INFECTION AND IMMUNITY. Feb. 1976, p. 365-372
Vol. 13, No. 2

Printed in U.S.A.

# *Streptococcus mutans* Dextransucrase: Mode of Interaction with High-Molecular-Weight Dextran and Role in Cellular Aggregation

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Received for publication 8 August 1975

The interaction between *Streptococcus mutans* dextransucrase (EC 2.4.1.5) and high-molecular-weight dextran was studied in both the presence and absence of substrate sucrose. Equivalent weight-percent solutions of primer dextrans that differed 200-fold in molecular weight were found to be equally efficient in priming new dextran synthesis. Sodium borohydride reduction of dextran had no effect on its priming ability. These results suggest that dextran synthesis proceeds by addition of glucosyl residues to nonreducing termini of primer dextrans and that several enzyme molecules simultaneously bind to single high-molecular-weight dextran molecules. Kinetic data suggested that dextransucrase contains only one dextran binding site per enzyme molecule. The nature of the commonly observed highly aggregated state of dextransucrase was also studied. Two types of enzyme aggregates were distinguished: (i) oligomeric enzyme aggregates that formed in the absence of dextran and were dissociated by 1 M KCl; and (ii) dextran-induced enzyme aggregates that were stable to 3 M salt. Oligomeric enzyme aggregates were obtained from supernatants of fructose-grown cultures, whereas dextran-induced enzyme aggregates appeared to be present in glucose-grown cultures. The molecular weight of the smallest species of dextran-free dextransucrase observed in solutions of 1 M KCl was estimated to be 40,000 by gel column chromatography. Addition of dextran to primer-dependent dextransucrase resulted in formation of complexes that were stable in CsCl density gradients and exhibited a buoyant density of 1.382 g/cm$^3$ as compared with a buoyant density of 1.302 g/cm$^3$ exhibited by dextransucrase. The enzyme-dextran complexes observed in CsCl density gradients contained about 25% dextran. This corresponded to 150 enzyme molecules (molecular weight, 40,000) per dextran molecule (molecular weight, $2 \times 10^6$). The implication of these results to the mechanism of sucrose- and dextran-induced aggregation of *S. mutans* is discussed.

*Streptococcus mutants* elaborates a dextransucrase (EC 2.4.1.5) that exhibits a high affinity for dextran (4, 10) and is present in both an extracellular and cell surface-associated location (12, 15, 17, 38). The cell-associated and extracellular enzymes do not differ significantly with respect to optimal pH, temperature, or kinetic parameters (25) and are probably alternate states of the same enzyme (25, 29). The addition of either dextran or sucrose to broth cultures or washed-cell suspensions of *S. mutans* results in a rapid, dramatic cellular aggregation (14, 17). Dextran-induced aggregation results from dextran cross-linking of cells (14). Sucrose-induced aggregation depends upon synthesis of dextran by dextransucrase (17) and subsequent cellular binding of the product. The ability of *S. mutans* to synthesize and bind dextran has been recognized to be of special significance for colonization of the smooth enamel surfaces of teeth (13-15). Since dextransucrase contributes to (i) the cellular aggregation phenomenon and (ii) adherence of the organism to smooth surfaces, it is important to define the mode of enzyme association with dextran. In this communication we present evidence that dextransucrase interacts with multiple sites on individual dextran molecules and discuss the implication of this mode of association to the mechanism(s) of the sucrose and dextran aggregation phenomenon.

## MATERIALS AND METHODS

**Bacteria.** *S. mutans* strain 6715 and its derivative S19 (42) were grown as previously described (10)

except that fructose was substituted for glucose in some cases as noted below.

**Dextransucrase.** Enzyme assay and ammonium sulfate precipitation have been described (4, 11). Samples of 100 to 200 μl of ammonium sulfate-precipitated enzyme in 0.01 M sodium acetate buffer, pH 5.5, were applied to columns (0.9 by 14 cm) of Bio-Gel A 1.5 M (Bio-Rad Laboratories, Richmond, Calif.) and eluted with 0.01 M sodium acetate buffer (with or without 1 to 3 M KCl). Column fractions (0.22 ml) were assayed for dextransucrase for 30 to 180 min, depending upon the activity present.

