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Specificity of the Glucan-Binding Lectin of *Streptococcus cricetus*

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The specificity of the glucan-binding lectin (GBL) of *Streptococcus cricetus* AHT was determined. Examination of the kinetics of aggregation of cell suspensions with glucans containing various percentages of α-1,6, α-1,4, α-1,3, and α-1,2 anomeric linkages revealed that only glucans with at least 80% α-1,6 linkages promoted strong aggregation. Moreover, only linear glucans with molecular weights greater than 5×10^5 were capable of causing rapid aggregation of the bacteria. The lectin was observed to be present on *S. cricetus* strains, on *Streptococcus sobrinus*, and on several *Streptococcus mutans* strains. Preincubation of suspensions of *S. cricetus* AHT with glucan T10 (molecular weight of 10,000) before the addition of high-molecular-weight glucan resulted in competitive inhibition in a concentration-dependent manner. Inhibition was achieved also with isomaltopentaose, isomaltohexaose, and isomaltooctaose, but at higher concentrations than glucan T10. In contrast, no inhibition was observed with maltoheptaose, providing additional evidence for the specificity of GBL. Treatment of suspensions of *S. cricetus* AHT with trypsin before and after aggregation with high-molecular-weight glucan revealed a substantial level of protection of GBL when in a bound state. Collectively, these results indicated that GBL has an absolute affinity for glucans rich in α-1,6 linkages and possesses an active site which recognizes internal sequences and accommodates isomaltosaccharides of at least nine residues. This unusual specificity may contribute to the colonization of *S. cricetus*, *S. sobrinus*, and *S. mutans* in glucan-containing plaque in the oral cavity.

Many bacteria have evolved surface lectins which are capable of complexing with simple sugars or complex saccharides (reviewed in reference 34). The lectins probably play a role in the adhesion of the bacteria to surfaces. Oral streptococci also seem to adhere to surfaces, such as saliva-coated teeth, mucosa, and the dorsum of the tongue (3, 12, 15, 20, 26, 45). In 1969, Gibbons and Fitzgerald (13) reported that dextrans were capable of agglutinating *Streptococcus mutans* and suggested that a surface dextran-binding protein was responsible for this phenomenon. Later, glucan-binding proteins (GBPs) were purified from oral streptococci in two different studies. Russell and colleagues (40, 42) isolated three GBPs from a serogroup c *S. mutans* strain. Two of these proteins, however, exhibited glucosyltransferase (GTF) activity. McCabe et al. (29) were able to purify a single dextran-binding protein (74,000 molecular weight) from a cariogenic strain of *Streptococcus sobrinus* using affinity chromatography. More recently, GBP was cloned into *Escherichia coli* by using a bacteriophage vector (41). A 76,500-molecular-weight protein was shown to be antigenically identical to purified GBP. However, no additional characterization of the cloned GBP was reported.

It has been suggested that the surface glucan-binding lectin (GBL) is a virulence factor in promoting the colonization of the mutans streptococci (13, 25, 29, 30, 38). Because dextrans (glucans rich in α-1,6 anomeric linkages) are synthesized from sucrose by several oral streptococci (4, 7, 14, 17, 20, 33), specific interactions with the GBL may be important in adherence and accumulation processes, resulting in an extracellular matrix firmly bound to tooth surfaces (11, 13, 15, 22–25, 30, 37, 44).

Landale and McCabe (25) recently provided evidence to show that isomaltosaccharides possessing at least eight hexose residues were required to optimally fit the active site of the GBL of *S. sobrinus* 6715-49. Landale and McCabe (25) used purified GBL and affinity electrophoresis to establish the size of the combining site. In this report, we show that the agglutination of intact bacteria can be inhibited by isomaltopentaose and higher isomaltooligosaccharides. An assay based on inhibition of aggregation suggested that the active site of GBL accommodates 9 to 10 residues of an α-1,6-linked glucan. In addition, the introduction of branches into linear isomaltoglucans results in a diminution of aggregation. Further experiments established that several types of oral streptococci possess a surface-associated GBL.

(Preliminary accounts of this research have appeared previously [D. Drake, K. G. Taylor, and R. J. Doyle, J. Dent. Res. **66**:226, 1987].)

MATERIALS AND METHODS

Bacteria and growth conditions. *Streptococcus cricetus* AHT (serotype a) was the primary strain used in this study. Additional strains used were *S. cricetus* E49 and HS-6 (serotype a), *S. sobrinus* ATCC 33748 (serotype h) and 6715 (serotype g), *S. mutans* OMZ175 (serotype f), *S. rattus* BHT and *S. rattus* FA-1 (serotype b), and *S. mutans* Ingbritt, Ingbritt 162 and 175, NG-5, 10449, and GS-5 (serotype c) and V100 and B14 (serotype e). Freeze-dried preparations of each strain were maintained as permanent stocks. Stocks were also kept on brain heart infusion broth (Difco Laboratories, Detroit, Mich.)–yeast extract (Difco)–$CaCO_3$ plates at room temperature. All strains were routinely subjected to API metabolic strip analyses and serological testing to confirm authenticity of phenotypes (1, 6). For daily use, bacteria were stored as turbid suspensions in Trypticase soy broth (TSB; BBL Microbiology Systems, Cockeysville, Md.) at 4°C. To eliminate possible sucrose contamination, we incubated TSB with yeast invertase (Sigma Chemical Co., St. Louis, Mo.) at a concentration of 5 mg of enzyme preparation per g of dry medium for 2 h at 55°C. Sterile media (75 ml in 250-ml flasks) were inoculated with 0.5-ml

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volumes of the refrigerated stock. Bacterial cultures were incubated statically in a 5% CO_2 incubator at 37°C overnight. Bacteria were harvested by centrifugation (6,000 × *g*) and suspended in phosphate-buffered saline (PBS; 20 mM potassium phosphate, 0.15 M NaCl, pH 7.2).

