Carbohydrate Research, 127 (1984) 95–107
Elsevier Science Publishers B.V., Amsterdam – Printed in The Netherlands

THE FORMATION OF α-D-(1→3) BRANCH LINKAGES BY A D-GLUCANSUCRASE FROM *Streptococcus mutans* 6715 PRODUCING A SOLUBLE D-GLUCAN*

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(Received October 1st, 1983; accepted for publication, October 22nd, 1983)

ABSTRACT

An exocellular D-glucansucrase that synthesizes a water-soluble, α-D-(1→6)-linked D-glucan having a high proportion of α-D-(1→3) branches was purified from the culture broth of *Streptococcus mutans* 6715. The rate of incorporation of D-[^{14}C]glucose from [^{14}C]sucrose into D-glucan of high molecular weight by this enzyme was increased (stimulated) by the presence of exogenous *Leuconostoc mesenteroides* B-512F dextran, and it was found that this dextran could act as an acceptor. A highly branched dextran, containing 45–50% of α-D-(1→3) branch linkages, did not stimulate the enzyme nearly so much as B-512F dextran, which has a low degree (5%) of α-D-(1→3) branches. We interpret this as evidence that the stimulating effects of dextran are not due to priming. If they were, the more highly branched dextran should have produced the greatest stimulation per unit weight, because a much greater number of nonreducing-end, priming sites would be available. We show that the D-glucansucrase was capable of transferring D-glucosyl groups from sucrose to B-512F dextran to form α-D-(1→3) branches, thereby rendering the dextran more resistant to hydrolysis by endodextranase. The presence of 1.6M ammonium sulfate caused the enzyme to synthesize a D-glucan having a much higher percentage of α-D-(1→3) linkages.

INTRODUCTION

Streptococcus mutans has been shown to be the primary organism responsible for dental caries[1,2]. Its ability to form insoluble plaque on tooth surfaces is attributed to its production of exocellular polysaccharides, which mediate cell–cell and cell–tooth adherence[1,2]. Most cariogenic strains of *S. mutans* produce two different exocellular polysaccharides, a water-insoluble D-glucan, linked predominantly α-D-(1→3), and a water-soluble D-glucan, linked mainly α-D-(1→6) with various pro-

*Supported by Grant No. DE-03578 from the National Institute of Dental Research, National Institutes of Health, U.S. Public Health Service.
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0008-6215/84/$ 03.00

portions of α-D-(1→3) branch linkages[1-17]. Both D-glucans are synthesized from sucrose by exocellular enzymes known as D-glucansucrases[1], which are often referred to as GTF-I (D-glucosyltransferase-insoluble, which produces the water-insoluble D-glucan), and GTF-S (D-glucosyltransferase-soluble, which produces the water-soluble D-glucan[12,18]). The two D-glucansucrases can interact with either, or both, of the two D-glucans, so that, in a mixture produced by the two enzymes, a wide range of polysaccharide structures may be possible[1,5,6,10,12,19-33]. In addition *S. mutans* produces an exocellular dextranase that can further affect the composition and structure of the D-glucans produced[22,34-38].

We now describe our studies on the mechanism of formation of α-D-(1→3) branch linkages in the soluble D-glucan produced by GTF-S from *S. mutans* 6715, which is reported to contain 27% of α-D-(1→3) branches[39], and the interaction of this enzyme with so-called, "primer" dextran.

EXPERIMENTAL

Enzymes. — Streptococcus mutans 6715 was grown in the medium described by Ciardi *et al.*[24] Cells were removed by using a Millipore (Bedford, MA) Pellicon membrane cassette system with an HVLP Durapore microfiltration membrane. The filtrate was then concentrated ten-fold by using a Millipore PTGC membrane cassette, which has a nominal, molecular-weight cutoff of 10,000. This material was further concentrated five-fold by using an Amicon ultrafiltration cell with an XM-100 membrane. The concentrate was then chromatographed on a column of Bio-Gel A-15m (Bio-Rad, Richmond, CA) as described by Shimamura *et al.*[39]. This was followed by separation of the GTF-I and GTF-S on DEAE-cellulose (Sigma Chem. Co., St. Louis, MO) in a manner similar to that of Germaine *et al.*[22], except that the chromatography was conducted at pH 6.8 in 20mM potassium phosphate buffer, using a sodium chloride gradient of 0 to 0.2M. The fraction that contained only GTF-S activity (see ref. 22) was rechromatographed on a DEAE-Bio-Gel A column in the same phosphate buffer, with the same sodium chloride gradient. The final purification step consisted of an affinity procedure in which the enzyme, in 0.02% sodium azide, was bound to a column of Sephadex G-50 (Pharmacia Fine Chem., Uppsala, Sweden), and then eluted with 4M guanidine hydrochloride, in a manner similar to that of Hamelik and McCabe[38]. After buffer exchange by dialysis against 50mM sodium acetate buffer, pH 5.5, containing 0.02% of sodium azide, the purified GTF-S contained 50 ±7 IU/mg of protein, when assayed in the presence of 3.3 mg of B-512F dextran/mL and 57 μg of carbohydrate per mg of protein.

