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D-GLUCOSYLTRANSFERASE OF *Streptococcus mutans*: ISOLATION OF TWO FORMS OF THE ENZYME THAT BIND TO INSOLUBLE DEXTRAN

WILLIAM R. FIGURES AND JOHN R. EDWARDS
Department of Chemistry, Villanova University, Villanova, Pennsylvania 19085 (U.S.A.)
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ABSTRACT

The D-glucosyltransferase from *Streptococcus mutans* 6715 has been separated into three enzymic fractions that differ in their binding to dextran and in their synthesis of dextran from sucrose. One enzymic fraction (AFF-I) does not bind to insoluble dextran, and it produces an insoluble D-glucan. Fraction AFF-IIU was eluted from a dextran affinity-column by either dextran or urea, whereas fraction AFF-IID was eluted only by dextran. Both of these fractions produce insoluble D-glucans from sucrose.

INTRODUCTION

The exocellular glucosyltransferase [(1→6)-α-D-glucan:D-fructose 2-glucosyl transferase (EC 2.4.1.5)] produced by *Streptococcus mutans* has been studied in terms of its purification, the mechanism of catalytic action, and the role of dextran in the colonization of the tooth surface by the organism. Multiple schemes for the purification of this enzyme have appeared in the literature[1–7], including a procedure, described by this laboratory, in which the enzyme was purified in the presence of the detergent Tween 80 and potassium chloride[8]. The utilization of the detergent and potassium chloride minimized the aggregation of the enzyme. This procedure yielded a preparation of the D-glucosyltransferase that demonstrated a high affinity for insoluble D-glucan.

The present work describes a further fractionation of the previously described AFF-II glucosyltransferase (GTase) activity[8] into two GTase activities that demonstrate distinctly different catalytic properties. These results support the proposed mechanism of growth of the dextran from the nonreducing end by one of these fractions.

MATERIALS AND METHODS

Organisms and culture conditions. — *Streptococcus mutans* 6715 was obtained from F. P. Mertz (Eli Lilly and Co., Indianapolis, IN). For long-term storage, the organisms were lyophilized, and kept at −20°. Each culture was initiated from this

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primary stock. This procedure was designed to minimize the possibility of mutation of the organism due to prolonged subculture.

For bulk cultures, *S. mutans* was grown under conditions previously described[8]. Briefly, the culture medium consisted of Trypticase Soy Broth that had been treated with yeast invertase, and then filtered through an Amicon PM-10 ultrafilter. The medium was supplemented with Tween 80 [0.05% (w/v)] and D-fructose (1.1%).

Buffer. — The buffer used in most of the chromatographic separations consisted of 0.05M sodium phosphate (pH 6.0), Tween 80 (0.05%), and M potassium chloride, and was designated PKT buffer. Sodium phosphate buffer (0.05M, pH 6.0) was used for most other purposes.

Isolation of exocellular-GTase activities. — The (cell-free culture) supernatant liquor containing the GTase activities was concentrated by utilizing an Amicon PM-10 ultrafilter. The concentrate was then partially purified by gel-permeation chromatography on Bio-Gel A1.5m in the presence of 0.05% (w/v) Tween 80 and M potassium chloride. These methods have been described in detail in a previous report[8].

The Bio-Gel A1.5m–insoluble D-glucan, affinity column used in a previous purification scheme to obtain the AFF-II GTase preparation was prepared as before[8]. The column was charged with 15–20 I.U. of the Bio-Gel A1.5m-purified preparation. The column was then eluted with PKT buffer until no further, ultraviolet-absorbing material (280 nm) could be detected in the eluate. Instead of eluting the column with D-glucan of low molecular weight, as previously described, the column was first eluted with PKT buffer containing urea (6M). Fractions eluted by using this procedure were dialyzed against PKT buffer (in order to remove urea) and then assayed for GTase activity. The column was washed with PKT buffer to remove the urea, and then eluted with PKT buffer containing low-molecular-weight D-glucan (mol. wt. ~10,000; 1 mg/mL). Fractions eluted with low-molecular-weight D-glucan were pooled, and the D-glucan was removed by continuous diafiltration on an Amicon PM-30 membrane using PKT buffer. This procedure removed all of the low-molecular-weight D-glucan within 3 to 5 h. The material that was eluted with urea was labeled AFF-IIU, and that eluted with low-molecular-weight D-glucan was labeled AFF-IID.