Polysaccharides. Several high-molecular-weight glucans, other polysaccharides, and carbohydrates were used in this study. Glucans with molecular weights ranging from 10,000 to 2 × 10^6 were obtained from Sigma. Glucans with known anomeric linkages were kindly supplied by M. E. Slodki, Agricultural Research Service, Peoria, Ill. Cellobiose, inulin, pullulan, lichenan, nigeran, laminarin, isomaltose, and isomaltotriose were obtained from Sigma. Maltoheptaose was supplied by Boehringer Mannheim Biochemicals, Indianapolis, Ind. Isomaltopentaose, isomaltohexaose, and isomaltooctaose were supplied by Cristi Granath of Pharmacia A.B., Uppsala, Sweden.

Aggregation assays. Four different procedures were used to measure aggregation of oral streptococci induced by the addition of high-molecular-weight glucans. In what was designated as an endpoint assay, suspensions of *S. mutans* (in PBS) were adjusted to an optical density (540 nm) of approximately 0.75 to 0.85 (Spectronic 20 spectrophotometer; Bausch & Lomb, Inc., Rochester, N.Y.). Samples of 3 ml were dispensed into a series of test tubes (13 by 100 mm), and the initial optical densities were measured. Glucans prepared in PBS were added in 50-μl volumes to appropriate tubes for a final glucan assay concentration of 10 μg/ml. Control tubes received PBS. Each suspension was immediately vortexed for 5 s and subsequently statically incubated for 2 h at 37°C. Optical density values were again determined after incubation. The extent of aggregation was expressed as the average percent decrease of turbidity of at least three replicate tubes.

In the centrifugation-aggregation assay, various oral streptococci were washed and suspended in glycine-NaOH buffer (25 mM glycine, pH 8.6). Samples of 3 ml were dispensed into glass centrifuge tubes. High-molecular-weight (2 × 10^6) glucan T2000 was added at a final concentration of 5 mg/ml, and the tubes were vortexed for 5 s. Control tubes received distilled H_2O and were vortexed in an identical manner. The suspensions were centrifuged at 6,000 rpm (8,200 × *g*) at 4°C for 15 min, and the supernatants were discarded. The bacteria were suspended in 4 ml of glycine-NaOH buffer. Smears from each sample were prepared on glass slides and stained with crystal violet. Photographs were taken on a Zeiss microscope at ×100 magnification under bright-field optics.

The kinetics of the decrease in turbidity was measured by two different procedures. For the standard rate assay, bacterial suspensions were adjusted to an optical density of 0.75 to 0.85, and 3-ml suspensions were added to test tubes (13 by 100 mm). High-molecular-weight glucan was added at a final concentration of 10 μg/ml, and the suspensions were vortexed for 5 s. Control tubes received PBS. The decrease in optical density was continuously monitored spectrophotometrically for 5 min. The micro-rate assay was similarly performed. Bacterial suspensions were adjusted to an optical density (500 nm, 1.25 cm) of 0.85 to 0.95 (Junior Coleman II spectrophotometer). The assay mixture consisted of 0.65 ml of cell suspension, 0.30 ml of PBS, and 0.05 ml of high-molecular-weight glucan (final concentration, 0.5 μg/ml). The decrease in optical density was continuously monitored for 1 min, with values recorded every 15 s. Rate constants for both assays were obtained from the slopes of first-order plots of ln A/A_0 (A = observed optical density, A_0 = optical density at time zero) versus time in minutes. Each sample was assayed at least three times. The best-fitting curves were obtained through simple linear regression of average values. Correlation coefficients averaged ≥0.90.

Aggregation inhibition assays. Carbohydrates used as inhibitors were glucose, maltose, fructose, isomaltose, isomaltotriose, isomaltopentaose, isomaltohexaose, isomaltooctaose, maltoheptaose, and glucan T10 (10,000 molecular weight). For the standard rate assay, prospective inhibitors were prepared in PBS. An appropriate inhibitor (50 μl) was added to 3-ml suspensions of *S. cricetus*. The tubes were subsequently vortexed for 5 s and incubated statically at 37°C for 30 min. Control tubes received 50 μl of PBS and were incubated under identical conditions. In the micro-rate assay, inhibitors were carefully weighed and added directly to the assay tube containing 0.30 ml of PBS. The bacterial suspension was then added, and each tube was vortexed for 5 s. The rate assays were then performed as described above. The extent of inhibition was determined by the following formula: percent inhibition = [1 − (percent decrease of experimental/percent decrease of positive control)] percent × 100. Negative controls consisted of bacterial suspensions incubated with PBS as a mock inhibitor and also as a negative promoter of aggregation. Positive controls consisted of bacterial suspensions with PBS again as a mock inhibitor, followed by the addition of glucan T2000 (2 × 10^6 molecular weight).

Protease treatment of *S. cricetus*. *S. cricetus* was cultured in invertase-treated TSB and suspended in PBS as described above. Trypsin (type XI; Sigma) was prepared in PBS and added to bacterial suspensions (3 ml) at a final protease concentration of 10 μg/ml. The tubes were subsequently incubated statically for 30, 60, and 90 min at 37°C. At each time point, standard rate assays were conducted on duplicate tubes with glucan T2000. Control tubes received PBS and were incubated and assayed in an identical manner. Bacterial suspensions already aggregated with glucan T2000 (10 μg/ml) were assayed similarly. Trypsin at 10 μg/ml or PBS was added to the aggregated cells, and each sample was incubated statically at 37°C for 30, 60, and 90 min. Samples were withdrawn at each time point, and standard rate assays were conducted.

