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Characterization of Salivary α-Amylase Binding to *Streptococcus sanguis*

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The purpose of this study was to identify the major salivary components which interact with oral bacteria and to determine the mechanism(s) responsible for their binding to the bacterial surface. Strains of *Streptococcus sanguis*, *Streptococcus mitis*, *Streptococcus mutans*, and *Actinomyces viscosus* were incubated for 2 h in freshly collected human submandibular-sublingual saliva (HSMSL) or parotid saliva (HPS), and bound salivary components were eluted with 2% sodium dodecyl sulfate. By sodium dodecyl sulfate-polyacrylamide gel electrophoresis and Western transfer, α-amylase (EC 3.2.1.1) was the prominent salivary component eluted from *S. sanguis*. Studies with ^{125}I-labeled HSMSL or ^{125}I-labeled HPS also demonstrated a component with an electrophoretic mobility identical to that of α-amylase which bound to *S. sanguis*. Purified α-amylase from human parotid saliva was radiolabeled and found to bind to strains of *S. sanguis* genotypes 1 and 3 and *S. mitis* genotype 2, but not to strains of other species of oral bacteria. Binding of [^{125}I]α-amylase to streptococci was saturable, calcium independent, and inhibitable by excess unlabeled α-amylases from a variety of sources, but not by secretory immunoglobulin A and the proline-rich glycoprotein from HPS. Reduced and alkylated α-amylase lost enzymatic and bacterial binding activities. Binding was inhibited by incubation with maltotriose, maltooligosaccharides, limit dextrins, and starch.

Streptococcus sanguis, one of the first species of bacteria to colonize a clean tooth surface (10, 61), is associated with gingival and dental health (22, 42, 58). Successful colonization of the oral cavity by *S. sanguis*, as with other bacteria, initially depends on bacterial adherence to host surfaces (23). Previous studies have demonstrated that bacterial adherence is greatly affected by the presence of host secretions, such as saliva. One way in which salivary components are thought to exert their effects on microbial colonization is by the formation of surface biofilms or pellicles (39). For example, electron microscopic studies have demonstrated a distinct, electron-dense layer, presumed to be the acquired salivary pellicle, interposed between the tooth and adherent dental plaque (18, 23, 41). In vitro studies have demonstrated a significant effect of saliva on bacterial adherence to various substrates, such as extracted teeth, powdered enamel, or synthetic hydroxyapatite (11, 12, 27, 55). *Streptococcus mutans* has been found to adhere in lower numbers to saliva-coated hydroxyapatite compared with the numbers that adhere to untreated hydroxyapatite, while *S. sanguis* and *Actinomyces viscosus* attach in higher numbers to saliva-coated hydroxyapatite (11).

In addition to the acquired enamel pellicle, the formation of a salivary coat or film on the bacterial surface must also be considered in the interaction of bacteria with oral surfaces. Some types of bacteria aggregate when they are suspended in saliva (21, 37, 55), suggesting that salivary components interact with the bacterial surface. In vivo studies have also indicated that the implantation of bacteria in the oral cavity of humans is modified by pretreatment of the bacteria with saliva (60). Collectively, these studies suggest that salivary components bound to the bacterial surface may facilitate the clearance of bacteria from the oral cavity or foster their adherence to oral surfaces.

A number of salivary glycoproteins and proteins have been shown to interact with various oral bacteria in vitro. These include mucins (20, 28, 37, 38), proline-rich glycoprotein (5, 56), secretory immunoglobulin A (sIgA) (40, 64), a fucose-rich glycoprotein from parotid saliva (16), lysozyme (25, 52), β_2-microglobulin (15), and others (3, 4). The goal of the present study was to ascertain to what extent salivary components interact with *S. sanguis* and other bacteria and to determine the mechanism(s) responsible for their binding to the bacterial surface.

MATERIALS AND METHODS

Materials. The following materials were obtained from the indicated sources: Trypticase soy broth (TSB) and agar (BBL Microbiology Systems, Cockeysville, Md.); yeast extract (Difco Laboratories, Detroit, Mich.); crude human salivary α-amylase, *Bacillus amyloliquefaciens* α-amylase, *Aspergillus oryzae* α-amylase, porcine pancreatic α-amylase, *Triticum aestivum* (wheat seed) α-amylase inhibitor, D-glucose, D-galactose, lactose, D-mannose, maltose, maltotriose, maltooligosaccharide, pullulan (limit dextrin), starch, sIgA, rabbit anti-human salivary amylase, bovine serum albumin (BSA; A-7906), chloramine-T, Coomassie brilliant blue (B-0630), phenylmethylsulfonyl fluoride (type XI), sodium borohydride, sodium *meta*-periodate, and trypsin (T-1005) (Sigma Chemical Co., St. Louis, Mo.); Bio-Gel P-6DG, Bio-Gel P60, horseradish peroxidase-conjugated affinity-purified goat anti-rabbit IgG (heavy and light chain specific), and 4-chloro-1-naphthol (HRP color development reagent) (Bio-Rad Laboratories, Richmond, Calif.); dialysis tubing (Spectra/Por 3; Spectrum Medical Industries, Los Angeles, Calif.); molecular weight protein markers (phosphorylase *b* [94,000], BSA [67,000], ovalbumin [43,000], carbonic anhydrase [30,000], and trypsin inhibitor [20,100]), Sephadex G-25, Sephadex G-200, and Mono-Q HR5/5 prepacked columns (Pharmacia Fine Chemicals, Piscataway, N.J.); *N*-succinimidyl-3-(4-hydroxyphenyl)propionate (Pierce

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Chemical Co., Rockford, Ill.); and $Na^{125}I$ (17 Ci/mg; Amersham Corp., Arlington Heights, Ill.).

Bacterial strains and culture conditions. Fresh bacterial isolates were obtained from a healthy 30-year-old male with no history of systemic antibiotic use in the previous year. Supragingival plaque, which was allowed to form overnight, was taken with a sterile curette from a single site (buccal surface of the upper left second premolar) and placed into sterile Ringer solution. After vortexing for 30 s, appropriate dilutions were plated onto Trypticase soy agar (1.5%) plates containing 5% fresh sheep blood. After anaerobic incubation for 5 days in an atmosphere of 85% N_2–10% H_2–5% CO_2, 30 colonies were randomly selected and subcultured onto blood agar. A strain of *S. sanguis* genotype 1, designated FAS4, was identified by its characteristic Gram stain, colony morphology on mitis salivarius agar, production of ammonia from arginine, hydrolysis of esculin (46), and other biochemical features, as determined by the API Rapid Strep System (Analytab Products, Plainview, N.Y.). A strain of *A. viscosus*, designated FAS24, was identified by its colony morphology on blood agar, characteristic Gram stain, production of catalase (29), production of succinic acid from glucose, and other biochemical features determined by the API An-Ident System (Analytab Products). Other well-characterized strains used in this study included *S. sanguis* genotype 1 strains G9B (kindly provided by B. Rosan, University of Pennsylvania), NCTC 7868 (Challis), ATCC 10558, and Blackburn (kindly provided by A. Coykendall, University of Connecticut); *S. sanguis* genotype 2 strains ATCC 10556 and HPC-1 (A. Coykendall); *S. sanguis* genotype 3 strains CR3 and CR311 (A. Coykendall); *S. mitis* genotype 1 strains 10557 (A. Coykendall) and KS32AR (45); *S. mitis* genotype 2 strains OP51 and 10712 (A. Coykendall); *S. mutans* NCTC 10449 (A. Coykendall); *A. viscosus* T14v (kindly provided by J. Cisar, National Institute of Dental Research); nontypeable *Haemophilus influenzae* 955 and 84; *Bacteroides gingivalis* 7A1-28 and 18-10; *Bacteroides intermedius* 25611; and *Bacteroides levii* 29147 (all kindly provided by B. Loos, State University of New York at Buffalo); and *Candida albicans* DS1 (kindly provided by M. Edgerton, State University of New York at Buffalo). Streptococci were classified by the scheme of A. Coykendall (12a) by using the API Rapid Strep System (Analytab Products). Strains were stored in 20% glycerol broth at −70°C.

