that is identical to the mechanism we proposed for B-512F dextran synthesis. A similar mechanism may be suggested for the synthesis of the insoluble α-(1→3)-glucan synthesized by GTF-I, but, instead of the primary 6-hydroxyl group of the glucosyl unit, in stereochemical position to make the nucleophilic attack on C-1 of the glucosyl unit of the growing glucan, the 3-hydroxyl group is positioned to make the attack, and thus form an α-(1→3), instead of an α-(1→6), bond.

We have also found that the addition of increasing amounts of B-512F dextran produces a decrease in the amount of insoluble glucan formed, and a concomitant increase in the amount of soluble glucan formed. Moreover, the total amount of glucan synthesized (soluble plus insoluble) is increased 1.6 times over the amount of insoluble glucan formed when no exogenous dextran is added. This stimulation by dextran had previously been observed[19,31,32].

Germaine and Schachtele[31] and Figures and Edwards[19] studied this stimulation by using two dextrans of different molecular weights, 10^4 and 2×10^6. They argued that the activation of the glucosyltransferases by equal weight-percent solutions of the two dextrans should differ by a factor equal to the ratio of their molecular weights (*i.e.*, 200-fold) if the synthesis were from the reducing end, but, as they observed that the activation was identical for the two dextrans, they concluded that the synthesis could not be from the reducing end, and hence must be from the nonreducing end. This line of reasoning is based on the premise that the activation by exogenous dextran must be by a primer mechanism in which the transfer of the D-glucosyl group from sucrose is to the nonreducing end, or to the reducing end of the exogenous, dextran molecules. Our data indicate, however, that, during synthesis, both glucose and glucan are covalently attached to the enzyme, and that the glucose is transferred to the reducing end of the growing, covalently attached glucan, and is not transferred either to the reducing end or nonreducing end of exogenous glucan. Exogenous primer, therefore, is not involved, and the glucan chain can only grow linearly when it is attached to the active site of the enzyme; once it is released, elongation is terminated[29]. The mechanisms that we proposed[20] for the synthesis of dextran,

and now for the synthesis of *S. mutans* insoluble and soluble glucans, are consistent with these facts.

The enzymes studied by Germaine and Schachtele[31] and Figures and Edwards[19] synthesized glucan without the addition of dextran, and, hence, also do not require a primer for action. Mayer *et al.*[33] specifically addressed the question as to whether *S. sanguis* dextransucrase required added dextran, for synthesis of glucan, by treating enzyme and substrate with dextranase and α-D-glucosidase. They found that there was no effect on the ability of dextransucrase to synthesize glucan from sucrose under these conditions, and, hence, that there was no requirement for a primer.

Robyt and Corrigan[32] showed that the activation by exogenous dextran cannot be by a nonreducing-end, primer mechanism. They chemically modified 60% of the nonreducing ends of dextran by attaching tripsyl groups to the (nonreducing) primary 6-hydroxyl groups, which would be the required sites for the addition of glucose units to the nonreducing ends. At the lowest concentration of modified, exogenous dextran (300 μg/mL), well below the maximum stimulating concentration, there should have been a 60% diminution in the amount of stimulation, if the exogenous dextran were acting as a nonreducing-end primer, but they found essentially no lessening in the amount of stimulation. Robyt and Corrigan[32] suggested two alternative possibilities for the stimulation by exogenous dextran: (*a*) an allosteric effect on the enzyme, and (*b*) an acceptor action whereby the exogenous dextran displaces the glucosyl units, or the glucanosyl chains, or both, from the active sites of the enzymes.

In the present study, we found that the mechanism of decreasing the formation of insoluble glucan and the concomitant formation of soluble glucan, by the addition of exogenous dextran to GTF-I-sucrose digests, is by an acceptor reaction in which α-(1→3)-glucanosyl chains are transferred to the exogenous, soluble dextran, and are attached by branch linkages at various positions along the dextran chain.

Montville *et al.*[34] commented that the application of *Leuconostoc* models to *S. mutans* must be qualified. They stated that the *Leuconostoc* model for branching[28] suggests that the addition of increasing amounts of soluble dextran would produce an increased amount of α-(1→3) branching, and, hence, the addition of dextran to the *S. mutans* enzyme system should produce more, instead of less, insoluble glucan, as is observed. However, Montville *et al.*[34] clearly misinterpreted the role of the α-(1→3) linkage in producing insoluble glucan. The formation of α-(1→3) branch-linkages will not impart insolubility as they implied, but rather, will actually produce an increased solubility, as observed. The insoluble character of the *S. mutans* glucan, synthesized by GTF-I, is imparted by the contiguous sequences of α-(1→3) linkages, and not by α-(1→3) branch-linkages[35]. As the *Leuconostoc* model[28,32] would predict, an increase in the concentration of soluble dextran would facilitate the transfer of shorter and shorter segments of α-(1→3)-glucan from the GTF-I by the formation of α-(1→3) branch-linkages to the soluble dextran. The formation of this graft polymer thus makes the insoluble glucan soluble.

In summary, the mechanism of action of the two glucosyltransferases from

S. mutans 6715 is identical with the mechanism of action of dextransucrase from *L. mesenteroides* B-512F, namely, there is both a glucosyl- and a glucanosyl-enzyme complex formed during synthesis, and the glucan is synthesized by the transfer of a glucosyl group to the reducing end of the glucanosyl chain, which remains covalently attached to the active site of the enzyme until polymerization is terminated by an acceptor reaction. Insoluble glucans are produced by the synthesis of contiguous α-(1→3) linkages by GTF-I, and soluble glucans are produced by the synthesis of contiguous α-(1→6) linkages by GTF-S. Branching occurs by acceptor reactions when exogenous glucan displaces a growing glucan from the active site of the enzyme. If a soluble α-(1→6)-glucan displaces an insoluble α-(1→3)-glucan, a soluble graft-polymer results.

The similarities of action of the glucosyltransferases (glucansucrases) from the two genera *Leuconostoc* and *Streptococcus* are not surprising, in that the enzymes are elaborated by two very closely related, bacterial genera that have more similarities than differences[3,6]. All three of the enzymes use the same substrate, sucrose, as the high-energy donor of a D-glucosyl group to synthesize glucans that are composed of either α-(1→6) or α-(1→3) linkages as either main-chain, contiguous linkages or branch linkages.

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