**CsCl density gradient centrifugation.** CsCl solutions containing samples of ammonium sulfate-prepared enzyme in either the presence or absence of dextran T2000 (Pharmacia) were adjusted to a final density of 1.424 g/cm$^3$ and centrifuged for 20 h at 15 C in a Beckman SW50.1 rotor in a Sorvall OTD-2 ultracentrifuge at 48,000 rpm. Gradient fractions were collected by pumping from the bottom of the tube and assayed for enzyme activity. The density gradients established in each tube were determined by refractive index measurements on every fifth fraction and translated into density by reference to a standard curve prepared in this laboratory. Control experiments indicated that incubation of enzyme in CsCl (1.424 g/cm$^3$) for 20 h resulted in loss of 25 to 50% of activity.

**Dextran reduction.** Sodium borohydride reduction of commercial dextrans T10 and T2000 was accomplished by incubating 750 μl of a 1-mg/ml solution in 0.1 M NaOH with 10 mg of $NaBH_4$ for 2 to 6 h at 23 C followed by 18 h at 4 C. The excess reagent was destroyed by addition of acetic acid and the borate was removed by repeated evaporations of methanolic solutions. The dextran preparations referred to as control (see Fig. 2) were incubated in only 0.1 M NaOH for 2 to 6 h at 23 C followed by 18 h at 4 C. Next, 10 mg of $NaBH_4$ was added to acetic acid and this solution was combined with the alkaline dextran solution. This procedure was devised in order to equalize exposure to alkali and final borate concentration to obviate differences between reduced and control samples that might have resulted from either the alkaline incubation (34, 47) or possible incomplete removal of borate from the reduced sample. After removal of borate, the dried dextrans (reduced and control) were dissolved in 750 μl of 0.05 M sodium acetate buffer, pH 5.5.

## RESULTS

**Interaction of dextransucrase and dextran in the primed synthesis of new dextran.** Glucans produced from sucrose by *S. mutans* have been suggested to comprise a mixed population of dextran containing variable proportions of α(1 → 3) branch points and a branched glucan in which α(1 → 3) linkages predominate in the backbone of the molecule (1, 3, 8, 16, 17, 27, 40, 41). Dextrans obtained from commercial sources (Pharmacia) are products of *Leuconostoc* dextransucrase preparations and contain up to 10% branch points, the majority of which are α(1 → 3) linked (26, 40). Thus, dextran molecules from either bacterial source contain multiple nonreducing termini and a single reducing terminus.

Since the proportion of α(1 → 3) branch points is similar for dextrans T10 and T2000 (whose molecular weights approximate $10^4$ and $2 \times 10^6$, respectively), the molar concentration of reducing ends in identical weight-percent solutions of T10 and T2000 should differ by a factor equal to the ratio of their molecular weights (about 200-fold). In contrast, the molar concentration of nonreducing ends should be equivalent. Therefore, if nonreducing ends are substrates, the priming efficiency of T10 and T2000 will be similar when identical weight-percent solutions are compared. Conversely, if reducing ends are substrates, the efficiency of priming by T10 will always be greater than that of T2000 when compared as identical weight-percent solutions. Such a comparison of priming abilities of T10 and T2000 using primer-dependent dextransucrase is shown in Fig. 1. It is evident that equal priming responses are observed. The solid line represents the response expected with T2000, as compared with the T10 data, if reducing ends are substrates. Furthermore, a comparison of the priming ability of untreated and $NaBH_4$-reduced dextran preparations revealed that borohydride-reduced dextrans exhibited undiminished priming activity (Fig. 2). These data suggest that nonreducing ends of the primer dextrans serve as substrate for new dextran synthesis.

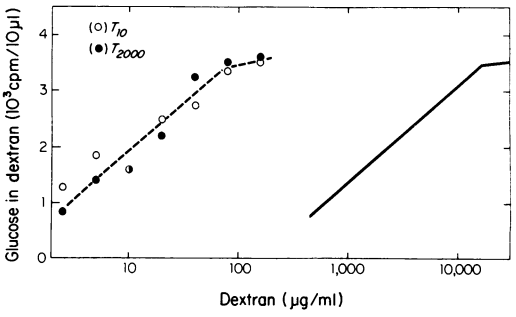


FIG. 1. *Priming of dextran synthesis by T10 and T2000. Primer-dependent dextransucrase was concentrated 50-fold by ammonium sulfate precipitation of the supernatant from fructose (0.75 M)-grown S. mutans S19 cultures (harvested when culture turbidities reached about 300 Klett units (no. 66 filter)). Ten microliters of the dextransucrase preparation was combined with 30 μl of reaction mix (devoid of primer dextran) and 10 μl of dextran (T10 or T2000 at appropriate concentrations). After 20 min at 37 C, 10-μl samples were removed and processed as before (11). See text for explanation of solid line.*