Poly(acrylamide) gel-electrophoresis. — Poly(acrylamide) gel-electrophoresis of the AFF-IIU and AFF-IID preparations was conducted in the presence of Tween 80, as previously described[8,11] with one additional modification. Samples were not pretreated with low-molecular-weight D-glucan (which had improved resolution). Instead, urea at a final concentration of 6M was present in the samples, in the gel matrix, and in the electrode buffers. The resolution of GTase activities was greatly improved by the addition of urea.

Enzyme assays. — GTase activity was determined by measuring (*a*) the formation of insoluble D-glucan by utilizing the spectrophotometric method previously described[8], or (*b*) the release of free fluoride from α-D-glucopyranosyl fluoride (Glc-1-F) with a fluoride-specific ion-electrode[12]. Typically, the activity of the enzyme fraction was determined after a 30-min incubation. Soluble-D-glucan formation was

determined by measurement of incorporation of D-[^{14}C]glucose from specifically labeled sucrose (D-[^{14}C(U)]glucosyl D-fructoside) into material insoluble in 70% ethanol. Determinations of soluble D-glucan were performed after removal of insoluble D-glucan by filtration through 0.45-μm filters (Schleicher and Schuell, A. H. Thomas Co., Philadelphia, PA). Free D-glucose was determined by the D-glucose oxidase–peroxidase method[13]. One unit of enzymic activity will convert one μmol of sucrose into dextran per min in phosphate buffer (0.05M, pH 6.0).

Source of materials. — Invertase, Tween 80, dextran, D-glucan (mol. wt. ~10,000, and 193,000), D-glucose oxidase–peroxidase, and sucrose were purchased from Sigma Chemical Co., St. Louis, MO. D-[^{14}C(U)]Glucosyl D-fructoside (0.6 mCi/mg) was obtained from New England Nuclear, Boston, Mass. Trypticase Soy Broth (without D-glucose) is a product of Baltimore Biological Laboratories, Baltimore, MD. All other chemical compounds used in this study were of reagent grade.

RESULTS

Isolation of two GTase activities having high affinity for insoluble D-glucan. — When the insoluble D-glucan–Bio-Gel A1.5m affinity-column was charged with the Bio-Gel A1.5m GTase preparation, the AFF-I GTase was eluted in the wash with PKT buffer, as previously described[8] (see Fig. 1, peak I). The column was then eluted with PKT buffer containing urea (6M). The fractions were dialyzed against PKT buffer to remove urea, and each fraction was assayed for GTase activity. A peak (IIU) of GTase activity was detected having an elution volume that was slightly greater than that observed when the GTase had been eluted with low-molecular-weight D-glucan in a previous study[8]. The activity in this peak constituted ~47% of the