The dextranase used in these experiments was an endodextranase produced by *Penicillium funiculosum,* and was purchased from Sigma Chem. Co. It had a specific activity of 55 IU/mg, and the solution used in these experiments contained 100 IU/mL. Unless stated otherwise, one volume of dextranase was used to hydro-

lyze ten volumes of D-glucan, so that the dextranase content of the digests was 9 IU/mL.

Carbohydrates. — Dextran T-2000 was purchased from Pharmacia. [U-^{14}C]Dextran was produced from [U-^{14}C]sucrose (New England Nuclear, Boston MA) by dextransucrase from *Leuconostoc mesenteroides* NRRL B-512F[40]. Native, high-molecular-weight B-512F dextran was purchased from Sigma Chem. Co., and *L. mesenteroides* NRRL B-742 fraction S and B-1142 dextrans[41] were kindly supplied by Dr. Morey Slodki of the U.S. Department of Agriculture's Northern Regional Research Center, Peoria, IL. *L. mesenteroides* NRRL B-1355 fraction S D-glucan[41] (alternan) was prepared as previously described[42]. ^{14}C-Labeled *S. mutans* 6715 water-soluble D-glucan was prepared from 0.1M [U-^{14}C]sucrose by purified GTF-S. Pullulan was obtained from the laboratories of the late Professor Dexter French, and rabbit-liver glycogen was purchased from Sigma Chem. Co.

Analytical methods. — Total carbohydrate analysis was performed by the phenol–sulfuric acid method[43], and protein concentration was determined by the Coomassie Blue method of Bradford[44].

D-Glucansucrase activity was assayed by the procedure described by Germaine *et al.*[45]. Susceptibility of ^{14}C-labeled D-glucans to dextranase hydrolysis was measured in an analogous manner: dextranase was added to a sample of D-[^{14}C]glucan, and the amount of ^{14}C-labeled material rendered methanol-soluble was determined by pipetting 40-μL aliquots onto 1.5-cm squares of Whatman No. 3MM filter paper. The paper squares were immediately dropped into a beaker of stirred methanol, and washed in five changes of methanol, for 10 min each time. After being dried, the papers were counted for ^{14}C by liquid scintillation in toluene cocktails. The percentage of methanol-insoluble ^{14}C was calculated by comparing the amount of ^{14}C on methanol-washed papers to that on paper squares that had not been washed. The hydrolysis was judged to be complete when no further decrease in methanol-insoluble ^{14}C was observed, even after the addition of more dextranase to the digest.

Proton-decoupled, ^{13}C-nuclear magnetic resonance spectra were obtained on ~100–150 mg of polysaccharide in deuterium oxide at 80°, using a JEOL FX-90Q Fourier-transform n.m.r. spectrometer at 22.5 MHz. Approximately 10,000 scans were obtained for each spectrum.

Production of enzymically modified D-glucans. — ^{14}C-Labeled *L. mesenteroides* B-512F dextran was enzymically modified by methods similar to those previously described[46]. Three different sets of conditions were employed: in the first, 1 mL of *S. mutans* 6715 GTF-S (3.2 IU/mL when assayed in the presence of 3.3 mg of B-512F dextran/mL) was incubated with 0.9 mL of [^{14}C]B-512F dextran (22 mg/mL, 55 c.p.m./μg) and 0.11 mL of 1.7M sucrose in 50mM sodium acetate buffer, pH 5.5, at 25° for ~36 h. The resulting polysaccharide was precipitated with ethanol (1.5 vol.) and redissolved in sodium acetate buffer (3 mL). In the second procedure, 2 mL of GTF-S was mixed with 2 mL of [^{14}C]B-512F dextran solution, and the mixture was placed in a dialysis bag. The sealed bag was then placed in a stirred

vessel containing 1.4 L of 10mM sucrose in sodium acetate buffer, and allowed to react for ~36 h at 25°. The polysaccharide inside the bag was precipitated with ethanol and redissolved in sodium acetate buffer (10 mL).