Hill plots of dextransucrase kinetic data (with respect to sucrose) suggest that the enzyme contains two substrate sites (A. M. Chludzinski, G. R. Germaine, and C. F. Schachtele, in press), a donor (sucrose) site and an acceptor (dextran) site. In the absence of dextran, the acceptor site will accommodate sucrose ($n$ = 1.7). In the presence of dextran, sucrose only occupies the donor site ($n$ = 1.1). If dextran is considered the substrate in a Hill plot, the value of $n$ is found to be 0.77 (Fig. 3). Thus, it seems likely that *S. mutans* dextransucrase contains only one dextran binding site. These characteristics of the *S. mutans* dextransucrase are similar to those of the *Leuconostoc mesenteroides* dextransucrase (see references 40, 44).

**Dextran binding by dextransucrase in the absence of dextran synthesis. (i) Dextransucrase from glucose-grown cultures.** Dextransucrase activity is eluted from molecular exclusion gels as a very high-molecular-weight (>$10^6$) species (12, 25, 32). We have further noted (10) that enzyme activity from glucose-grown cultures is largely independent of the addition of primer dextran. We suggested earlier (4, 10) that sufficient quantities of contaminating sucrose are present in many of the commonly used medium components (2, 31, 37, 43) to allow the synthesis of small amounts of dextran during early culture growth. It is possible that such dextrans promote aggregation of the extracellular dextransucrase and increase the proportion of total enzyme that is cell associated. This might explain both the extraordinarily high molecular weights associated with enzymatic activity and the lack of a requirement for primer dextran (10). An analogous problem of contamination of media and substrate with primer required for amylosucrase-catalyzed synthesis of $\alpha(1 \rightarrow 4)$ glucans is illustrated in reference 35. To study enzyme-dextran complexes, one requires a dextransucrase preparation that is initially free of contaminating dextran. The criteria we use to evaluate the dextran-free status of dextransucrase preparations are (i) demonstration of its primer dependency and (ii) the absence of high-molecular-weight enzyme aggregates. For example, dextransucrase prepared by ammonium sulfate precipitation from culture supernatants of glucose-grown cultures is only poorly stimulated by addition of primer dextran (10) and elutes in the void volume of Bio-Gel A 1.5 M columns (Fig. 4). The molecular weight of enzyme activity is not reduced by preincubation and column chromatography of the enzyme with buffer containing up to 3 M KCl (Fig. 4). Thus, neither criterion is satisfied by crude enzyme preparations from glucose-grown cultures.

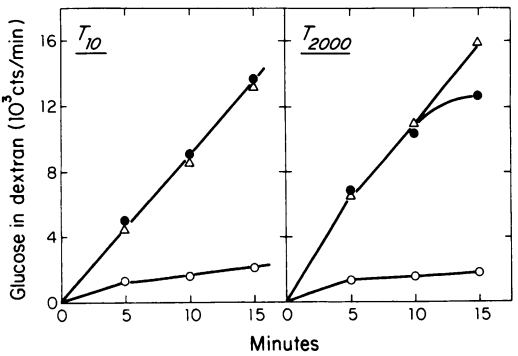


FIG. 2. *Comparison of priming ability of sodium borohydride-reduced and nonreduced dextrans T10 and T2000. Dextransucrase (25 μl) from the preceding experiment was combined with dextran-free reaction mix (75 μl) and T10 or T2000 solutions (25 μl). At 5-min intervals 25-μl samples were removed and treated as before (11). Final concentrations of dextrans were: T10 nonreduced, 180 μg/ml; reduced, 184 μg/ml: T2000 nonreduced, 187 μg/ml; T2000 reduced, 177 μg/ml. Symbols: (○) No dextran; (●) nonreduced dextran; (△) sodium borohydride-reduced dextran.*