For routine use, strains were subcultured by placing a hot loop in the frozen cultures, and then the loop was streaked onto blood agar plates. Following incubation for 12 to 48 h at 37°C, several well-isolated colonies were picked for growth in TSB supplemented with 5% yeast extract (TSB-Y) and cultured statically at 37°C. In some cases, these cultures were used to inoculate larger batch cultures (1% [vol/vol]) in TSB-Y or chemically defined medium (62). Broth used for *Haemophilus* cultures was supplemented with 1 μg of NAD per ml and 5 μg of hemin per ml, and broth used for *Bacteroides* cultures was supplemented with 5 μg of hemin per ml and 0.5 μg of menadione per ml. Streptococci, *C. albicans* and *H. influenzae* were routinely cultured aerobically at 37°C for 18 h, while *A. viscosus* was grown aerobically for 24 h, and *Bacteroides* species were grown anaerobically for 48 h.

Collection and handling of saliva. Human parotid saliva (HPS) was collected by using a modified Carlsen-Crittenden apparatus (13), and human submandibular-sublingual saliva (HSMSL) was collected with a permplastic custom-made collector (6). Salivary flow was stimulated by the application of 2% citric acid every 20 to 30 s to the tongue. Saliva samples (20 to 25 ml) were collected in chilled tubes containing 2 ml of enzyme inhibitor solution (0.1 M Tris hydrochloride [pH 7.5], 2% disodium EDTA, 10% *n*-propanol, and 2 mM phenylmethylsulfonyl fluoride). Saliva samples were diluted 1:1 with 0.154 M NaCl and stirred at 10°C for 30 min prior to use in experiments.

Salivary mucin-containing fractions were obtained by gel filtration of HSMSL on columns of Sephadex G-200 as described previously (37, 53, 57). These fractions contained sIgA, high-molecular-weight mucin, and low-molecular-weight mucin. The proline-rich glycoprotein was purified from HPS as described previously (5).

Analytical methods. Protein determinations were done by the method of Lowry et al. (44) or by using an amino acid analyzer (model 6300; Beckman Instruments, Inc., Fullerton, Calif.) after hydrolysis of samples in 6 N HCl for 24 h at 110°C. For determination of hexosamines, samples were hydrolyzed in 2 N HCl for 6 h at 100°C and analyzed on the amino acid analyzer.

Sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) was performed by the method of Laemmli (35). Samples were prepared for electrophoresis by heating them at 100°C for 3 to 5 min in sample solubilizing solution (0.064 M Tris buffer [pH 6.8] containing 2% SDS, 10% glycerol, and 5% 2-mercaptoethanol). Anionic PAGE was performed by the method of Ornstein (49). Gels were stained for protein with Coomassie brilliant blue. Molecular weights were estimated from standard plots of the log molecular weights of standard proteins versus their relative mobilities.

Western transfer was performed by the methods of Burnett (9) and Towbin et al. (63). Nitrocellulose-bound antigens were incubated for 30 min in blocking buffer (0.01 M sodium phosphate buffer [pH 7.2] containing 1% BSA with 0.05% Tween 20) followed by incubation with the appropriate dilution of the rabbit anti-human salivary α-amylase in blocking buffer for 30 to 60 min. After washing in blocking buffer, membrane-bound antigens were incubated with horseradish peroxidase-conjugated, affinity-purified goat anti-rabbit IgG for 1 h; washed three times; and developed in 3% 4-chloro-1-naphthol in methanol and 0.018% hydrogen peroxide at a 5:1 ratio. In control experiments, HSMSL, HPS, and bacterial extracts did not react with normal rabbit serum.

Purification of α-amylase isoenzymes. About 200 to 300 mg of lyophilized HPS collected from a single donor was suspended in 8 to 10 ml of buffer (0.1 M Tris hydrochloride 0.1 M NaCl, and 0.01% NaN_3 [pH 7.5]) for 30 to 60 min at 6°C and then centrifuged at 12,000 × *g* for 30 min at 6°C. The supernatant was chromatographed at 6°C on columns (2.5 by 120 cm) of Bio-Gel P60 equilibrated in buffer at 1 to 2 ml/h (33, 34). Protein elution was monitored by determining the A_{280}, and α-amylase was localized by determining the reactivities of the fractions with rabbit anti-human salivary α-amylase. Two major families, A and B, of α-amylase (EC 3.2.1.1) were found (Fig. 1). These were pooled, dialyzed exhaustively against distilled water, and lyophilized. The A and B families represented 5 to 10% and 7 to 11% by weight, respectively, of the material recovered from the column. Hexosamine analysis indicated that family A contained the glycosylated isoenzymes. Family B, 0.5 to 1 mg, representing the nonglycosylated forms, was suspended in 1 ml of 0.01 M Tris hydrochloride (pH 8.0; buffer A) and subjected to fast protein liquid chromatography (FPLC) (Pharmacia) by using a Mono-Q anion-exchange column. Four peaks were obtained by elution with buffer A in a shallow gradient from 0 to 0.1 M NaCl at a flow rate of 1 ml/min (Fig. 2). Fractions

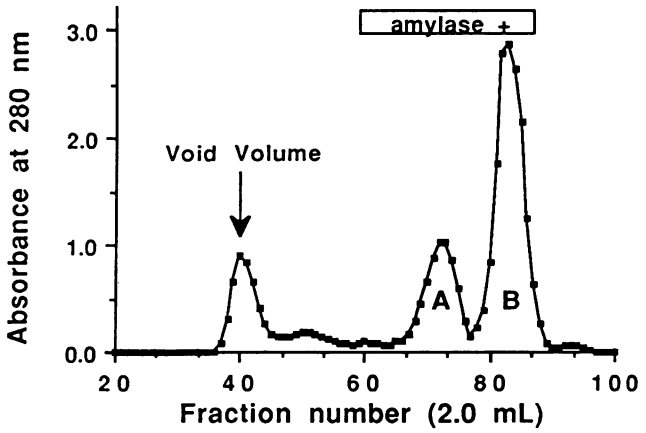

FIG. 1. Chromatography of HPS on Bio-Gel P60. α-Amylase-containing fractions were identified by their reactivities with rabbit anti-salivary α-amylase serum. Family B (fractions 80 to 93) were pooled for further purification by FPLC.

were monitored for protein at 280 nm, pooled, dialyzed against cold distilled water, and lyophilized. The major peak (B1; Fig. 2), which eluted at 0.02 M NaCl, was found to be homogeneous by SDS-PAGE, anionic PAGE, and SDS-PAGE–autoradiography of its iodinated derivative (Fig. 3). Its amino acid composition was comparable to that published previously for α-amylase (Table 1). Hexosamines were not detected, indicating that this isoenzyme is nonglycosylated. B1 possessed approximately 0.326 U of α-amylase activity per μg of protein and represented the major nonglycosylated isoenzyme, which was comparable to isoenzyme 4 described previously (34), and was used in further bacterial binding experiments. The second peak (B2; Fig. 2), which eluted at 0.03 M NaCl, also possessed α-amylase enzymatic activity and had an amino acid com-