RESULTS

Aggregation of *S. cricetus* by high-molecular-weight glucans with various percentages of anomeric linkages. A number of glucans with known contents of anomeric linkages were used in an attempt to determine the specificity of the GBL of *S. cricetus*. The ability of the respective glucans to induce aggregation of *S. cricetus* AHT was determined in endpoint assays (Table 1). Only glucans with at least 80% α-1,6 anomeric linkages were capable of promoting strong aggregation of the bacteria. Glucan B-1298, with 64% α-1,6 linkages, induced minimal aggregation, resulting in only a 16% decrease in turbidity. However, increasing the concentration of B-1298 or B-1355 fourfold resulted in strong aggregation, signifying the specificity for the α-1,6 linkage (data not shown). No agglutination was observed when glucans with 45 to 57% α-1,6 linkages (10 μg/ml) were used. Glucans such as laminarin (β-1,3, β-1,6), pullulan (α-1,4, α-1,6), starch (α-1,4), glycogen (α-1,4, α-1,6), and nigeran (α-1,3, α-1,4) along with inulin (β-2,1), sugars, and saccharides such as glucose, maltose, isomaltose, and fructose were incapable of promoting agglutination as well. Therefore, these data suggest that only high-molecular weight

TABLE 1. Role of the α-1,6 glucosidic bond in the agglutination of *S. cricetus* AHT[a]

Glucan	% α-1,6	% Other linkages	% Decrease in absorbance
B-1208	95	5	76
B-1225	90	10	68
B-1255	82	18	71
B-1298	64	36	16
B-742	57	43	0
B-1299	50	50	0
B-1355(s)	45	55	1

[a] Glucans with known molar percent anomeric linkages were used to determine the specificity of the GBL. Each glucan was prepared in PBS at a final assay concentration of 10 μg/ml. Glucans such as lichenan [(α-1,4)$_2$α-1,3], pullulan (α-1,4, α-1,6), amylose (α-1,4), laminarin (β-1,3, β-1,6), maltoheptaose (α-1,4), glycogen (α-1,4, α-1,6), along with carbohydrates such as glucose, maltose, isomaltose, isomaltopentaose, isomaltooctaose, nigeran (α-1,3, α-1,4), and fructose were incapable of promoting agglutination.

glucans rich in α-1,6 linkages are capable of promoting aggregation of *S. cricetus*.

Molecular weight effect of glucan-mediated aggregation of *S. cricetus*. The following experiments were done to determine whether glucan molecular weight is important in the promotion of bacterial aggregation. Glucans ranging in molecular weight from 10,000 to 2×10^6 were mixed at a final concentration of 10 μg/ml with suspensions of *S. cricetus* AHT. Analysis of the kinetics of decrease in turbidity revealed a distinct dependency on the molecular weight of glucan used in the assay (Fig. 1). Incorporation of glucan T2000 (2×10^6 molecular weight) resulted in a rate constant of 3.17×10^{-1} min^{-1}. It has been previously shown (13) that glucan T2000 will mediate the rapid clumping of *S. mutans*, and therefore this glucan was used throughout this study as

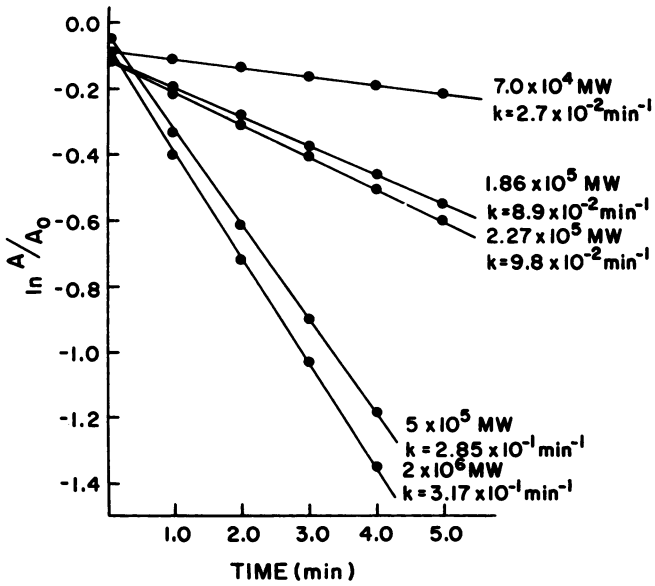

FIG. 1. Agglutination of *S. cricetus* AHT is mediated by high-molecular-weight glucans. *S. cricetus* AHT was cultured in sucrose-free TSB in a 5% CO_2 atmosphere at 37°C. Cell suspensions in PBS were assayed by the standard rate assay. Data points represent averages of at least three replicates. Simple linear regression was used to generate best-fitting lines. Rates constants (k) are slopes calculated from the linear regression statistical program. A, Absorbance at each time point; A_0, absorbance at time zero; MW, molecular weight.

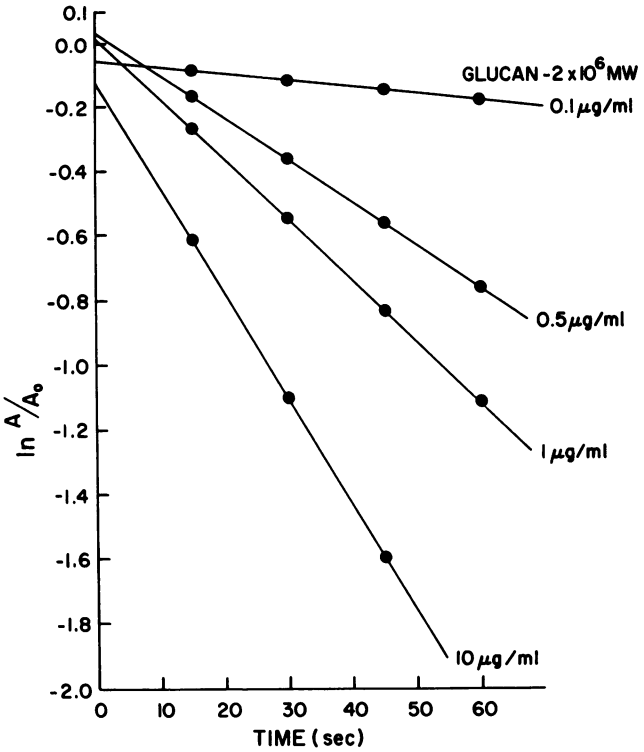

FIG. 2. Effect of glucan concentration on aggregation of *S. cricetus*. The data based on micro-rate assays were subjected to a linear regression statistical program. MW, Molecular weight.

a positive control. Addition of a fourfold-lower-molecular-weight glucan (5×10^5) also induced aggregation of cells, but to a somewhat lesser extent. However, considerable decreases in rates of turbidity loss were observed when glucans with a molecular weight of 2.27×10^5 were used. A rate constant of only 2.7×10^{-2} min^{-1} was obtained with a 70,000-molecular-weight glucan, 11.7 times lower than that observed upon the addition of T2000. Furthermore, aggregation of *S. cricetus* AHT did not occur after the addition of glucans with molecular weights of less than 70,000. Thus, these studies indicated that molecular weight is critical in the ability of glucans to cause aggregation of *S. cricetus*, with an apparent molecular weight of at least 5×10^5 needed for the rapid and strong aggregation.