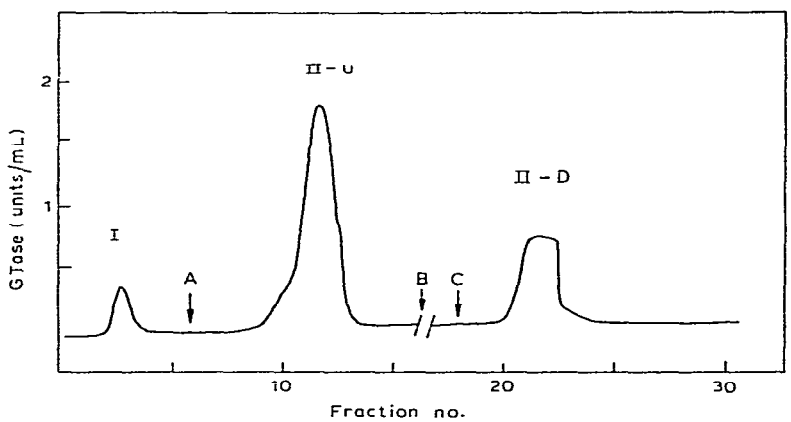

Fig. 1. Affinity chromatography of the Bio-Gel A1.5m-purified GTase on a column of Bio-Gel A1.5m–insoluble D-glucan. [The GTase (20 I.U.) was applied to the column, and the nonadsorbing peak (I) was eluted with PKT buffer. The column was then eluted with PKT buffer containing 6M urea (arrow A). The column was next washed with PKT buffer, to remove urea (arrow B). The column was then eluted with PKT buffer containing dextran (mol. wt. 9,400, arrow C). GTase activity was determined by turbidimetric assay of formation of insoluble D-glucan from sucrose.]

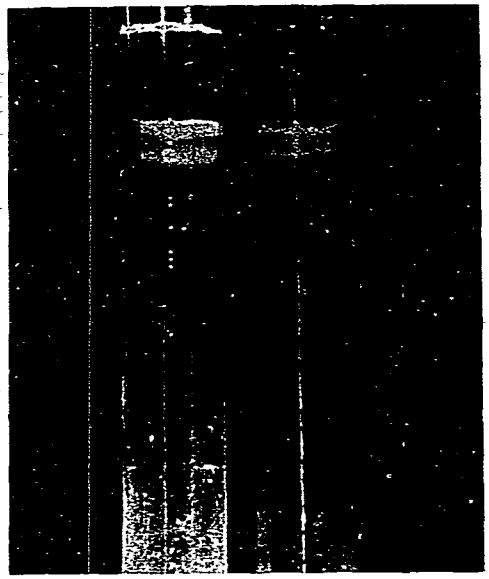

Fig. 2. Poly(acrylamide)-gel electrophoresis of AFF-IIU and AFF-IID GTase activities obtained from affinity chromatography on a column of Bio-Gel A1.5m–insoluble D-glucan. Electrophoresis was conducted in the presence of Tween 80 (1%) and urea (6M). [After electrophoresis, the gels were incubated in sucrose (5%), to make the bands of insoluble D-glucan visible. The AFF-IID preparation is on the left, and the AFF-IIU preparation, on the right.]

GTase activity applied. The column was next eluted with PKT buffer, to remove the urea, and then with PKT buffer containing low-molecular-weight D-glucan. A third peak of activity was found to be eluted with the D-glucan (see Fig. 1, peak II-D) and to constitute 21% of the total activity applied. The total recovery (68%) of the AFF-II forms of the enzyme was somewhat lower than that reported previously, in which the total AFF-II fraction had been eluted completely with low-molecular-weight D-glucan[8]. The loss of GTase activity is a result of a cooperative mechanism of action between AFF-IIU and AFF-IID in the synthesis of insoluble D-glucan, as demonstrated later in this report.

Poly(acrylamide) gel-electrophoresis of the AFF-IIU and AFF-IID preparations. — The AFF-IIU and AFF-IID preparations were subjected to electrophoresis in the presence of Tween 80 (1%) and urea (6M). After completion of electrophoresis, the gels were incubated in phosphate buffer (0.2M, pH 6.0) containing sucrose (5%), in order to make the bands of GTase activity visible. Each preparation of GTase exhibited two bands of activity on the gels (see Fig. 2). Close examination of the bands revealed similar electrophoretic mobilities for each preparation. However, the bands produced by the AFF-IIU preparation appeared to be much sharper than those produced by the AFF-IID preparation, but this was not due to an overloading of the gel, as identical patterns and band appearance were observed in gels loaded with ~0.5 of the amount of material (data not shown).