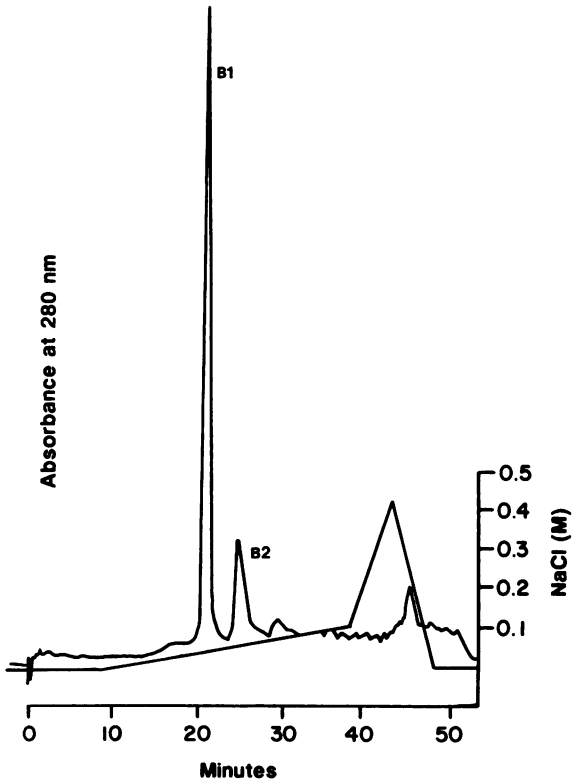

FIG. 2. FPLC (Mono Q) anion-exchange chromatography of nonglycosylated α-amylase. Family B (1 mg) was chromatographed in 0.01 M Tris hydrochloride (pH 8.0) on a Mono-Q HR5/5 column, bound materials were eluted with an NaCl gradient, and 1-ml fractions were collected at a flow rate of 1 ml/min.

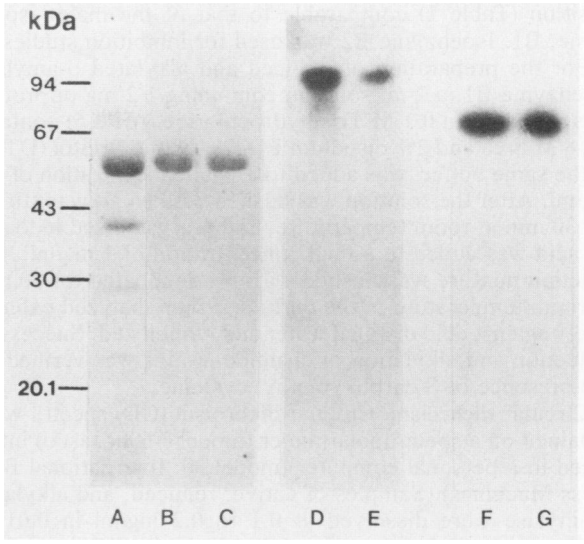

FIG. 3. Electrophoresis of parotid α-amylase by SDS-10% PAGE (values in brackets are micrograms of protein per lane) (lane A, HPS [25 μg]; lane B, family B of α-amylase [25 μg]; lane C, isoenzyme B1); by anionic PAGE (lane D, family B of α-amylase [25 μg]; lane E, isoenzyme B1 [25 μg]); by SDS-10% PAGE–autoradiography (lane F, ^{125}I-labeled isoenzyme B1; lane G, ^{125}I-labeled isoenzyme B1 eluted from *S. sanguis* with SDS). kDa, Kilodaltons.

TABLE 1. Amino acid compositions of purified α-amylase isoenzymes

Amino acid	No. of residues/1,000 amino acid residues in:			
	Isoenzyme		Literature value[a]	DNA sequence[b]
	B1	B2		
Aspartic acid	150	160	152	149
Threonine	41	45	40	46
Serine	72	49	63	65
Glutamic acid	81	78	67	61
Proline	45	44	51	44
Glycine	130	117	98	103
Alanine	59	58	47	50
Half-cystine	19	16	18	23
Valine	72	74	71	70
Methionine	15	11	20	21
Isoleucine	50	56	53	55
Leucine	50	53	49	53
Tyrosine	36	39	40	41
Phenylalanine	51	55	51	57
Lysine	48	51	45	48
Histidine	25	26	22	23
Arginine	55	68	58	55
Tryptophan	ND[c]	ND	55	37

[a] Data are from reference 31.
[b] Data are from reference 47.
[c] ND, Not determined.

position (Table 1) comparable to that of the major isoenzyme, B1. Isoenzyme B2 was used for inhibition studies.

For the preparation of reduced and alkylated α-amylase isoenzyme B1, a 2-ml solution containing 1.2 mg of protein was prepared in 0.1 M Tris hydrochloride (pH 8.5) containing 8 M urea and 2% disodium EDTA. Dithiothreitol (DTT), in the same buffer, was added to a final concentration of 0.5 mg/ml. After the solution was flushed with N_2, it was stirred for 30 min at room temperature, and recrystallized iodoacetic acid was added to a final concentration of 1 mg/ml. The reaction mixture was flushed with N_2, incubated for 30 min at room temperature in the dark, and then dialyzed exhaustively against cold distilled water and lyophilized. Successful reduction and alkylation of disulfide groups was verified by the presence of S-carboxymethyl cysteine.

Circular dichroism. Circular dichroism (CD) spectra were obtained on a spectrapolarimeter (model J-600; Jasco) interfaced to a personal computer (model 30; International Business Machines). Samples of native, reduced, and alkylated α-amylase were dissolved at 0.1 to 0.2 mg/ml in 0.01 M sodium phosphate containing 0.154 M NaCl (PBS; pH 7.5). All spectra were corrected for solvent and represent the average of three scans run at room temperature. The effect of DTT on the CD spectra was assessed after it was added (0.5 mg/ml) to the α-amylase solutions. The effect of guanidine hydrochloride (GuHCl) on the CD spectra was determined by preparing a solution of α-amylase in PBS containing 8 M GuHCl. The effect of a combination of both DTT and GuHCl on the CD spectra was determined by the addition of DTT (0.5 mg/ml) to a solution of α-amylase in PBS–8 M GuHCl.

Assays of α-amylase activity. Following anionic PAGE of α-amylase preparations, the gels were rinsed in distilled water and then washed for 1 h in 1% soluble starch containing 1 mM $CaCl_2$ at room temperature. The gels were rinsed (three times with 50 ml of distilled water each time) and developed with 10 mM KI–10 mM I_2 in distilled water. Isoenzymes of α-amylase are visualized as yellow bands on a blue background (24). The Amylase 3 single-reagent system (Sigma) was used at room temperature to quantitate the α-amylase enzymatic activity of the purified preparations.

Initial characterization of saliva-bacterium interactions. Bacteria were harvested from 400 ml of TSB-Y by centrifugation at 4,000 × *g* for 30 min at 4°C. The cells were washed three times with 50 ml of cold PBS (0.01 M sodium phosphate buffer and 0.154 M NaCl with 0.01% NaN_3 [pH 7.0]). The final pellet was suspended in 5 ml of PBS and vortexed for 1 min to disperse the bacterial cells.

Experimental suspensions were prepared by adding 1 ml of the final bacterial cell suspension to 5 ml of HSMSL, HPS, or PBS. Suspensions were gently vortexed and incubated statically at 27°C for 2 h, and the mixtures were scored for aggregation by visual examination. The cells were then pelleted by centrifugation as described above, and the supernatant (depleted saliva) was removed, dialyzed extensively against cold distilled water, and lyophilized. The cell pellets were washed once in 10 ml of cold PBS and centrifuged as described above, and the supernatants were discarded. Both experimental and control cell preparations were suspended in 2 ml of 2% SDS in PBS, vortexed for 1 min, and incubated for 12 to 18 h at 27°C with gentle mixing on an orbital shaker (Junior Orbit Shaker; Lab Line, Melrose Park, Ill.). The cell suspensions were again vortexed for 1 min, and the cells were removed by centrifugation. The resulting materials were dialyzed, first against 10% ethanol to remove SDS (17) and then against cold distilled water, and lyophilized. Experiments were conducted at least twice for each strain tested.