The third procedure was the same as the second, except that the sucrose concentration outside the dialysis bag was 0.1M, and the precipitated polysaccharide was redissolved in 15 mL of buffer.

RESULTS

Table I gives the effects of a number of α-D-glucans on the relative rate of synthesis of D-glucan by *S. mutans* 6715 GTF-S. Both T-2000 dextran and native B-512F dextran, which are only 5% branched[41], activated the enzyme to the greatest extent. *L. mesenteroides* B-1142 dextran, with 28% of α-D-(1→3) branches[41], activated GTF-S to a lesser degree, and *L. mesenteroides* B-742 fraction S dextran, with almost every D-glucosyl residue branched [45% of α-D-(1→3) branches[41]], activated GTF-S even less. The other α-D-glucans (pullulan, glycogen, and alternan), which differ from dextran in that they contain no consecutive sequences of α-D-(1→6)-linked D-glucosyl residues[47], did not stimulate GTF-S to any significant extent.

Table II shows that *S. mutans* 6715 GTF-S is capable of modifying B-512F dextran to make it more resistant to hydrolysis by dextranase. It also shows that B-512F dextran, modified by GTF-S in the dialysis bag, was more resistant to dextranase action than was dextran that had been modified in the test-tube experiments, and that a higher concentration of sucrose outside the dialysis bag favored a greater degree of modification. It was, in fact, possible to modify B-512F dextran so that it was more resistant to dextranase hydrolysis than was *S. mutans* 6715 sol-

TABLE I

STIMULATION OF *S. mutans* 6715 GTF-S ACTIVITY BY α-D-GLUCANS

α-D-*Glucan*[a]	*GTF-S activity*[b]		*Methylation data (from refs. 41 and 47)*					
	IU/mL	*Relative (%)*	*2,3,4*	*2,3,4,6*	*2,4*	*2,4,6*	*2,3,6*	*2,3*
T-2000	3.0	100	90	5	5	0	0	0
B-512F, native	2.9	97	90	5	5	0	0	0
B-1142	2.6	87	44	28	28	0	0	0
B-742 S	2.1	70	4	46	50	0	0	0
B-1355 S (alternan)	0.20	7	47	7	11	35	0	0
Pullulan	0.20	7	33	<1	0	0	66	0
Glycogen	0.15	5	0	6	0	0	89	5
None	0.10	3	—	—	—	—	—	—

[a]All D-glucans were at a concentration of 0.5 mg/mL. [b]Assayed at 37° with 0.1M [^{14}C]sucrose (7.2×10^6 c.p.m./mL).

TABLE II

ENDODEXTRANASE HYDROLYSIS OF MODIFIED AND UNMODIFIED, ^{14}C-LABELED α-D-GLUCANS

α-D-Glucan (^{14}C-labeled)	*Sucrose conc. in the modification reaction* (M)	*Percent of ^{14}C remaining insoluble in methanol after dextranase hydrolysis*	
		Mean ±*S.D.*	*Number of replicates*
6715 S D-glucan[a] (unmodified)	—	28.1 ±0.6	4
B-512F dextran[a] (unmodified)	—	5 ±1.5	7
B-512F dextran (modified by GTF-S in a test tube)	0.09	12 ±1.0	3
B-512F dextran (modified by GTF-S in a dialysis bag)	0.01	19.7 ±0.4	3
B-512F dextran (modified by GTF-S in a dialysis bag)	0.10	34 ±1.6	3

[a][^{14}C]Dextrans were synthesized with 0.1M [^{14}C]sucrose.