Properties of the AFF-IIU and AFF-IID preparations. — As noted in a previous

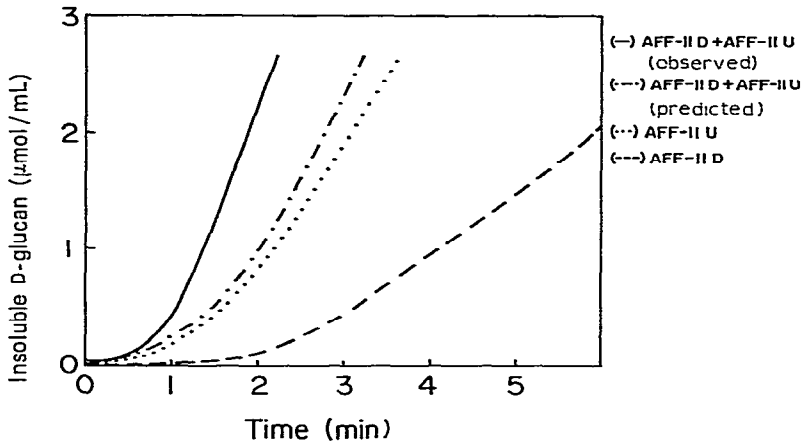

Fig. 3. Time-course study of the synthesis of insoluble D-glucan by AFF-IIU and AFF-IID GTase preparations. [AFF-IID (– –), AFF-IIU (...), predicted rate by summation of AFF-IID and AFF-IIU (-.-), and AFF-IIU plus AFF-IID (—).]

section, the recovery of the AFF-IIU and AFF-IID preparations respectively constituted 47 and 21 % of the total GTase applied to the affinity column; that is, ~82 % of the recovered activity previously reported when the affinity column was eluted with low-molecular-weight D-glucan in order to obtain the total AFF-II preparation[8]. As it had been determined that neither urea (6M) nor low-molecular-weight D-glucan would irreversibly denature the GTase activities[8], a study was undertaken to determine the cause of the apparent, lower recovery.

The AFF-IIU and AFF-IID preparations were mixed in the same proportions in which they had been recovered from the affinity column, and the mixture was assayed for its ability to convert sucrose into insoluble D-glucan. The initial rate of reaction was ~20 % greater than the sum of the activities when determined separately (see Fig. 3), indicating that some type of synergism existed between the two fractions.

Synergistic behavior between these fractions was also indicated by the general morphology of the insoluble D-glucan synthesized by each fraction. The AFF-IID fraction synthesized an insoluble polymer having a very fine, granular appearance (see Fig. 4 C). The AFF-IIU fraction synthesized an extremely coarse, granular product (see Fig. 4 B). When the two fractions were mixed in incubation mixtures, a gel-like material was produced after extended incubation times (see Fig. 4 A). Neither of the individual fractions synthesized the gel, even after a 24-h incubation with sucrose.

A clue to the mechanism of cooperativity was obtained by a series of heat-denaturation studies of the enzyme fractions. Both the AFF-IIU and AFF-IID fractions could be irreversibly inactivated by heat (3 min at 100°). When the heat-inactivated AFF-IID GTase was added to a reaction mixture containing sucrose and