Preparation of ^{125}I-labeled saliva samples, [^{125}I]sIgA, and [^{125}I]α-amylase. Saliva samples were prepared for radiolabeling by a modification of the method of Bolton and Hunter (7). HSMSL and HPS (2 to 3 mg [dry weight]) were suspended in 100 μl of sodium borate buffer (pH 8.5) for 30 min followed by centrifugation at 12,000 × *g* at room temperature for 1 min to remove insoluble material. The supernatants were derivatized by adding 10 μl of 10 mg of *N*-succinimidyl-3-(4-hydroxyphenyl)propionate per ml in dioxane with incubation at 4°C for 15 min. The procedure was repeated twice more, after which the mixtures were desalted on columns (10-ml disposable pipettes) containing Sephadex G-25 (fine) equilibrated in 0.1 M NH_4HCO_3 (pH 8.0). Fractions of 1 ml were collected and monitored at 280 nm. Void volume fractions were pooled and lyophilized. *N*-Succinimidyl-3-(4-hydroxyphenyl)propionate-derivatized saliva samples, sIgA, and purified α-amylase B1 (50 to 100 μg of protein) were iodinated by the chloramine-T method in 100 μl of PBS (26). Iodinated materials were separated from free iodine and reagents by gel filtration on columns (10-ml disposable pipettes) of Sephadex G-25 or Bio-Gel P6DG equilibrated in PBS. Fractions of 1 ml were monitored for radioactivity with a gamma counter (model 5500; Beckman). Void volume materials were used for the subsequent studies. The specific activities (counts per minute per microgram of protein) obtained were 3.5×10^6 for HSMSL, 2.9×10^6 for HPS, 3.5×10^6 for sIgA, and 1×10^6 to 3×10^6 (5.5×10^4 to 16.5×10^4 cpm/pmol) for α-amylase.

In vitro interaction of ^{125}I-labeled saliva with bacteria. Bacteria cultured in TSB-Y were washed in PBS (pH 7.0) and suspended to an A_{600} of 10. The bacterial suspension (1 ml) was added to 1.5-ml microcentrifuge tubes coated with 1% BSA in PBS. ^{125}I-labeled HSMSL or ^{125}I-labeled HPS was added (approximately 10^6 cpm), and the suspensions were mixed for 30 min at 27°C on an orbital shaker. The bacteria were harvested by centrifugation as described above and washed three times in PBS. The pellets were extracted with 50 μl of sample solubilizing solution for 1 h at 45°C, and the extracts were subjected to SDS-PAGE. Autoradiography was performed on dried gels at −70°C for 24 to 48 h with X-Omat AR film (Eastman Kodak Co., Rochester, N.Y.) by using an intensifying screen (Cronex Lightning-Plus; E. I. du Pont de Nemours & Co., Inc., Wilmington, Del.). Autoradiographs were analyzed by using a laser densitometer (model 2202; LKB Instruments, Inc., Rockville, Md.) interfaced to a computer (model IIe; Apple Computers).

Binding of [^{125}I]α-amylase to bacteria. Bacteria were suspended to an A_{600} of 0.1 or 1 in PBS containing 0.1% lipid-free BSA. These suspensions correspond to 1×10^8 to 2×10^8 or 1×10^9 to 2×10^9 bacteria per ml, respectively, as extrapolated from standard curves derived from plots of the A_{600} versus cell numbers (cells were counted with a Petroff-Hauser counter). Experiments were performed in polypropylene microcentrifuge tubes precoated with 1.0% BSA to reduce the nonspecific interaction of ligand with the tube walls. Reaction mixtures contained 0.1 ml of the bacterial suspension, various amounts of [^{125}I]α-amylase, and buffer added to a total volume of 1.0 ml. For screening experiments, reaction mixtures contained 0.9 ml of the bacterial suspension (1×10^9 to 2×10^9 bacteria per ml) and 0.1 ml of [^{125}I]α-amylase. Duplicate samples were incubated at 27°C for 30 min, after which the reaction was terminated by centrifugation followed by three 1-ml washes with PBS.

The amount of radioactivity bound to the bacteria was measured with a gamma counter (model 5500; Beckman). Negligible radioactivity (less than 0.01%) was recovered in the microcentrifuge tubes from reaction mixtures which did not contain bacteria.

Inhibition of [^{125}I]α-amylase binding to bacteria by unlabeled α-amylase, other proteins, or various sugars was also examined. Reaction mixtures, containing 0.1 ml of the bacterial suspensions, 0.1 ml of various concentrations of the inhibitor, and 0.7 ml of buffer, were allowed to incubate at room temperature for 30 min. This was followed by the addition of 0.1 ml of [^{125}I]α-amylase and incubation for an additional 30 min. The reaction was terminated as described above. When sugars were used as inhibitors, the bacteria were heated at 80°C for 30 min before the sugars were added to inhibit acid production by the bacteria. The effects of calcium and EDTA were assessed by the addition of 0.1 ml of a 10 or 100 nM solution of $CaCl_2$ or trisodium EDTA to the reaction mixtures instead of the inhibitor described above. In place of PBS-BSA, 0.1 M Tris hydrochloride-buffered saline with BSA (pH 7.0) was used.

The reversibility of α-amylase–bacterium interactions was evaluated by adding a 200- to 400-fold excess of unlabeled α-amylase to bacteria with bound [^{125}I]α-amylase. Following incubation for 2 to 8 h at room temperature, the reaction was terminated as described above.

Modification of *S. sanguis* G9B. For trypsinization, bacteria were cultured in TSB-Y, washed, and suspended in PBS at an A_{600} of 1.14. This suspension (1 ml) was mixed with trypsin (1.25 mg, containing 18250 BAEE units, in 0.5 ml of PBS) and incubated at 37°C in a shaking water bath for 3 h. The cells were then washed three times in PBS, passed 10 times through a 25-gauge needle, and heated at 80°C for 30 min to inactivate the enzyme. Bacteria in 1.5 ml of buffer alone served as a control. A total of 0.1 ml of each bacterial suspension was used in binding experiments with [^{125}I]α-amylase as described above. To ascertain whether treatment at 80°C for 30 min inactivated trypsin, trypsin or heated trypsin was mixed with an equal volume of PBS containing 0.1% BSA. After incubation for 3 h at 37°C, portions of each mixture were mixed with SDS-PAGE solubilizing solution and boiled for 2 min. SDS-PAGE indicated that trypsin cleaved BSA into low-molecular-weight fragments, while the heat-treated enzyme did not.

For periodate studies, bacteria were cultured in TSB-Y, washed, and suspended in 0.05 M sodium acetate buffer (pH 4.5) containing 0.9% NaCl to an A_{600} of 2.6. A total of 1 ml of this suspension was mixed with 0.5 ml of the same buffer containing 0.15 M sodium *meta*-periodate. After 60 min at room temperature, the pH was adjusted to 8.0, and sodium borohydride was added to 10 mg/ml. After 3 h at room temperature, the cells were washed three times in PBS, passed 10 times through a 25-gauge needle, and adjusted to an A_{600} of 1.0. As controls, bacteria were treated with sodium *meta*-periodate only, sodium borohydride only, sodium acetate buffer alone, or PBS. One hundred microliters of each bacterial suspension was then used in the α-amylase-binding assays.