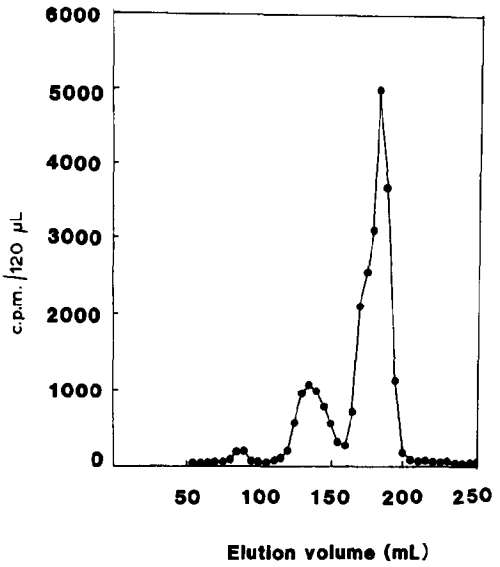

Fig. 1. Bio-Gel P-2 chromatography of endodextranase hydrolyzate of ^{14}C-labeled *L. mesenteroides* B-512F dextran. [Void volume, 85 mL; total included volume, 190 mL. Column, 2.5 × 53 cm; eluant, 0.02% NaN_3, and temp., 25°.]

uble D-glucan, although it must be noted that the *S. mutans* 6715 D-glucan in Table II was synthesized under a different set of conditions, *i.e.*, in a test tube, rather than in a dialysis bag.

The dextranase digests in Table II were each chromatographed on Bio-Gel P-2 in order to demonstrate that the degree of methanol-insolubility was directly related to the amount of higher-molecular-weight material remaining after dextranase hydrolysis. Fig. 1 shows that nearly all of the B-512F dextran is de-

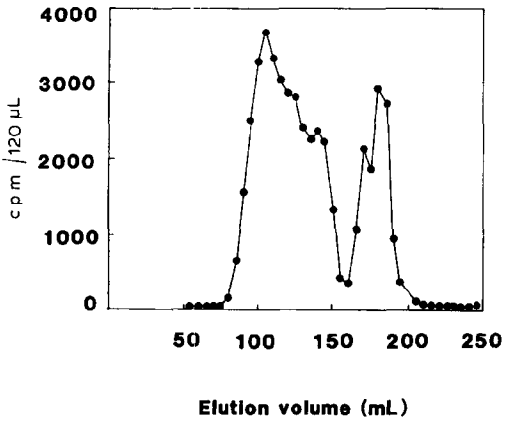

Fig. 2. Bio-Gel P-2 chromatography of endodextranase hydrolyzate of ^{14}C-labeled *S. mutans* 6715 soluble glucan. (See Fig. 1 for details.)

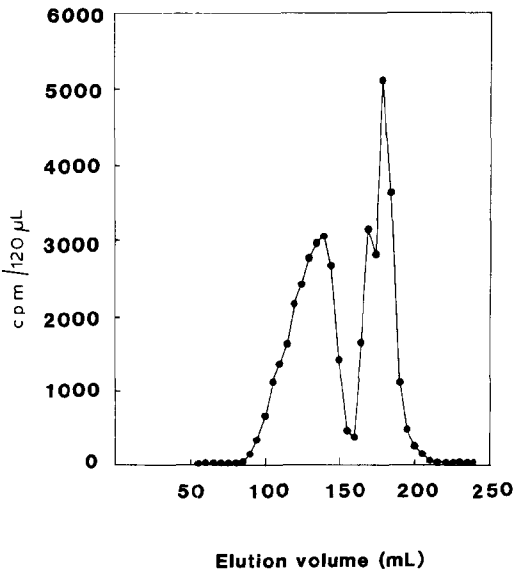

Fig. 3. Bio-Gel P-2 chromatography of endodextranase hydrolyzate of ^{14}C-labeled B-512F dextran that had been modified by *S mutans* 6715 GTF-S with 0.09M sucrose in a test tube. (See Fig. 1 for details).

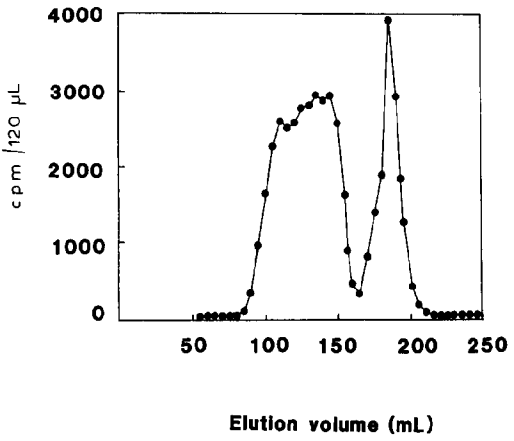

Fig. 4. Bio-Gel P-2 chromatography of endodextranase hydrolyzate of ^{14}C-labeled B-512F dextran that had been modified by *S. mutans* 6715 GTF-S in a dialysis bag, which was placed in 0.01M sucrose. (See Fig. 1 for details.)