Effect of concentration of high-molecular-weight glucan on aggregation of *S. cricetus*. In addition to the influence of molecular weight on aggregation, it was necessary to determine the optimum concentration of glucan for inducing strong aggregation and concurrently the minimum amount suitable for inhibition studies. The results of these experiments are illustrated in Fig. 2. Rate constants were obtained with different concentrations of high-molecular-weight glucan T2000 in the microassay. Because of the smaller volume of cells, rate constants were always much higher than those obtained from the standard assay. Incorporation of 10 μg of T2000 per ml resulted in a very high rate of decrease of turbidity easily detectable within 1 min of mixing. A 10-fold-lower amount of glucan (1.0 μg/ml) caused only a 43% decrease in the rate. Addition of a 50-fold-lower amount (0.5 μg/ml) of T2000 resulted in a 59% rate decrease, but strong aggregation of the cells was still observed. Decreasing the amount of high-molecular-weight glucan T2000 100-fold to 0.1 μg/ml resulted in a substantial 94% decrease in the rate

TABLE 2. Agglutination of oral streptococci by glucan T2000[a]

Organism	Serotype[b]	Rate constant[c]
S. cricetus AHT	a	3.2×10^{-1}
S. cricetus E49	a	3.1×10^{-1}
S. cricetus HS-6	a	3.1×10^{-1}
S. mutans OMZ175	f	0.0[d]
S. mutans 10449	c	0.0[d]
S. mutans V100	e	0.0[d]
S. mutans GS-5	c	0.0
S. mutans Ingbritt	c	0.0
S. mutans Ingbritt 162	c	0.0
S. mutans Ingbritt 175	c	0.0[d]
S. mutans NG-5	c	0.0
S. mutans B14	e	0.0
S. rattus FA-1	b	0.0
S. rattus BHT	b	0.0
S. sobrinus ATCC 33748	h	1.1×10^{0}
S. sobrinus 6715	g	6.7×10^{-1}

[a] Bacteria were statically cultured overnight in invertase-treated TSB at 37°C in 5% CO_2.

[b] Serotype according to Bratthall (2).

[c] Slopes from best-fitting curves derived from a linear regression analysis. Units are min^{-1}.

[d] Macroscopic agglutination was not readily measurable by light scattering. After centrifugation, these strains were observed to be agglutinated by glucan T2000 (Fig. 3).

constant. However, a considerable number of cells in the reaction mixture remained in suspension in a nonaggregated state. Therefore, a glucan T2000 concentration of 0.5 μg/ml was chosen to provide for both strong promotion of aggregation and nonsaturating conditions for appropriate microassay inhibition studies.

Aggregation rate constants of various mutans streptococci. The majority of the studies reported herein utilized *S. cricetus* AHT (serotype a). To determine whether other serotype strains of the mutans streptococci aggregated in the presence of high-molecular-weight glucans in a similar manner, we conducted standard rate assays on representative strains of Bratthall (2) serotypes a, b, c, e, f, and g. Strains E49 and HS-6, also of serotype a, exhibited rate constants essentially indistinguishable from that of AHT (Table 2). Interesting differences were observed with strains ATCC 33748 and 6715, representative of serotypes h and g, respectively. Extensive, rapid aggregation of the cell suspensions occurred upon addition of glucan T2000 with rate constants 2 and 3.6 times higher, respectively, than that observed with AHT. All the classical *S. mutans* strains failed to aggregate upon addition of glucan T2000 at concentrations of 10 to 40 μg/ml in the standard rate assay. However, if these strains were centrifuged in the presence of high-molecular-weight glucan and suspended in glycine-NaOH buffer, a small but significant amount of aggregation was observed. Representative micrographs of these experiments are shown in Fig. 3. Aggregates were not observed if cells were centrifuged with distilled water (Fig. 3A). In contrast, numerous small aggregates occurred in suspensions centrifuged with high-molecular-weight glucan T2000 (5 mg/ml) (Fig. 3B). The addition of 5 to 10 mg of low-molecular-weight glucan T10 resulted in the dispersion of these aggregates (data not shown), illustrating competitive inhibition similar to that observed in other aggregation rate assays. It is concluded from these studies that the GBL may be expressed on the cell surface of some of the classical *S. mutans* serogroup strains.

Inhibition of glucan T2000-induced aggregation of *S. cricetus* with low-molecular-weight glucan T10. To further characterize the specificity of the GBL, it was important to determine whether the high-molecular-weight glucan-mediated aggregation was inhibitable by a low-molecular-weight glucan. It was shown above that glucans with a molecular weight of 70,000 were not capable of inducing aggregation of cell suspensions (Fig. 1). To determine whether a representative low-molecular-weight glucan would serve as a prospective inhibitor, we conducted inhibition studies by examining the kinetics of turbidity decrease (Fig. 4). Glucan T2000 (10 μg/ml) induced strong aggregation of suspensions