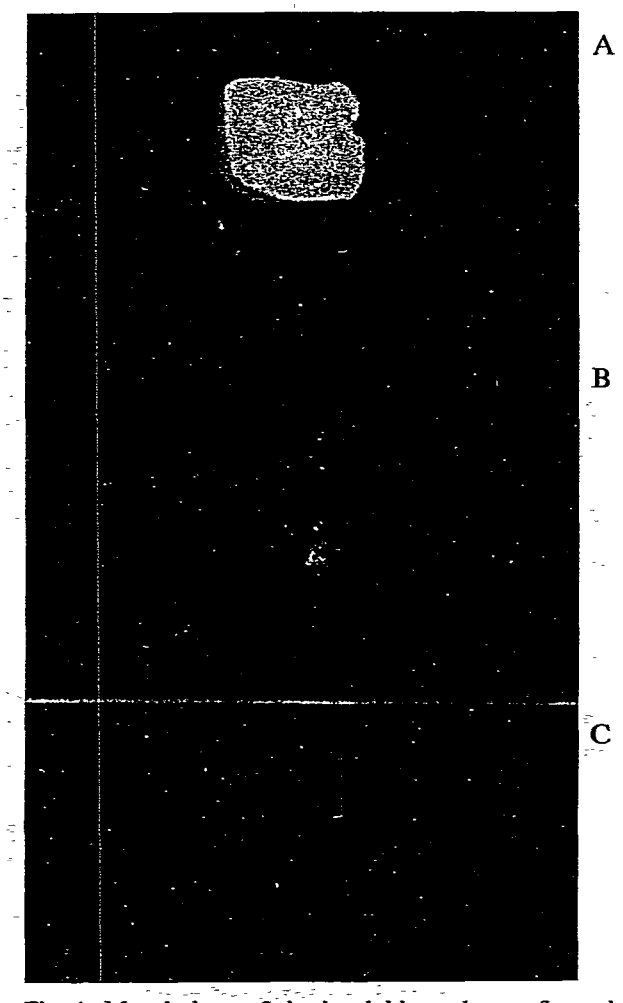

Fig. 4. Morphology of the insoluble D-glucans formed by the GTase activities obtained from the Bio-Gel A1.5m–insoluble D-glucan, affinity column. [Mixture of AFF-IIU and AFF-IID (top), AFF-IIU alone (middle), and AFF-IID alone (bottom).]

the active form of the other enzyme, there was no net increase in activity (see Table I). However, when the AFF-IID preparation was allowed to react with sucrose for a short period of time (2–3 min) prior to heat inactivation, and then added to active AFF-IIU, a 25% increase in the reaction rate was observed over that measured for AFF-IIU control. When the AFF-IIU was first incubated with sucrose followed by heat inactivation, and then added to active AFF-IID and sucrose, there was no stimulation of activity; this indicated that the mechanism of cooperativity between AFF-IIU and AFF-IID could be based on the activation of the AFF-IIU fraction by a heat-stable product formed by the AFF-IID and sucrose reaction-mixture. Presumably, this activator was product D-glucan.

TABLE I

THE EFFECT OF THE ADDITION OF HEAT-INACTIVATED INCUBATION-MIXTURES TO THE PURIFIED GTases

GTase preparation	*Control (sucrose only)*	*Incubation mixtures added after heat inactivation*	
		AFF-IIU	*AFF-IID*
		(nmol of D-glucan formed per min)	
AFF-IIU	60	62	75
AFF-IID	44	38	39

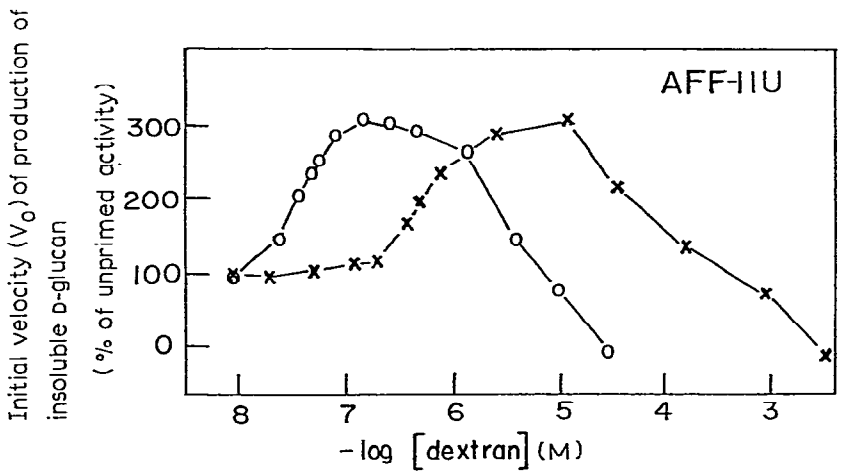

Fig. 5. The effect of soluble D-glucan on the initial velocity of production of insoluble D-glucan by the AFF-IIU preparation. [The AFF-IIU preparation was incubated with sucrose (20mM) and various concentrations of soluble D-glucans. The rates of formation of insoluble D-glucan were determined by using the turbidimetric assay; dextran, mol. wt. 193,000 (–○–), and dextran, mol. wt. 10,000 (–×–).]