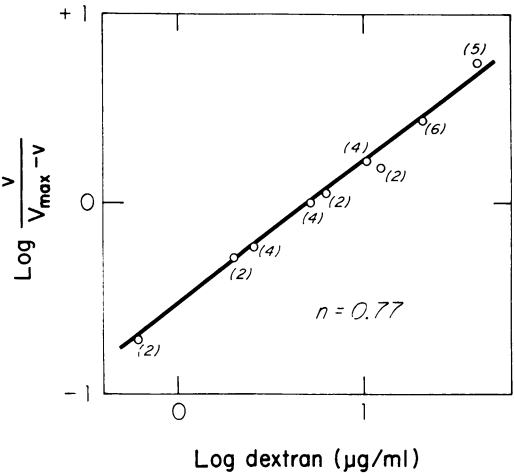


FIG. 3. *Hill plot of primed dextran synthesis. The results are the average of a number of independent experiments (numbers in parentheses) utilizing dextrans T10 or T2000 as primer source.*

**(ii) Dextransucrase from fructose-grown cultures.** We reported earlier (4) that fructose was a competitive inhibitor of sucrose in dextran synthesis. We have further observed that sucrose-induced aggregates of washed suspensions of fructose-grown cells developed more slowly and to a lesser extent than observed with glucose-grown cells, and we have consistently found up to fourfold greater quantities of enzyme in culture supernatants from fructose-

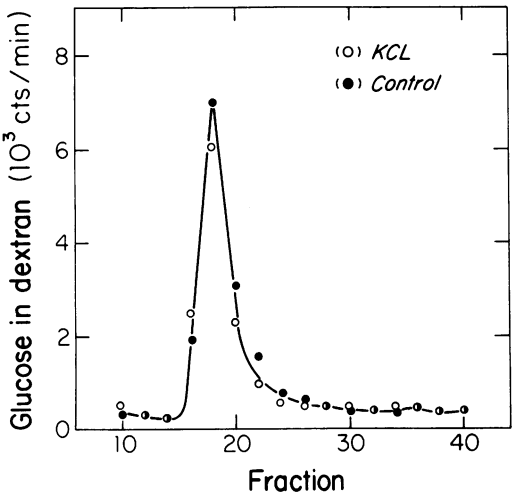


FIG. 4. *Bio-Gel A 1.5M chromatography of dextransucrase. Ammonium sulfate-precipitated dextransucrase from glucose-grown culture supernatants of strain 6715 was either applied directly (●) or preincubated with 3 M KCl (○). The column was eluted with 0.01 M sodium acetate buffer, pH 5.5, alone (●) or containing 3 M KCl (○). Pharmacia blue dextran was used to determine the void volume (fraction 18).*

grown cultures as compared with glucose-grown cultures (data not shown). Finally, the enzyme activity from fructose-grown cultures is primer dependent (ratio of activity of primed/unprimed, >10) in contrast to enzyme from glucose-grown cultures (primed/unprimed, <1.5). Taken together, these observations suggest that fructose promotes an increase in the proportion of enzyme that is extracellular, an effect that is most likely due to fructose inhibition of dextran synthesis during culture growth. Dextransucrase from fructose-grown cultures eluted just after the void volume of Bio-Gel A 1.5 M columns (Fig. 5b). Thus enzyme aggregates were present; however, if the enzyme was preincubated in 1 M KCl and passed through a KCl-equilibrated column by elution with KCl-containing buffer, high-molecular-weight enzyme aggregates were not observed (Fig. 5b). Rather, two principle peaks of activity were seen whose molecular weights were estimated to be about 40,000 and 80,000 (perhaps representing enzyme monomer and dimer). Oligomeric dextransucrase aggregates have also been noted with enzyme preparations from *Leuconostoc* (22). Dextransucrase from fructose-grown cultures of *S. mutans*, therefore, may be prepared in a nonaggregated primer-dependent form.