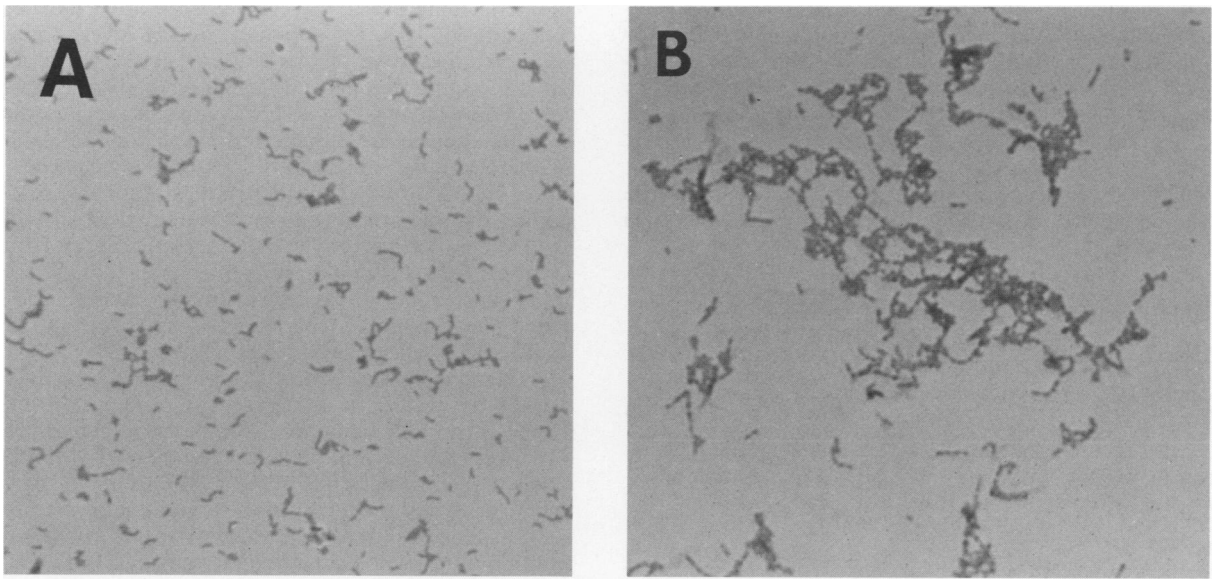

FIG. 3. Agglutination of classical *S. mutans* in a centrifugation assay. Oral streptococci were cultured in TSB in 5% CO_2 at 37°C and harvested by centrifugation. Bacterial suspensions were prepared in glycine-NaOH buffer. (A) Distilled water was added to the cell suspension. Smears were prepared on glass slides and stained with crystal violet. Magnification, ×100. (B) High-molecular-weight glucan T2000 was added at a final concentration of 4 mg/ml. Slides were prepared as described above. Magnification, ×100.

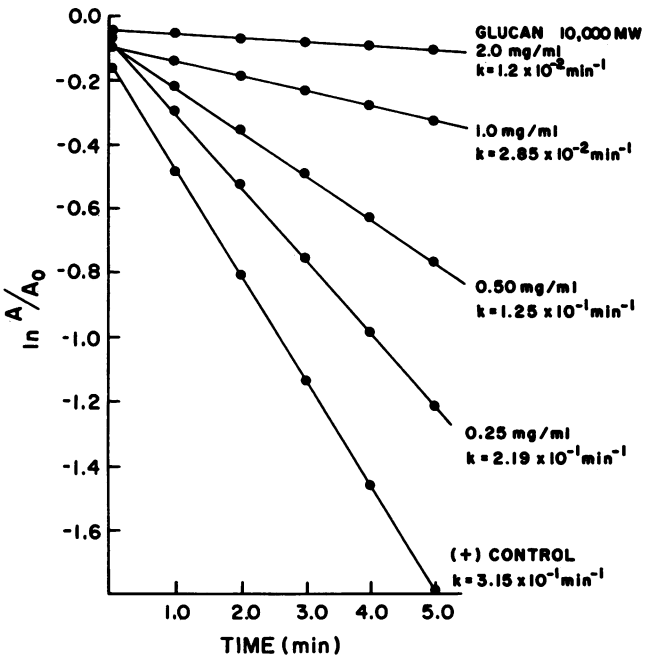

FIG. 4. Inhibition of aggregation of *S. cricetus* with a low-molecular-weight glucan. *S. cricetus* AHT was cultured in TSB and prepared in PBS. The standard rate assay was performed with glucan T10. Glucan T10 was incubated with cell suspensions for 30 min at 37°C before the addition of glucan T2000. Data were subjected to a linear regression statistical program. MW, molecular weight; *k*, rate constant.

of *S. cricetus* as observed above. Plots of ln A/A_0 versus time gave rise to a rate constant of 3.15×10^{-1} min^{-1}. However, incubation of bacterial suspensions with 0.25 mg of glucan T10 per ml (10,000 molecular weight) caused a 30% inhibition of aggregation. Moreover, further increases in glucan T10 concentration resulted in concomitant decreases in the rates of aggregation obtained, with a maximum of 96% inhibition achieved upon incorporation of 2.0 mg of inhibitor per ml in the assay. Washing the cells in PBS after incubation with various concentrations of glucan T10 resulted in the complete restoration of GBL activity (data not shown), suggesting that the binding of low-molecular-weight glucans is reversible. Of particular interest are the findings that significantly lower levels of inhibition of aggregation were achieved with glucan T10 if cells were aggregated with T2000

TABLE 3. Inhibition of aggregation of *S. cricetus* AHT with low-molecular-weight glucan T10

Glucan T10 concn (mg/ml)[a]	% Inhibition[b]	
	Before T2000 addition	After T2000 addition
Control[c]	0.0	0.0
0.25	30.5	12.0
0.50	60.3	34.0
1.0	91.0	74.2

[a] Final assay concentration

[b] Glucan T10 was added to a suspension of *S. cricetus* AHT before or after the cells were aggregated with high-molecular-weight glucan T2000.

[c] Cell suspensions received PBS and were assayed under identical conditions.

before the addition of the inhibitor (Table 3). Incorporation of 0.50 mg of glucan T10 per ml resulted in 60.3% inhibition before the addition of T2000, whereas only 34.0% inhibition was observed with previously aggregated cells. This intercellular multiple binding of high-molecular-weight glucan by GBL resulting in the formation of aggregates would possibly account for the observed increased difficulty in inhibition by glucan T10. Therefore, the results indicate that although low-molecular-weight glucans are incapable of inducing aggregation, they are able to bind to GBL in a competitive manner and thereby cause a pronounced inhibition of glucan T2000-mediated aggregation.