Subsequent studies of the effect of soluble D-glucan on the rate of synthesis of insoluble D-glucan indicated that the AFF-IIU preparation could be activated by low concentrations of soluble D-glucans. Soluble D-glucans of two different molecular weights (9,400 and 193,000) were able to raise the rate about 3 fold for formation of insoluble D-glucan by the AFF-IIU preparation (see Fig. 5). The difference in molar concentration necessary to obtain maximal activation for the two soluble D-glucans was approximately equivalent to a factor of the difference of their respective molecular weights (~20-fold). These data are in agreement with the results of Germaine and Schachtele[9]. The rate of formation of insoluble D-glucan by the AFF-IID preparation was not raised by addition of D-glucan (see Fig. 6). At higher concentrations of exogenous D-glucan, both preparations exhibited marked decreases in the rate of production of insoluble D-glucan. However, this decrease in the synthesis

tion in the reaction[12]. In order to evaluate the method of activation of the AFF-IIU GTase by exogenous, soluble D-glucan, it was important to determine the effect of the added D-glucan on the rate of substrate conversion into polymer. When the AFF-IIU preparation was incubated with Glc-1-F at saturating concentration (30mM) of substrate, the rate of fluoride release was ~2.5 times that of synthesis of insoluble D-glucan (see Table II). Addition of soluble D-glucan (mol. wt. 193,000) to the reaction mixture at a concentration (200nM) that produced the maximum rate of synthesis of insoluble D-glucan caused little change in the rate of release of fluoride (see Table II). These data presented a dilemma, in terms of the fate of the "extra" D-glucosyl units, which was reflected by the excess in the release of fluoride over the synthesis of D-glucan in the absence of exogenous D-glucan. This material could not be accounted for by the release of free D-glucose due to hydrolysis of Glc-1-F, or by synthesis of soluble D-glucan as measured by the incorporation of D-[^{14}C]glucose into material precipitable with 70% ethanol, or into nondialyzable product. These results can best be explained by the formation of oligosaccharides of low molecular weight that would be undetectable by either of these assays. As shown by the data in Table II, addition of exogenous, soluble D-glucan to the reaction mixture results in the formation of insoluble D-glucan in amounts equivalent to that of fluoride release. It is probable that the observed activation of the AFF-IIU GTase by soluble D-glucan results from a re-direction of D-glucosyl units from oligosaccharide synthesis (in the absence of exogenous primer) towards addition to the exogenous primer. The driving force for this mechanism may be a result of a smaller K_m value for the (larger) D-glucan molecule than that for oligosaccharides. The AFF-IID fraction was not affected by the addition of exogenous dextran, although the rate determined by fluoride release was greater than that for polymer formation.

DISCUSSION

S. mutans glucosyltransferases can be isolated in two forms which demonstrate high affinity for insoluble D-glucan. Although the two GTases are similar in electrophoretic mobility, they appear to have different catalytic activities. The synthesis of insoluble D-glucan by the AFF-IIU GTase is activated by addition of exogenous, soluble D-glucan, whereas AFF-IID is not affected in this way. The product afforded by the AFF-IID preparation also raises the rate of production of insoluble D-glucan by AFF-IIU, and it is, presumably, a soluble D-glucan. In addition, the AFF-IID GTase was inhibited by sucrose, whereas the AFF-IIU preparation was not. The insoluble D-glucans formed by each preparation differed in appearance, and, with a mixture of the two GTases, a gel-like material was formed after extended incubation (6 h).