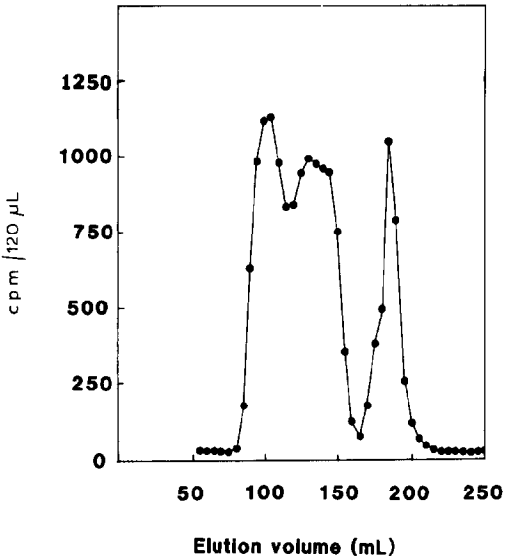

Fig. 5. Bio-Gel P-2 chromatography of endodextranase hydrolyzate of ^{14}C-labeled B-512F dextran that had been modified by *S. mutans* 6715 GTF-S in a dialysis bag, which was placed in 0.1M sucrose. (See Fig. 1 for details.)

graded to low-molecular-weight material by dextranase hydrolysis. In contrast, *S. mutans* 6715 soluble D-glucan contains a large proportion of higher-molecular-weight material that is resistant to dextranase hydrolysis, as shown in Fig. 2. Figs. 3–5 show that the modified B-512F D-glucans also contain this higher-molecular-

weight, dextranase-resistant material, and that those which were more resistant to dextranase contain more of the higher-molecular-weight compounds.

These results suggest that the conditions of synthesis play a role in determining the proportions of α-D-(1→3) branching in the D-glucan formed by GTF-S. However, the ^{13}C-n.m.r. spectrum of the D-glucan formed from 0.1M sucrose by GTF-S in a test tube (see Fig. 6) appeared to be almost identical to a spectrum of that which was formed in a dialysis bag that had been placed in 0.1M sucrose. However, small differences may not be observable by n.m.r. spectroscopy alone. One variable that did significantly affect the proportion of α-D-(1→3) linkages formed was the presence of a high concentration of ammonium sulfate. Fig. 7 shows a ^{13}C-n.m.r. spectrum of the D-glucan formed from 0.1M sucrose by GTF-S in the presence of 1.6M ammonium sulfate. It is clear that the D-glucan formed in the presence of such a high concentration of ammonium sulfate contains a much greater percentage of α-D-(1→3) linkages, as indicated by the relative intensity of peak 1 compared to that of peak 2. In Figs. 6 and 7, peak 1 arises from anomeric carbon atoms of D-glucose units involved in α-D-(1→3) linkages, whereas peak 2 is due to anomeric carbon atoms engaged in α-D-(1→6) linkages. It should be noted that, in Fig. 6, the intensity of peak 1 is ~60% of that of peak 2, whereas, in Fig. 7, peak 1 is of approximately the same intensity as peak 2, indicating a relatively greater proportion of α-D-(1→3) linkages.

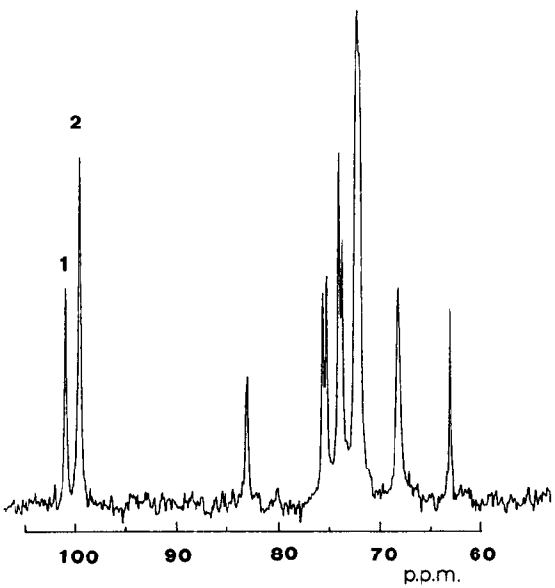

Fig. 6. ^{1}H-Decoupled, ^{13}C-n.m.r. spectrum of D-glucan formed by *S. mutans* 6715 GTF-S from 0.1M sucrose in a test tube [Peak 1 is due to C-1 linked to C-3, and peak 2 is due to C-1 linked to C-6.]