Inhibition of glucan T2000-mediated aggregation of *S. cricetus* with isomaltosaccharides. Inhibition studies with isomaltosaccharides consisting of glucose residues in α-1,6 linkages were performed to obtain information on the size of the active site of the GBL. The micro-rate assay was developed particularly for these compounds owing to the very small amounts available. The results of these experiments are shown in Fig. 5. As described above, incorporation of glucan T10 in cell suspensions before the addition of glucan T2000 resulted in inhibition of aggregation in a concentration-dependent manner. Isomaltooctaose also inhibited aggregation, although higher concentrations were needed to achieve a 50% level of inhibition. These data are not surprising considering that isomaltooctaose has a molecular weight approximately seven times lower than that of T10. Isomaltohexaose and isomaltopentaose, consisting of six and five glucose residues, respectively, were also able to inhibit glucan T2000-induced aggregation, but only at much higher concentrations. A 50% level of inhibition was achieved with isomaltohexaose and isomaltopentaose at concentrations 5,600 and 7,200 times that of T2000, respectively. No inhibition was observed, however, with isomaltotriose at concentrations as high as 33 mg/ml (data not shown). Also, no inhibition occurred with maltoheptaose, an oligosaccharide consisting of seven glucose residues in α-1,4 linkage, even at a concentration 18,000 times that of glucan T2000. These observations, in combination with previous results (Table 1), confirm the affinity of the GBL for α-1,6 anomeric linkages. If these data are plotted as the glucan concentration required to achieve a 50% level of inhibition, an interesting curve results (Fig. 6). A steep curve results when the inhibition data of isomaltopentaose, isomaltohexaose, and isomaltooctaose are compared. It is pertinent to point out the substantial difference in 50% inhibition levels of isomaltohexaose and isomaltooctaose, saccharides which differ by only two glucose residues. Moreover, a gentle sloping line occurs beyond isomaltooctaose, an 8-glucose-residue compound, to glucan T10, consisting of 55 glucose residues. Collectively, these data suggest that the GBL has an absolute affinity for α-1,6 anomeric linkages and has an active site which accommodates isomaltosaccharides of at least 9 to 10 hexose residues.

Protection by saccharide ligand against inactivation by trypsin. It is known that salivary proteases from other microbiota are abundant in the oral cavity. Therefore, it was of interest to determine the susceptibility of GBL to proteolysis in the presence and absence of specific ligand. Experiments were designed to examine inhibition via rate assays of cell suspensions treated with trypsin before or after the addition of high-molecular-weight glucan T2000. The results of these studies are presented in Fig. 7. Cell suspensions of *S. cricetus* AHT (Fig. 7A) were treated with trypsin (10 μg/ml) before the addition of glucan T2000 and incubated for 90 min at 37°C. Control suspensions were incubated with PBS

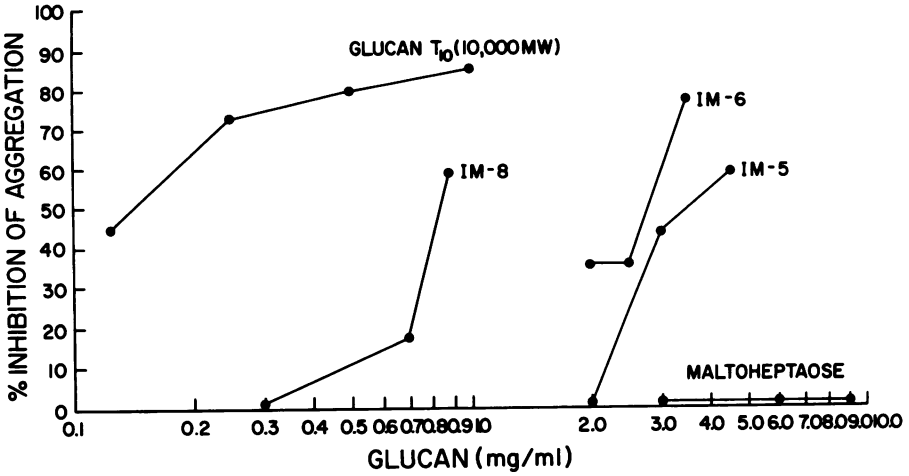

FIG. 5. Inhibition of glucan T2000-mediated aggregation of *S. cricetus* by isomaltosaccharides. Suspensions of *S. cricetus* AHT were incubated with the prospective inhibitors and assayed for glucan T2000-induced aggregation by the microassay procedure. IM-8, Isomaltooctaose; IM-6, isomaltohexaose; IM-5, isomaltopentaose; MW, molecular weight.

under identical conditions. In Fig. 7B, cell suspensions were first aggregated with glucan T2000 and then incubated with trypsin for 90 min at 37°C. Controls again received PBS, but only after the cells were aggregated. A considerable difference in the susceptibility of GBL to proteolysis was observed between the site-saturated and site-unoccupied states. Comparison of the rate curves generated revealed that cells previously aggregated with glucan T2000 were relatively resistant to proteolysis, whereas cells not aggregated with glucan exhibited a substantial loss of aggregation capacity. When these data are displayed in terms of GBL activity versus time of incubation, the susceptibility differences are more strikingly apparent (Fig. 8). After 60 min of incubation in the presence of trypsin, nonaggregated cells exhibited a 35% loss of GBL activity compared with only a 10% loss with the glucan-bound cells. At 90 min, only 36% of the original GBL activity remained with the nonaggregated cells, whereas greater than 70% of GBL activity was present with cells previously aggregated with glucan T2000. These studies demonstrate the significantly higher level of resistance of the GBL to proteolysis when complexed with glucan.