RESULTS

Characterization of in vitro-formed bacterial pellicles. All of the streptococci examined in this study were aggregated with HPS or HSMSL, whereas bacteria in PBS alone did not aggregate. This aggregation was visible within 20 to 30 min. In some cases, the streptococci settled to the bottom of the test tube by 2 h, leaving a clear supernatant. In contrast, *A. viscosus* T14v and FAS24 exhibited only weak aggregation in HSMSL or HPS. To identify the components in saliva which bound to bacteria, cells exposed to saliva were extracted with 2% SDS. These extracts were compared by SDS-PAGE with intact HSMSL, HPS, and 2% SDS extracts of bacteria exposed to PBS alone (control). Only a few salivary components from HSMSL and HPS were seen to interact with *S. sanguis* and the other oral bacteria. These components were not detected in the SDS extracts of bacteria which were incubated with PBS. *S. sanguis* G9B, FAS4, and Challis, but not strain 10556, bound two components of 53 and 58 kilodaltons from HSMSL (Fig. 4, lane C) and HPS. The remaining components in HSMSL and HPS were recovered in the depleted saliva samples (Fig. 4, lane A). Western transfer analysis with rabbit anti-α-amylase serum identified these components as α-amylase isoenzymes (Fig. 5). None of the other strains examined (*S. mitis* KS32AR, *S. mutans* 10449, or *A. viscosus* T14v or FAS24) appeared to bind α-amylase. This finding was not inherent to the saliva of a single donor since similar results were obtained with *S. sanguis* FAS4 and *A. viscosus* FAS24 with HSMSL and HPS from another individual. The binding of α-amylase to *S. sanguis* was unaffected by the type of culture medium used, as *S. sanguis* FAS4 cultured in chemically defined medium also bound α-amylase from HPS and HSMSL.

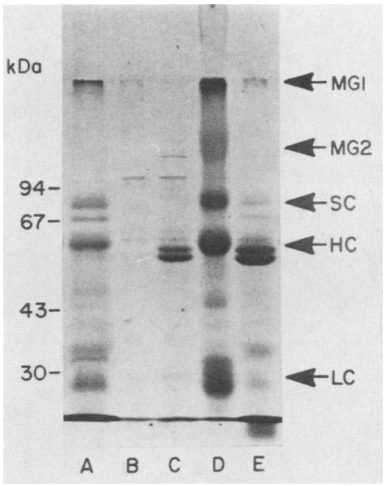

FIG. 4. SDS-10% PAGE of HSMSL-derived pellicles (micrograms of protein per lane). Gels were stained with Coomassie brilliant blue. Lane A, HSMSL after depletion by *S. sanguis* G9B (40 μg); lane B, *S. sanguis* G9B control incubated in PBS (40 μg); lane C, *S. sanguis* G9B HSMSL-derived pellicle (40 μg); lane D, mucin- and sIgA-containing materials from HSMSL obtained by Sephadex G-200 fractionation (40 μg); the relative mobilities of the high-molecular-weight mucin (MG1), the low-molecular-weight mucin (MG2), secretory component (SC), heavy chain (HC), and light chain (LC) from the sIgA in this preparation are noted (53); lane E, intact HSMSL (40 μg). kDa, Kilodaltons.

^{125}I-labeled saliva-bacterium interactions. To examine further the selective nature of saliva-bacterium interactions, iodinated saliva samples were used. In a typical experiment, only 2 to 3% of the iodinated materials added were recovered in the bacterial pellet, and approximately 75 to 80% of the bound radioactivity could be removed by a single extraction with sample solubilizing solution. A component with a mobility similar to that of α-amylase was present in ^{125}I-labeled pellicles derived from *S. sanguis* G9B, FAS4, and

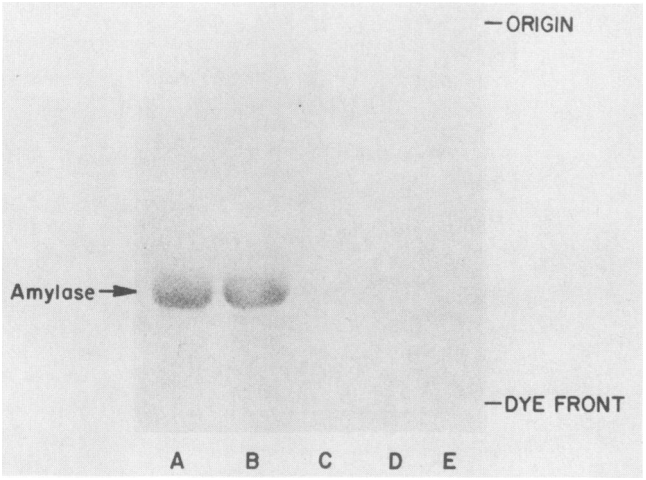

FIG. 5. SDS-7.5% PAGE and Western transfer with rabbit anti-α-amylase of HPS-derived pellicles. Lane A, Intact HPS; lane B, *S. sanguis* G9B HPS-derived pellicle; lane C, *S. sanguis* G9B control incubated in PBS; lane D, *S. mitis* KS32AR HPS-derived pellicle; lane E, *S. mitis* KS32AR control incubated in PBS.

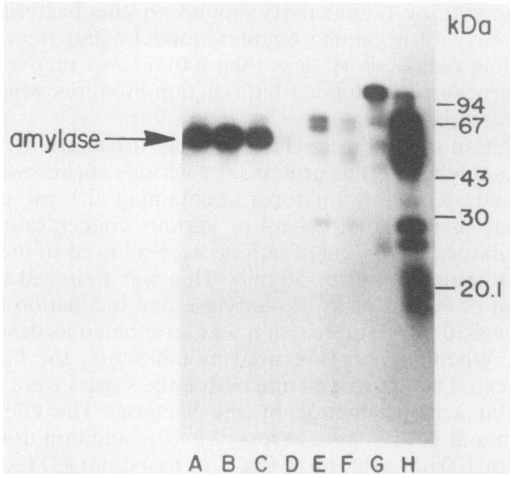

FIG. 7. SDS-10% PAGE–autoradiography of ^{125}I-labeled, HPS-derived pellicles. Lane A, *S. sanguis* G9B; lane B, *S. sanguis* FAS4; lane C, *S. sanguis* Challis; lane D, *S. mitis* KS32AR; lane E, *S. mutans* 10449; lane F, *A. viscosus* T14v; lane G, ^{125}I-labeled sIgA; lane H, ^{125}I-labeled HPS. kDa, Kilodaltons.

Challis (Fig. 6 and 7). Strains of *S. mitis*, *S. mutans*, and *A. viscosus* did not appear to bind appreciable amounts of this component. None of the strains tested appeared to bind materials with a mobility comparable to that of [^{125}I]sIgA (Fig. 7). All of the bacteria examined also bound component(s) with a molecular weight of ≤14,000 from ^{125}I-labeled HSMSL (Fig. 6). Scanning laser densitometry of autoradiographs of ^{125}I-labeled HSMSL revealed that the ratio of α-amylase to low-molecular-weight components was 0.57 in saliva. This ratio was 1.86 in pellicles of *S. sanguis* G9B treated with ^{125}I-labeled HSMSL, indicating that a much greater proportion of available α-amylase than low-molecular-weight components was bound to the bacteria. A ratio of α-amylase to low-molecular-weight components of 0.14 was observed in the pellicles of both *A. viscosus* T14v and *S. mitis* KS32AR, suggesting that α-amylase binds only feebly to these bacteria.