Based on the present data, the mechanism of activation of AFF-IIU by exogenous D-glucan may be regarded as involving a redirection of the D-glucosyl-transferring capacity of the enzyme, rather than an increase in the rate of conversion of substrate. This redirection might involve D-glucosyl transfer to a primer that can support

synthesis of insoluble D-glucan rather than to one that supports oligosaccharide synthesis. The K_m value for exogenous D-glucan, as determined by the concentration of dextran eliciting half-maximal activation, was ~40nM for a D-glucan of mol.wt. 193,000, and 800nM for a D-glucan of mol. wt. 9,400 (see Fig. 5). Based on weight-percent solutions of the two dextrans, identical activation curves would be expected if the polymer growth were from the reducing end. These results are in agreement with the work of Germaine and Schachtele[9], and support their mechanism of polymer growth from the nonreducing end. The results of Robyt and Corrigan[10] differed from this interpretation; they demonstrated activation of a GTase derived from *S. mutans* OMZ 176 with D-glucans that were chemically blocked at the reducing ends; however, they did not consider the possibility of an enzyme activity capable of forming new branch points in the D-glucan molecule. As they[10] worked with a relatively crude GTase preparation, prepared by ammonium sulfate precipitation of the enzyme from a cell-free, supernatant liquor, the possibility of the presence of "branching" activity is high, in light of the structural data reported[14–17] for a number of dextrans produced by *S. mutans*. McCabe and Smith[18] studied the role of a dextran acceptor with the GTase from *S. mutans* KI-R, and proposed a complex role for the soluble dextran in the synthesis of an insoluble dextran (using a crude enzyme preparation). It remains possible that "branching" activity may be the function of one of the GTases described here.

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OXIDIZED SACCHARIDES AS INHIBITORS OF α-GLUCAN SYNTHESIS BY *Streptococcus mutans* GLUCOSYLTRANSFERASE

KAZUHISA ONO, DONALD W. NUESSLE, AND ERIC E. SMITH*

Department of Biochemistry, University of Miami School of Medicine, P.O. Box 016129, Miami, Florida 33101 (U.S.A.)

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ABSTRACT

Specific inhibition by periodate-oxidized dextrans of the synthesis of α-glucan by *S. mutans* glucosyltransferase prompted a search for structurally related inhibitors that might be effective as anticaries agents. Clinical dextran derivatives in which from 5 to 50% of the D-glucose units were oxidized acted as potent and specific enzyme-inhibitors, as did 10%-oxidized derivatives of dextran fractions ranging in mol. wt. from 10^4 to 2×10^6. Within these limits, differences in oxidation or molecular weight did not significantly affect the high inhibitory potency of the derivatives. In contrast, periodate oxidation of (1→6)-α-, (1→3)-α-, and (1→4)-α-linked oligosaccharides containing $< \sim 15$ D-glucose units, and of sucrose and structurally related trisaccharides, yielded derivatives that were poor inhibitors. Enzymic hydrolysis of oxidized dextrans caused a loss of their inhibitory power and indicated that, to act as specific inhibitors, oxidized molecules must contain at least 16 to 20 D-glucosyl residues. The similar, minimum size required in order that unoxidized oligosaccharides may act as efficient acceptors in the glucosyltransferase reaction suggests that the inhibitory potencies of oxidized derivatives may reflect their relative abilities to bind at the acceptor site of the enzyme.

INTRODUCTION

The occurrence of dental plaque and carious lesions has been related to the synthesis of α-glucans from dietary sucrose by extracellular enzymes of oral streptococci[1–4]. *Streptococcus mutans* strains isolated from carious lesions[5] produce extracellular, water-insoluble α-glucans that enable the bacterial cells to adhere tenaciously to smooth, tooth surfaces[6–8], and so contribute to their high cariogenic potential. The α-glucans are synthesized by glucosyltransferases (EC 2.4.1.5) that polymerize the D-glucosyl group of sucrose, with the release of D-fructose. Studies on glycosyltransferases of *S. mutans* have been reviewed by Montville *et al.*[9].

We have described[10] the action of partially oxidized dextran as a potent and

*To whom correspondence should be addressed.