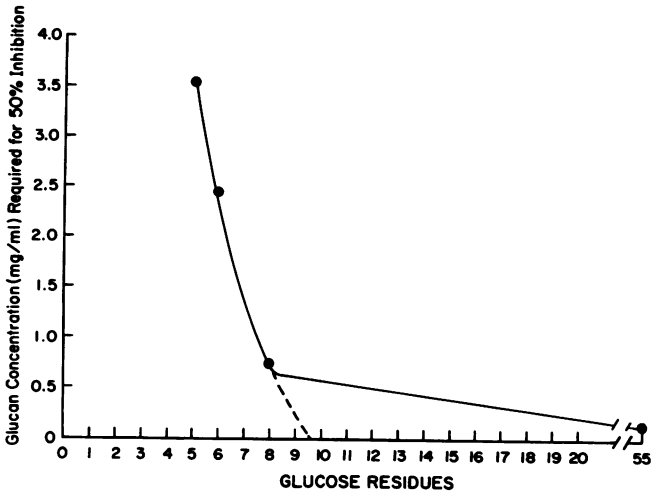

FIG. 6. Glucan concentration required for 50% inhibition of glucan T2000-mediated agglutination of *S. cricetus*. Inhibition data obtained with glucan T10 and the isomaltosaccharides were plotted in terms of the concentration of inhibitor needed to achieve a 50% level of inhibition versus the number of glucose residues of each inhibitor. Note that isomaltotriose (data not plotted) essentially represents infinity as no inhibition was observed even at concentrations up to 33 mg/ml.

DISCUSSION

The cell surface-associated lectin of *S. cricetus* exhibits several characteristics atypical of other lectins. Most lectins complex with terminal nonreducing sugar residues, but sometimes may also bind to penultimate residues (34). The glucan-binding site of the *S. cricetus* lectin is much larger, accommodating 9 to 10 hexose residues. In contrast to other lectins, there is no recognition of nonreducing termini by the GBL. Only glucans containing linear α-1,6 residues are recognized by the GBL. There may be an evolutionary advantage for such a large and stereospecific combining site for the GBL of an oral streptococcus. Components of the diet such as glucose, maltose, or starches would be unable to inhibit binding of the bacteria to α-1,6-linked glucans. Dietary components capable of complexing with the GBL may cause a reduced adhesion and colonization of the streptococci to teeth.

Using affinity electrophoresis, Landale and McCabe (25) reported that the size of the glucan-binding site of *S. sobrinus* 6715 was able to optimally accommodate eight hexose residues. Our value of 9 to 10 hexose residues for *S. cricetus* AHT is similar, but we used an aggregation rate assay. It is possible that small differences exist between species or between strains within a species. It will be necessary to use equilibrium dialysis or some similar method to ultimately determine the exact sizes of the active sites of the various GBLs.

S. cricetus is highly cariogenic in animals (19, 32) but is a species not commonly isolated from humans. However, this may be due to problems with existing primary isolation and plating techniques, resulting in low recoveries of viable bacteria. For example, it has been shown that a commonly used isolation medium restricts the growth of *S. cricetus* significantly owing to susceptibility to bacitracin (4, 16). Another possible explanation for the low isolation rate from humans resides in the interaction of *S. cricetus* with *Actino-*

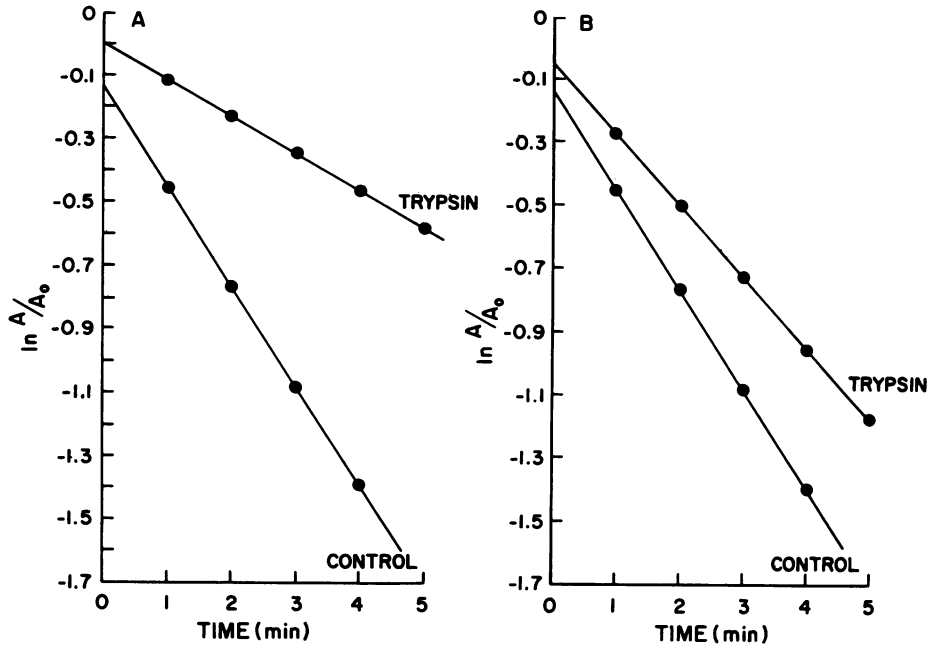

FIG. 7. Protection by glucan of trypsin inactivation of the GBP on *S. cricetus*. Suspensions of *S. cricetus* AHT were incubated with trypsin and then assayed for GBL activity by the standard rate assay. (A) Cells which received trypsin at a final concentration of 10 μg/ml and were incubated for 90 min at 37°C. After incubation, cells were assayed for glucan T2000-mediated aggregation. Control cells were incubated under identical conditions with PBS. (B) Cells which were aggregated with glucan T2000 initially and then incubated with trypsin (10 μg/ml) for 90 min at 37°C. After this incubation, cells were suspended by being vortexed for 5 s, and the standard rate assay was conducted. Control cells received PBS after aggregation with glucan T2000 and were incubated and assayed in an identical manner.