[^{125}I]α-amylase–bacterium interactions. Initial time course studies suggested that binding of a radiolabeled purified isoenzyme of α-amylase occurred promptly with *S. sanguis* G9B but not with *S. sanguis* 10556. This finding was consistent with our previous finding that α-amylase could be recovered from *S. sanguis* G9B but not from *S. sanguis* 10556 following incubation with saliva. Binding of α-amylase was 80 to 90% complete within 30 min. α-Amylase binding was maximal at 4°C, intermediate at 27°C, and least at 37°C (data not shown). For convenience, all subsequent experiments were conducted at 27°C for 30 min. A number of species of oral bacteria were tested for α-amylase binding (Table 2). Only strains of *S. sanguis* genotypes 1 and 3 and *S. mitis* genotype 2 bound α-amylase, while the other oral bacteria tested did not. Comparable binding of α-amylase to *S. sanguis* genotype 1 G9B and Challis were obtained with bacteria cultured in TSB-Y and chemically defined medium.

In order to determine the saturability of binding of α-amylase to *S. sanguis* G9B, experiments were performed in which both the number of bacterial cells and the amount of labeled α-amylase added were varied. Only when about 10^7 or fewer bacteria were incubated with increasing amounts of [^{125}I]α-amylase was saturation achieved, suggesting a rather strong interaction between α-amylase and *S. sanguis* (Fig. 8). Assuming that this interaction reached equilibrium, Scatchard analysis of data from six saturation experiments revealed that approximately 2 to 5 pmol of α-amylase was bound to 1×10^7 to 2×10^7 *S. sanguis* G9B isolates at saturation. Similar experiments performed with the other strains of *S. sanguis* revealed a similar degree of binding. For example, *S. sanguis* FAS4 bound 2.6 pmol of α-amylase, *S. sanguis* Challis bound 1.3 pmol and *S. mitis* OP51 bound 1.7 pmol. From these data, approximately 13,000 to 33,000 α-amylase molecules were calculated to be bound to each streptococcal cell. Further studies with disodium EDTA at final concentrations of 1 and 10 mM only slightly enhanced the binding of α-amylase to *S. sanguis* G9B compared with binding of the controls. The addition of $CaCl_2$ to a final concentration of 1 mM also did not alter the degree of α-amylase binding to the bacteria. Although previous studies have suggested that α-amylase enzymatic activity is calcium dependent, the binding of α-amylase to the bacterial surface did not appear to require Ca^{2+}

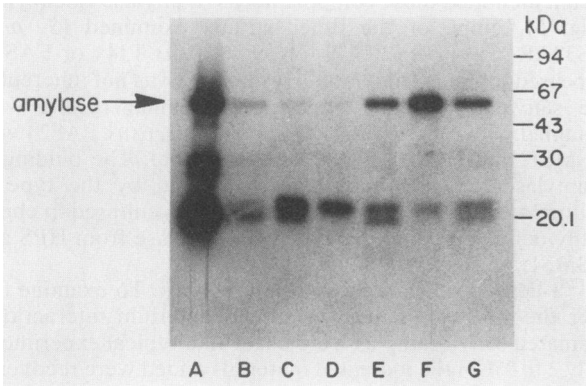

FIG. 6. SDS-10% PAGE–autoradiography of ^{125}I-labeled HSMSL-derived pellicles. Lane A, ^{125}I-labeled HSMSL; lane B, *A. viscosus* T14v; lane C, *S. mutans* 10449; lane D, *S. mitis* KS32AR; lane E, *S. sanguis* G9B; lane F, *S. sanguis* FAS4; lane G, *S. sanguis* Challis. kDa, Kilodaltons.

TABLE 2. Binding of [^{125}I]α-amylase to strains of bacteria[a]

Species	Strain	% Binding compared with G9B
S. sanguis		
Genotype 1	G9B	100.0[b]
	G9B (CDM[c])	123.2
	FAS4	109.3
	Challis	109.7
	Challis (CDM)	112.4
	10558	104.0
	Blackburn	78.0
Genotype 2	10556	0.7
	HPC-1	3.4
Genotype 3	CR3	101.8
	CR311	97.3
S. mitis		
Genotype 1	10557	3.5
	KS32AR	0.6
Genotype 2	OP51	101.3
	10712	106.8
S. mutans	10449	0.7
A. viscosus	T14v	0.9
H. influenzae	955	14.3
	84	8.2
B. gingivalis	7A1-28	0.8
	18-10	0.7
B. intermedius	25611	1.5
B. levii	29147	1.3
C. albicans	DS1	0.4

[a] A total of 1 to 2 pmol of [^{125}I]α-amylase was added to approximately 10^9 bacteria and incubated for 30 min at 27°C. After the pellet was washed three times in PBS, the radioactivity in the pellet was quantitated. Each value represents the average of duplicate samples.

[b] Typically, 50 to 75% of labeled α-amylase (50,000 cpm) bound to *S. sanguis* G9B.

[c] CDM, Chemically defined medium.

Attempts to reverse the binding of α-amylase from the bacterial surface by using as much as a 400-fold excess of unlabeled α-amylase for up to 8 h failed to remove more than 5% of the bound enzyme from the bacterial surface. If the bound radioactivity was eluted with solubilizing solution and examined by SDS-PAGE–autoradiography, the mobility of the eluted radiolabeled material was similar to that of [^{125}I]α-amylase, suggesting that the bound α-amylase is not altered by the bacteria (Fig. 3, lanes F and G).

To assess the specificity of α-amylase binding, streptococcal cells were preincubated with various amounts of unlabeled α-amylase isoenzymes or other secretory proteins. An approximate 25-fold excess of unlabeled α-amylase isoenzyme B1 was sufficient to inhibit the binding of [^{125}I]α-amylase by 90% (Fig. 9). Similar results were obtained when unlabeled isoenzyme B2 and the glycosylated (family A) and nonglycosylated (family B) families of parotid α-amylase were used as inhibitors (Table 3). These data suggest that the binding of α-amylase to streptococci is not mediated by the oligosaccharide units of this molecule. In contrast, the void volume materials (which contained sIgA and proline-rich glycoprotein) from Bio-Gel P60 chromatography of HPS as well as purified sIgA and proline-rich glycoprotein did not inhibit the binding of [^{125}I]α-amylase. Additional studies

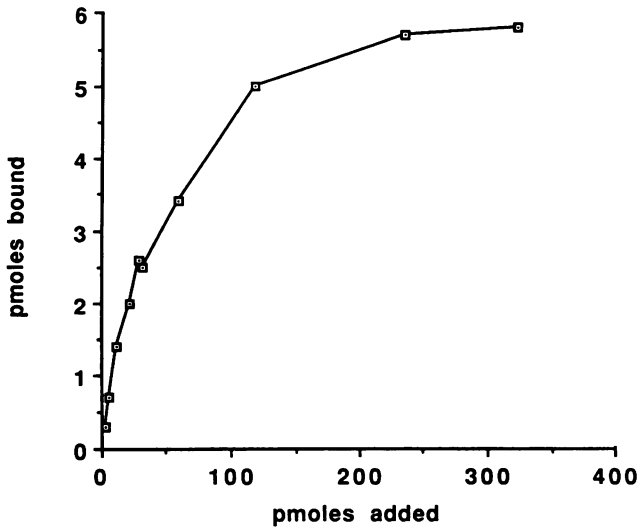

FIG. 8. Binding of [^{125}I]α-amylase to *S. sanguis*. Increasing amounts of [^{125}I]α-amylase were incubated with 10^7 bacteria, and the radioactivity bound to the cells was quantitated. Each point is the average of duplicate samples.

revealed that the interaction of [^{125}I]α-amylase with *S. sanguis* G9B was inhibitable by α-amylases from porcine pancreas and from sources as diverse as *Bacillus amyloliquefaciens* and *Aspergillus oryzae* (Table 3).