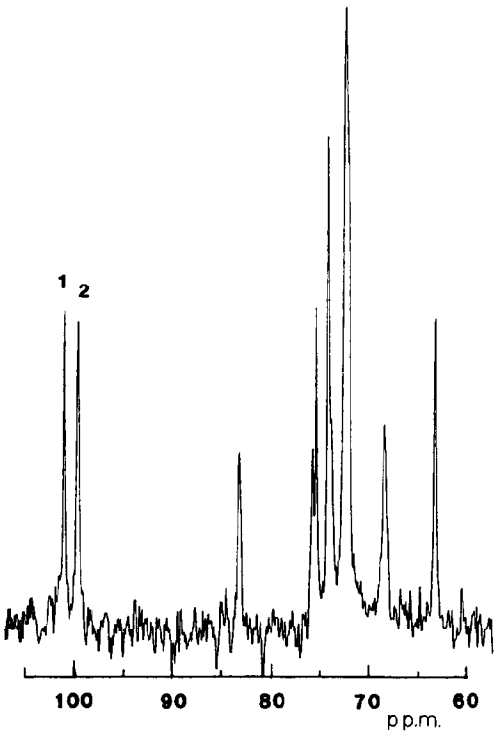

Fig. 7. ^{1}H-Decoupled, ^{13}C-n.m.r. spectrum of D-glucan formed by *S. mutans* 6715 GTF-S from 0.1M sucrose in a test tube, in the presence of 1.6M ammonium sulfate. [Peak 1 is due to C-1 linked to C-3, and peak 2 is due to C-1 linked to C-6.]

DISCUSSION

We have found, as have others[1,20,22,24,39], that synthesis of D-glucan by *S. mutans* 6715 GTF-S is stimulated by exogenous dextran. Dextrans from *L. mesenteroides* B-512F were the most effective activators, whereas the more-branched dextrans from strains B-1142 and B-742 (fraction S) were less effective. Alternan, the more-soluble D-glucan from *L. mesenteroides* B-1355, did not stimulate GTF-S to form D-glucan, nor did pullulan or glycogen. These results demonstrate that a D-glucan must contain linear, consecutive sequences of α-D-(1→6)-linked D-glucosyl units in order to activate GTF-S, and that the more-highly branched dextrans are less effective in their ability to activate GTF-S, indicating that this activation is not due to their acting as "primers", as some workers have suggested[20,22,33]. Were this activation due to the dextrans' acting as primers, *i.e.*, having D-glucosyl groups transferred to the nonreducing ends, the more-highly branched dextrans, which contain more nonreducing ends per unit of weight, would be expected to be more effective in stimulating the synthesis of D-glucan. However, just the opposite is actually observed, suggesting that it is the presence in these dextrans of unbranched

sequences of α-D-(1→6)-linked D-glucosyl residues that is one of the main causes of GTF-S activation.

Earlier, Robyt and Corrigan[21] had shown that the activation of *S. mutans* OMZ 176 dextransucrase by dextran and chemically modified dextran was not due to its acting as a "primer". They proposed that the stimulation could be due to its acting as an acceptor for the formation of α-D-(1→3) branch linkages. It should be pointed out that, although primers are a special type of acceptor, some acceptors are not primers. When dextran acts as an acceptor, to give rise to branch linkages, it is not acting as a primer in the generally understood sense, *viz.*, a primer is a necessary oligomeric or polymeric molecule whose end units (usually, if not always, the nonreducing ends) are required for the addition of new monomer units to give chain elongation. This was the definition of the term as originally used for phosphorylase[48,49]. Much confusion has arisen when the terms "acceptor" and "primer" have been used interchangeably[20,22,28,50–52]. It should be understood that, although dextran can act as an acceptor and activator for GTF-S, this is not the same as "priming".

Robyt and Taniguchi[53] showed that formation of α-D-(1→3) branches by dextransucrase from *L. mesenteroides* B-512F occurs by acceptor reactions with dextran. It was not, however, known whether this type of reaction could also occur with streptococcal D-glucansucrases, which produce soluble D-glucans having much higher proportions of α-D-(1→3) branches. Our work with *L. mesenteroides* B-742 S dextransucrase[46] has shown that, even in a very highly branched dextran, these branch linkages could be formed by acceptor reactions with dextran.