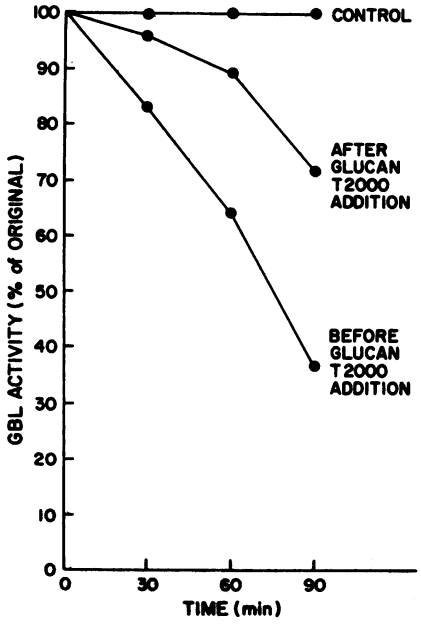

FIG. 8. Loss of aggregation by glucan during incubation of *S. cricetus* with trypsin. Suspensions of *S. cricetus* AHT were incubated with trypsin (10 μg/ml) either before or after the addition of glucan T2000. Controls received PBS. GBL activity was determined by using the rate constants generated from the trypsin-treated cells to calculate percent inhibition and subtracting these values from the controls.

myces species. Crowley et al. (6) showed that *S. cricetus* forms large coaggregates with various *Actinomyces* strains. It is conceivable that cellular aggregates are more susceptible to removal from the oral cavity (27). The scarcity of *S. cricetus* strains in supragingival plaque may also be attributed to a greater sensitivity to the salivary enzyme lysozyme. Iacono et al. (21) determined that growth of *S. cricetus* was inhibited by lysozyme at a concentration 100-fold less than the amount required to inhibit other oral streptococci. However, it is pertinent that *S. cricetus* has been isolated in significant numbers from humans as described in two different studies (2, 43). Of particular significance is the study of an Egyptian population in which *S. cricetus* was recovered from 50% of the patients compared with the 10% isolation rate for *S. mutans*.

It has been reported (5, 7, 20) that serotypes c, e, and f of classical *S. mutans* do not possess the GBL and therefore do not aggregate in the presence of high-molecular-weight α-1,6 glucans. The glucan-induced aggregation of various classical *S. mutans* strains (Fig. 3; Table 2) may be attributed to a GBL similar to that observed for *S. cricetus*. Aggregation was inhibited by glucan T10, and aggregates formed with a high-molecular-weight glucan were dispersed by the low-molecular-weight glucan T10. One possible surface protein distinct from GBL that could be responsible for glucan-mediated aggregation is Ag I/II or Spa A (1, 7). The high-molecular-weight surface protein has been described as having glucan-binding activity. However, it has recently been shown that *S. mutans* 10449 (serotype c) does not express Ag I/II (1), but this bacterium aggregates, albeit weakly, with glucan T2000 (Table 2). It therefore appears that *S. mutans* 10449 possesses a GBL, but the protein may be in such low density that macroscopic aggregation is not

readily achieved by the addition of glucan T2000 to cell suspensions. Several researchers have provided strong evidence that the glucan-mediated aggregation of oral streptococci is independent of surface GTFs (23, 24, 30, 31, 39). It is known that soluble dextran can act as an acceptor in the synthesis of high-molecular-weight glucan by GTF and therefore has the capacity to bind such compounds (35). Moreover, it has recently been shown that a glucan-binding domain can be isolated from purified GTF after treatment with proteases (36). However, these studies have utilized cell-free enzyme preparations, and these conditions may be dissimilar to conditions that exist with cell-bound GTF. In fact, studies examining the role of cell-bound GTF in adhesion of glucose-cultured *S. sobrinus* 6715 to preformed insoluble glucan layers have revealed the following. (i) Cell-bound dextransucrase cannot utilize soluble dextran as a receptor. (ii) Binding of cells to insoluble glucan is not inhibited by 0.5 M α-methyl-D-glucoside, a specific inhibitor of GTF activity. However, low-molecular-weight glucans totally inhibit adhesion of cells to glucan. (iii) Antiserum to purified GTF does not inhibit adhesion of cells to glucan nor does it inhibit glucan-induced aggregation. It is therefore unlikely that GTF may be contributing to the glucan-induced aggregation phenomena.

Another evolutionary advantage developed by oral streptococci may be the resistance of the GBL to proteolysis in the presence of specific ligand. It is known that the oral cavity contains various types of proteases (12, 15, 20) which could act on microbial proteins. The expected loss of GBL because of proteolysis would conceivably have a deleterious effect on adhesion and subsequent plaque formation. The relatively high level of resistance of binding-site-saturated GBL to proteolysis demonstrates a probable evolutionary adaptation to environmental conditions within the oral cavity.

The role of GBL as a virulence determinant is not well understood and is the subject of controversy. It has been suggested by some that plaque formation and subsequent caries development can be dissociated from glucan-mediated aggregation (8–10, 18). Others, however, have described mutants lacking GBL which were noncariogenic and unable to adhere to smooth surfaces in the presence of sucrose even though normal GTF activity was detected (22). Moreover, the importance of a glucan-binding receptor in binding to adherent glucan layers has been described (24, 30, 39). Olson (38) conducted studies in which isolated "dextran receptors" used as immunogens in rats afforded reduction in caries development. Also, antisera were able to inhibit glucan-mediated aggregation, and it was concluded that protection in vivo resulted from interference with bacterial binding of glucan (38, 39). Of particular relevance was the finding that multiple forms of the GBL exist (28). Coinciding with this is the important discovery that the expression of GBL may not be constitutive as commonly assumed, but under the influence of numerous environmental and physiological factors (D. Drake and R. J. Doyle, manuscripts in preparation). Therefore, the GBL may be an important component in colonization by the oral streptococci. The studies reported here describing the specificity and inhibition of aggregation of GBL-glucan interactions are not only important in further defining surface characteristics of the oral streptococci but may be an initial step in the development of compounds designed to prevent adhesion and colonization of these bacteria.

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