Incubation of the [^{125}I]α-amylase–bacterium mixtures in the presence of α-amylase substrates such as maltotriose appeared to inhibit the interaction of α-amylase with *S. sanguis* G9B (Fig. 10). This interaction was not inhibited to any appreciable extent by other sugars such as glucose, maltose, or lactose. Inhibition of α-amylase binding also occurred in the presence of 0.8% limit dextrins (95% inhibition), 0.1% starch (97% inhibition), and to a lesser extent, 1% maltooligosaccharides (55% inhibition).

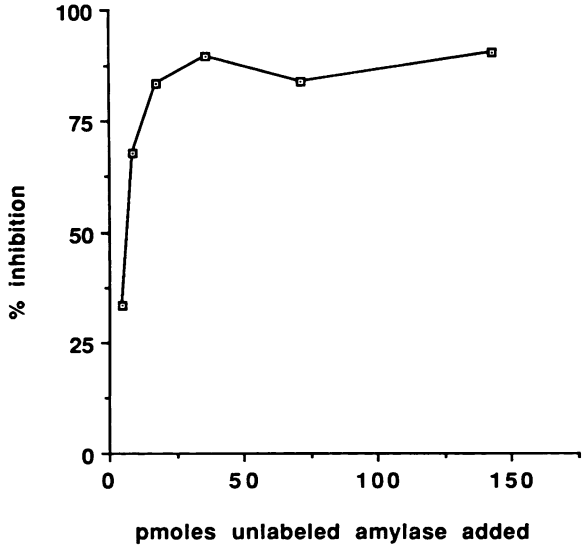

FIG. 9. Inhibition of binding of [^{125}I]α-amylase to *S. sanguis* G9B by unlabeled α-amylase. Increasing amounts of unlabeled α-amylase were incubated with 10^7 bacteria; this was followed by the addition of 1 pmol of [^{125}I]α-amylase. The radioactivity bound to the cells was then quantitated. Each point is the average of duplicate samples.

TABLE 3. Inhibition of [^{125}I]α-amylase binding to *S. sanguis* G9B by various proteins[a]

Inhibitor (no. of bacteria)	Protein (μg) added	Enzyme activity (U) added	% Bound
PBS		0	100
Bio-Gel P60 fractions[b]			
Excluded materials[c] (10^7)	96	ND[d]	97
Family A (10^7)	86	ND	6
Family B (10^7)	82	ND	10
sIgA (10^7)	76		91
Proline-rich glycoprotein	6		97
Amylase B1 (10^7)	10	3	3
Amylase B2 (10^7)	12	ND	4
Reduced and alkylated B1 (10^7)	6	0	101
Porcine pancreatic α-amylase (10^7)	12	13.1	5
Commercial salivary α-amylase (10^8)	12	8.4	7
Bacillus α-amylase (10^8)	12	6	7
Aspergillus α-amylase (10^8)	37	3.7	36
α-Amylase inhibitor[e] (10^7)	5		3

[a] Each reaction mixture contained 10^7 or 10^8 bacteria, the stated amount of inhibitor, and 1 pmol of [^{125}I]α-amylase B1 in a total of 1 ml. Each value is the average of duplicate samples.
[b] See Fig. 1.
[c] Fractions contain proline-rich glycoprotein and sIgA.
[d] ND, Not determined.
[e] A total of 1 mg of inhibitor protein was incubated for 30 min with 0.1 mg of radiolabeled α-amylase. This mixture was then tested in the binding assay and compared with results for a control sample without inhibitor.

Reduced and alkylated α-amylases lacked enzyme activity and were unable to inhibit the binding of native [^{125}I]α-amylase to *S. sanguis* G9B (Table 3). CD studies indicated that the reduced and alkylated materials lacked significant secondary structure compared with the native molecule (Fig. 11). A similar spectrum was obtained with α-amylase in GuHCl alone, suggesting that the loss of secondary structure was due to denaturation of the molecule. In contrast, the addition of DTT alone had no effect on the CD spectra of α-amylase, suggesting that the reduction of disulfide bonds alone is not sufficient to disrupt the overall conformation of the molecule. These results demonstrate that the biologic functions are dependent on the secondary structure, the tertiary structure, or both of the α-amylase molecule.

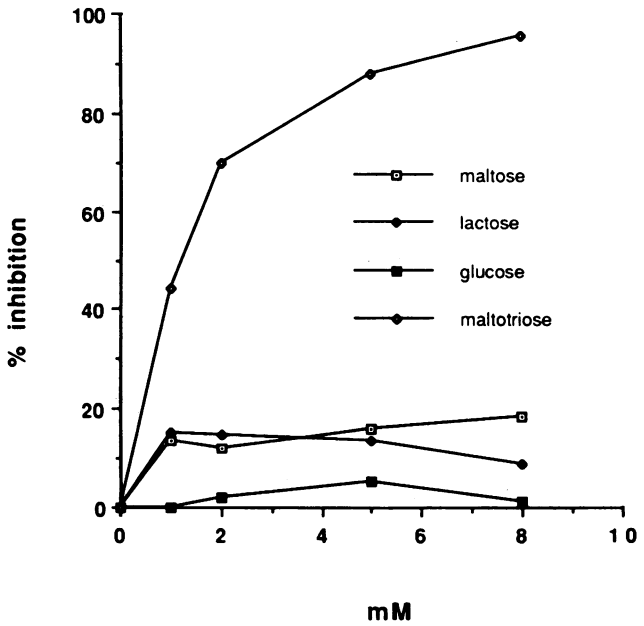

FIG. 10. Inhibition of binding of [^{125}I]α-amylase to *S. sanguis* G9B by various sugars. A total of one pmol of [^{25}I]α-amylase was added to approximately 10^8 bacteria (heated to 80°C for 30 min) in the presence of various sugars and incubated for 30 min at 27°C. After the bacteria were washed three times in PBS, the radioactivity bound was quantitated. Each point is the average of duplicate samples.

In order to explore the nature of the amylase receptor on the microbial surface, bacteria were subjected to several surface modifications. Heating of the bacteria to 80°C for 30 min had no effect on α-amylase binding to the bacteria. Exposure of *S. sanguis* G9B to trypsin, however, abolished the binding of α-amylase to the bacteria. Also, bacteria treated with sodium *meta*-periodate–sodium borohydride or sodium *meta*-periodate alone also abolished α-amylase binding. Sodium borohydride alone had no effect on the binding of the enzyme. Interestingly, the binding of α-amylase to bacteria incubated in sodium acetate-buffered saline (pH 4.5) was reduced compared with the binding to bacteria incubated in PBS, suggesting that the α-amylase receptor may be altered or extracted from the bacterial surface by a low pH or that α-amylase itself is altered at a low pH.

DISCUSSION

α-Amylase is a superfamily of enzymes isolated from phylogenetically diverse organisms. The enzyme hydrolyzes α-1,4-glucan bonds in polysaccharides containing three or more 1,4-linked D-glucose residues, with the major end products being glucose and maltose (31). Human salivary α-amylase is made up of two major families (families A and B); each family has several electrophoretic forms, or isoenzymes (33, 34). The isoenzymes of family A are glycosylated and have a single N-linked complex carbohydrate unit (65), while the isoenzymes of family B appear to be nonglycosylated. The isoenzymes within each family are thought to arise because of variations in the degree of amidation (32). It has long been thought that the major role of α-amylase involves the initial digestion of dietary starches. Recent evidence has pointed to an additional bacterium-interactive function of this salivary molecule. Although abundant in saliva (1), α-amylase has not been well studied in this regard. For example, the growth of both *Legionella pneumophila* (8) and *Neisseria gonorrhoeae* (45) has been reported to be inhibited by α-amylase. The results of the present study support and extend the finding of Douglas (14) that α-amylase selectively interacts with certain strains of oral streptococci. In fact, α-amylase was found to bind only to strains of *S. sanguis* genotypes 1 and 3 and *S. mitis* genotype 2, a finding that may be useful for differentiating strains of these species (12a). Radiolabeled enzyme promptly binds to the bacterial surface in a saturable manner, consistent with the binding of α-amylase to a limited number of receptors on the bacterial surface. This interaction is inhibited by the addition of unlabeled α-amylases from a variety of sources, but not by other salivary proteins, suggesting that the interaction is specific.