**(iii) Dextransucrase-dextran complexes.** Preincubation of the enzyme preparation from fructose-grown cultures with dextran T10 followed by gel chromatography in either the absence or presence of 1 M KCl resulted in a shift of enzyme activity to higher molecular weights (Fig. 5a) than those found in the absence of dextran (Fig. 5b). This experiment illustrates that dextran can induce formation of enzyme aggregates that are not dissociated by salt. Therefore, two types of enzyme aggregation

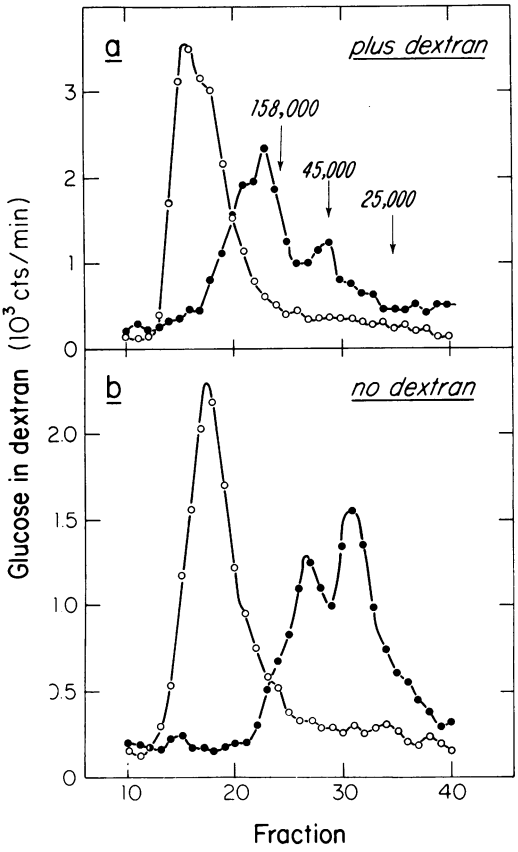


FIG. 5. *Bio-Gel A 1.5M chromatography of dextransucrase. Dextransucrase prepared by ammonium sulfate precipitation from culture supernatants of fructose (0.50 M)-grown S19 was preincubated with (●) or without (○) 1 M KCl in the absence (b) or presence (a) of T10 at a final concentration of 800 μg/ml before application to the column. In those cases where 1 M KCl was preincubated with the enzyme preparation, column elution was with 0.01 M sodium acetate buffer, pH 5.5, containing 1 M KCl. Those samples not exposed to KCl were eluted with buffer alone. The arrows (a) mark the elution positions of chymotrypsinogen A (molecular weight, 25,000), ovalbumin (molecular weight, 45,000), and aldolase (molecular weight, 158,000). Elution of standard proteins used buffer containing 1 M KCl. Void volume (blue dextran) corresponded to fraction 16.*

may be distinguished: (i) oligomeric enzyme aggregates that are dissociated by salt and formed in the absence of dextran (enzyme from fructose-grown cultures), and (ii) dextran-induced enzyme aggregates that are not dissociated by salt (enzyme from glucose-grown cultures or enzyme from fructose-grown cultures to which dextran was added).

Owing to their salt stability, we could demonstrate dextransucrase-dextran complexes in cesium chloride density gradients (Fig. 6). Dextransucrase from fructose-grown cultures exhibited a buoyant density of 1.302 g/cm$^3$. If the enzyme was preincubated with dextran T2000 before centrifugation, enzyme activity was found at a buoyant density of 1.382 g/cm$^3$. Since dextran T2000 had a buoyant density of 1.765 g/cm$^3$ (data not shown), the fact that enzyme activity was shifted to a higher density in the presence of dextran is further evidence for formation of salt-stable (in this case about 3 M CsCl) enzyme-dextran complexes. The CsCl data may be used to estimate the composition of the enzyme-dextran complexes. For example, the buoyant density of ribosomes in CsCl density gradients has been used to estimate their protein and ribonucleic acid composition (18). The method depends upon the large buoyant density difference between ribosomal ribonucleic acid and proteins and recognizing that the buoyant density of whole ribosomes is a reflection of the proportional buoyant density contribution of their ribonucleic acid and protein components. Thus, by analogy to the application of CsCl density gradient techniques to the compositional analysis of ribosomes (18), one can calculate from the CsCl buoyant densities of dextransucrase, dextran, and dextransucrase-dextran complexes that the complexes in Fig. 6 contain about 25% dextran. Since the dextran molecular weight is about $2 \times 10^6$, the molecular weight of the complex would be about $8 \times 10^6$. Thus, $6 \times 10^6$ daltons is contributed by the enzyme. One can calculate that about 150 enzyme molecules (assuming a monomer molecular weight of 40,000) are bound per dextran molecule under the conditions of this experiment. Therefore, data obtained in either the presence (Fig. 1) or absence (Fig. 6) of dextran synthesis lead to the same conclusion: several dextransucrase molecules may simultaneously bind to individual high-molecular-weight dextran molecules.