The experimental results given in Table II show that *S. mutans* 6715 GTF-S operates by a similar mechanism. Others have found that exogenous dextrans can act as acceptors, and are modified by GTF-S from a number of streptococcal strains[12,20,22,24,25], but the exact nature of this modification has not been ascertained.

The results herein show that this modification is due to the formation of α-D-(1→3) branches close enough to one another to cause segments of the dextran chain to become resistant to hydrolysis by endodextranase. The methanol-insolubility and the results of P-2 chromatography indicate that these dextranase-resistant fragments contain at least 8–12 D-glucosyl residues. The ability of GTF-S to form such highly branched segments solely by acceptor reactions means that the mechanism put forth by Robyt *et al.*[18,21,52–54] can explain the synthesis of even the most highly branched dextrans, regardless of whether they are produced by *L. mesenteroides* or by *S. mutans*.

L. mesenteroides B-742 S dextran contains an extremely high number of single D-glucosyl groups attached to the main chain by α-D-(1→3) branch linkages, and it is possible that D-glucosyl groups from sucrose could be transferred to them, to form either α-D-(1→6) or α-D-(1→3) linkages. Previous work had shown, however, that this is not the primary mechanism by which the D-glucan chains are elongated[18]. There is substantial evidence to support an insertion mechanism for dex-

tran formation, in which D-glucosyl units are added to the reducing end of a growing enzyme–dextranosyl chain[18,54]. In such a mechanism, acceptor reactions would play two roles: they could form branch linkages, and they could terminate chain elongation. Although we have demonstrated that GTF-S can form α-D-(1→3)-linked branches by acceptor reactions, it is possible that, as GTF-S produces the α-D-(1→6)-linked main-chain, D-glucosyl units are transferred to this chain from a separate D-glucosyl–enzyme site, to form single α-D-(1→3)-linked D-glucosyl branches[52]. This site could also transfer D-glucosyl groups to exogenous dextran, to form α-D-(1→3) branches by acceptor reactions.

There still remains a good deal to be learned about the mechanism of formation of α-D-(1→3) linkages by *S. mutans* 6715 GTF-S. As stated by Shimamura *et al.*[39], it is not known whether the formation of α-(1→6) and α-(1→3) linkages both occur at the same active site, at different active sites on the same enzyme molecule, or on different subunits of an enzyme consisting of two or more protein chains. There is some evidence that factors which may influence protein conformation and the aggregation state of the enzyme also affect the relative amounts of α-(1→3) linkages formed by other, similar D-glucansucrases from strains of *S. mutans*. Newman *et al.*[55] found that a high concentration (1.55M) of ammonium sulfate induced GTF-S from strain 3209 to produce an insoluble D-glucan that contained a significant proportion of consecutive α-(1→3) linkages. In addition, Mukasa *et al.*[56] described the effects of various salts on the relative amounts of soluble and insoluble D-glucans formed by GTF-I from strain 6715, and hypothesized that salts may affect the interactions between the enzyme and acceptor D-glucans. Our observation that 1.6M ammonium sulfate induces GTF-S from *S. mutans* 6715 to form a greater proportion of α-(1→3) linkages supports these findings, although the resulting D-glucan was not insoluble. We have not yet determined whether this increase is due to increased branching, or to linear sequences of α-(1→3)-linked D-glucosyl residues. Linear sequences of α-(1→3)-linked D-glucosyl residues tend to render a D-glucan insoluble, but D-glucans containing small proportions of α-(1→3) linear sequences may not necessarily be insoluble[12,57].

In summary, we have shown that the stimulation of GTF-S activity by dextrans is due, at least in part, to dextran acting as an acceptor for the formation of α-(1→3) branch linkages. Branch formation by acceptor reactions can account for the high degree of branching in the D-glucan formed by *S. mutans* 6715 GTF-S, but the details of such reactions are not yet known. These findings are consistent with the insertion mechanism proposed by Robyt *et al.*[18,54], and may account for the stimulation by dextran observed by others[20,22,24,39].

ACKNOWLEDGMENTS

We thank Dr. Morey Slodki for supplying the samples of *L. mesenteroides* B-742 and B-1142 dextrans, and Mr. Steve Eklund for his assistance in recording ^{13}C-n.m.r. spectra and in typing the manuscript.

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