We found that the radiolabeled α-amylase bound to the surface of *S. sanguis* could not be eluted by the addition of unlabeled α-amylase. In contrast, the labeled material was eluted with SDS, and a single band with a molecular weight identical to that of α-amylase was seen by SDS-PAGE–autoradiography. These data support the idea that α-amylase binding to the streptococcal surface is mediated by a nonco-

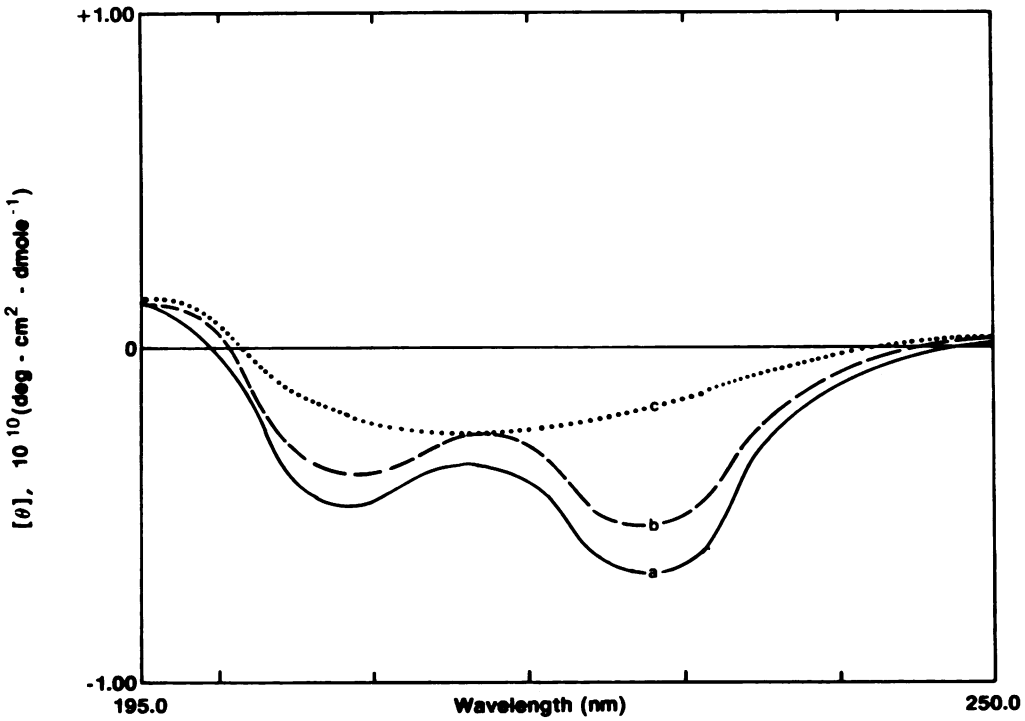

FIG. 11. CD of native salivary α-amylase (curve a), native salivary α-amylase in the presence of DTT (curve b), and reduced and alkylated salivary α-amylase (curve c).

valent, high-affinity mechanism. The large number of α-amylase molecules estimated to interact with each bacterium (13,000 to 33,000) is comparable to other significant bacterium-ligand interactions reported previously. For example, strains of *Streptococcus pyogenes* have been reported to have 1,000 fibronectin receptors on their surface (59), *Bacteroides gingivalis* is reported to possess up to 1,500 fibrinogen receptors on its surface (36), and 5,000 to 10,000 fibronectin molecules are reported to interact with strains of *Escherichia coli* O6:K2:M (19). It is interesting that the number of α-amylase molecules bound to *S. sanguis* is much larger than that calculated for another salivary molecule, proline-rich glycoprotein, which has been noted to have only 120 receptors per streptococcal cell (5). Assuming a smooth-surfaced streptococcal cell with a radius of 0.5 nm, we calculated the surface area of the streptococcal cell to be approximately 3×10^6 nm^2. Since the two-dimensional surface area occupied by a single mammalian α-amylase molecule is about 60 nm^2 (51), then the α-amylase bound at saturation would cover 25 to 63% of the cell surface. However, this is probably an overestimation, since the cell surface is certainly not smooth and thus has a considerably greater surface area.

The data reported here suggest that the streptococcal binding domain of α-amylase may overlap with, or is identical to, the catalytic site. For example, both bacterial binding and enzyme activities are destroyed by the denaturation of α-amylase, indicating that these activities are dependent on an intact secondary structure, tertiary structure, or both. Furthermore, binding of salivary α-amylase to the streptococcal surface could be inhibited by preincubation of the cells with α-amylases from a variety of sources; a protein α-amylase inhibitor from wheat seed (48); and enzymatic substrates including starch, maltotriose, and limit dextrins. The effect of these enzymatic substrates can be explained if the catalytic site and the streptococcal binding site are the same. Alternatively, these results can also be explained if bound substrate induces a conformational change in the α-amylase molecule which thereby alters a distinct streptococcal binding site. Continuing studies are being performed to distinguish between these possibilities. The chemical nature of the streptococcal receptor for α-amylase is less clear since initial studies have shown it to be sensitive to treatment with trypsin or periodate. Bacteria cultured in defined medium without sucrose bound to the same extent as bacteria cultured in complex medium, ruling out the possibility that α-amylase is bound to complex medium components or to sucrose-derived extracellular glucans (21–23). Studies are ongoing to define further the nature of the streptococcal α-amylase receptor.

The fact that the binding of salivary α-amylase to the streptococcal surface could be inhibited by preincubation of the cells with α-amylases from diverse phylogenetic sources supports the contention that certain structural features of α-amylase have been conserved through evolution. It is not surprising that pancreatic α-amylase inhibits the binding of salivary α-amylase to streptococci, since it shares 90% sequence homology with the salivary enzyme (30, 47). Even though considerably less sequence homology exists among more distantly related α-amylases, there are amino acid sequences conserved between α-amylases from sources as diverse as mammalian pancreas and *Bacillus amyloliquefaciens* (43, 54). It may be that these sequences code for the catalytic site as well as the streptococcal binding activity.

The biological role of the α-amylase–streptococcus interaction remains obscure. Since *S. sanguis* is an extremely successful colonizer of the oral cavity, this interaction may impart a selective advantage to *S. sanguis*, perhaps by promoting its colonization of the oral cavity. Several groups of investigators have identified α-amylase in the acquired

enamel pellicle (2, 50). This molecule may therefore provide receptors for bacteria to bind to on the tooth surface or on the surface of dental plaque. Alternatively, α-amylase may bind to bacteria while the α-amylase is in solution to facilitate the clearance of organisms from the oral cavity. Finally, α-amylase may also provide a nutritive function on the bacterial surface, perhaps by liberating saccharides from dietary starch for use by the bacteria.

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We thank Pamela Jones and Richard Ellen for careful review and helpful criticisms regarding this manuscript and A. Coykendall for discussion of recent taxonomic changes.

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ADDENDUM IN PROOF

It has been recently proposed that the group referred to in this paper as *S. sanguis* genotype 1 be designated *S. gordonii* and the group referred to as *S. mitis* genotype 1 be designated *S. oralis* (12a).

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