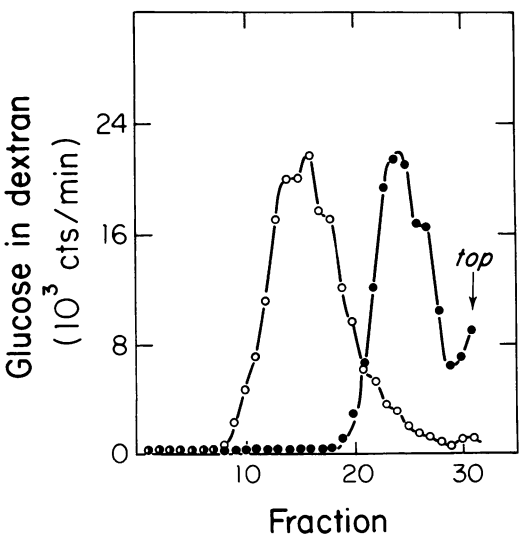


FIG. 6. *CsCl density gradient centrifugation of dextransucrase. Primer-dependent dextransucrase (see legend to Fig. 5) was either mixed directly with CsCl solution (●) or preincubated with T2000 (2.5 mg/ml) for 15 min at 24 C (○).*

## DISCUSSION

The data presented here (Fig. 1 and 6) suggest that dextransucrase from *S. mutans* interacts with a multiplicity of sites within individual dextran molecules. Furthermore, the finding that equal weight-percent solutions of dextrans T10 and T2000 exhibit identical priming efficiencies in new dextran synthesis strongly suggests that chain growth occurs from the multiple nonreducing termini present in dextran molecules. Koepsell et al. (23) observed the production of panose (4-$\alpha$-isomaltopyranosyl-D-glucose) when maltose and sucrose were incubated with *L. mesenteroides* dextransucrase. Synthesis of panose could only occur by addition of the sucrose-derived glucosyl moiety to the nonreducing end of maltose. Walker (46) has shown that oligoisomaltodextrins labeled in the nonreducing end are synthesized by *S. mutans* dextransucrase acting on [$^{14}$C]sucrose and isomaltose. Hehre (19) has long supported a nonreducing end mechanism and has pointed out that such a mechanism would allow for multiple propagation sites per individual dextran molecule (20). The above considerations are not consistent with models of dextran synthesis that incorporate a reducing end insertion mechanism (6, 7, 39).

Dextran binding by *S. mutans* may be mediated by two classes of cell-associated proteins, dextransucrase and a non-enzyme receptor (21, 30, 32, 36, 37, 43). The non-enzyme protein receptor may be proximal to the *a-d* site of the serogroup polysaccharide in group *d* strains (such as 6715) and antigenically distinct from the dextransucrase (30, 31). In a recent study it

was demonstrated that if cells of *S. mutans* are grown in medium that precludes or markedly reduces cell-associated dextransucrase, sucrose-induced aggregation is not observed whereas dextran-induced aggregation (albeit weak) occurs (43). Ability to aggregate upon sucrose addition required cell-associated dextransucrase. deStoppelaar et al. (5) described a mutant of *S. mutans* that produced only extracellular dextransucrase (i.e., cell-associated enzyme was not observed). The mutant failed to aggregate with either sucrose or dextran. Thus, it appears likely that a cell-associated dextran receptor other than dextransucrase is missing. In addition, the above-mentioned properties of this mutant severely diminish the possibility that an additional cellular receptor specific for dextransucrase exists. Thus, it appears that the association of dextransucrase with the cell surface is probably mediated by a dextran molecule which, in turn, is associated with a cell-surface dextran receptor protein (31). Involvement of dextransucrase in dextran-induced cellular aggregation (see reference 43) requires that either (i) dextransucrase possesses more than one dextran binding site, or (ii) dextransucrase aggregates formed through protein-protein (as opposed to dextran-protein) associations possess, as a unit, more than one dextran-binding site. The results in Fig. 3 tentatively suggest that dextransucrase contains only one dextran-binding site. A more conclusive study is needed, however, to determine with certainty the number of dextran-binding sites per enzyme molecule. Evidence that the enzyme from *L. mesenteroides* contains one dextran-binding site has appeared (40, 44). Results presented here indicate that the enzyme forms aggregates that differ from dextran-induced enzyme aggregates (Fig. 4 and 5) and that individual dextran molecules possess multiple sites for enzyme binding (Fig. 1 and 6). The model shown in Fig. 7 depicting the participation of dextransucrase in dextran-induced cellular aggregation seems most consistent with the evidence. The essential features of the model are as follows: (i) aggregates of dextransucrase are required for enzyme participation in dextran cross-linking of cells; and (ii) association of dextransucrase with the cell surface must be mediated by dextran. It should be noted that direct dextran cross-linking of cells through dextran binding only to the non-enzyme dextran receptor (see Fig. 7) is most likely quantitatively minor compared with dextransucrase-promoted dextran cross-linking. This follows from the study of Spinell and Gibbons (43). These considerations lead one to predict that mutants of *S. mutans* should not exist that are able to undergo sucrose-induced aggregation but never dextran-induced aggregation. Such mutants have not been reported. The model also predicts that

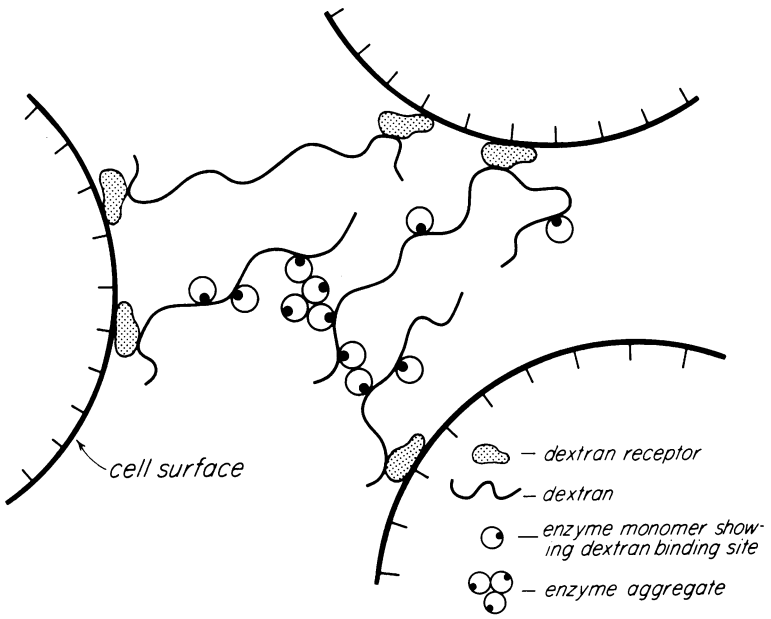


FIG. 7. *Schematic representation of the association of dextransucrase with the cell and its participation in cellular aggregation. Direct dextran cross-linking of cells through dextran receptor proteins (i.e., noninvolvement of dextransucrase) is also depicted.*

dextransucrase cannot singularly mediate either sucrose- or dextran-induced cellular aggregation since a dextran molecule that is anchored to the cell through a nonenzymatic dextran receptor protein is necessary for the formation of cell-associated dextransucrase. The mutant discussed earlier provides support for this contention (5).

Recently, mutants of *S. mutans* have been isolated that exhibited (i) marked loss of smooth surface virulence in pathogen-free and gnotobiotic rats, (ii) increased amounts of extracellular soluble glucans, and (iii) decreased cell-associated alkali-soluble glucans (9, 33, 45). Adherence ability, but not the ability to aggregate upon sucrose or dextran addition, correlated with synthesis of cell-associated alkali-soluble glucan. Thus, these mutants clearly confirm earlier suspicions (30, 36, 37) that aggregation and adherence phenomena are distinct processes and, furthermore, are dependent upon different glucan populations (9). Although dextransucrase has been implicated in sucrose-dependent adherence to smooth surfaces (24, 30, 31, 32), the mechanism(s) of synthesis and cellular binding of alkali-soluble glucans remains unknown. It is possible that non-dextran cell surface components such as capsular or serogroup polysaccharides or even peptidoglycan may act as acceptors during extended periods of dextran synthesis, with the resulting heteropolymers possessing adherent properties.

## ACKNOWLEDGMENTS

This work was supported by Public Health Service grants DE 03654 and DE 04171 from the National Institute of Dental Research. C.F.S. was the recipient of Public Health Service career development award K4-DE-42,859 from that institute.

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